

Published in final edited form as:

*Immunity*. 2011 May 27; 34(5): 715–728. doi:10.1016/j.immuni.2011.04.014.

## The Myeloid Transcription Factor KLF2 Regulates the Host Response to Polymicrobial Infection and Endotoxic Shock

Ganapati H. Mahabeleshwar<sup>1</sup>, Daiji Kawanami<sup>1</sup>, Nikunj Sharma<sup>1</sup>, Yoichi Takami<sup>1</sup>, Guangjin Zhou<sup>1</sup>, Hong Shi<sup>1</sup>, Lalitha Nayak<sup>1</sup>, Darwin Jeyaraj<sup>1</sup>, Robert Greal<sup>3</sup>, Mary White<sup>3</sup>, Ross McManus<sup>3</sup>, Thomas Ryan<sup>3</sup>, Patrick Leahy<sup>2</sup>, Zhiyong Lin<sup>1</sup>, Saptarsi M. Haldar<sup>1</sup>, G. Brandon Atkins<sup>1</sup>, Hector R. Wong<sup>4</sup>, Jerry B. Lingrel<sup>5</sup>, and Mukesh K. Jain<sup>\*,1</sup>

<sup>1</sup>Case 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, OH 44106, USA <sup>2</sup>Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, OH 44106 <sup>3</sup>Department of Clinical Medicine, Institute of Molecular Medicine, Trinity College Dublin, Ireland <sup>4</sup>Division of Critical Care Medicine, Cincinnati Children's Research Foundation and Department of Pediatrics, University of Cincinnati College of Medicine <sup>5</sup>Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, Cincinnati, OH 45267–0524

### SUMMARY

Precise control of myeloid cell activation is required for optimal host defense. However, this activation process must be under exquisite control to prevent uncontrolled inflammation. Herein, we identify the Kruppel-like transcription factor 2 (KLF2) as a potent regulator of myeloid cell activation in vivo. Exposure of myeloid cells to hypoxia and/or bacterial products reduced KLF2 expression while inducing hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), findings that were recapitulated in human septic patients. Myeloid KLF2 was found to be a potent inhibitor of nuclear factor-kappaB (NF $\kappa$ B)-dependent HIF-1 $\alpha$  transcription and, consequently, a critical determinant of outcome in models of polymicrobial infection and endotoxemia. Collectively, these observations identify KLF2 as a tonic repressor of myeloid cell activation in vivo and an essential regulator of the innate immune system.

### INTRODUCTION

Cells of the myeloid lineage are the principal effectors of the innate immune response to pathogen challenge. Under physiologic conditions, these cells circulate in the bloodstream in a quiescent state. However, in response to an infectious stimulus, these phagocytes are quickly activated and recruited to sites of injury where they engage in the elimination of invading microorganisms (Serbina et al., 2008; Serbina and Pamer, 2008). Indeed, myeloid cell activation is an exquisitely robust biological response that involves transcriptional

© 2011 Elsevier Inc. All rights reserved.

\*Address correspondence to: Mukesh K. Jain M.D., 2103 Cornell Road, Room 4-522, Cleveland, OH 44106, Tel: (216) 368-3607, Fax: (216) 368-0556, Mukesh.jain2@case.edu.

**Publisher's Disclaimer:** This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

### Supplementary

Supplemental information includes extended Experimental Procedure, six figures and associated figure legends.

alterations in gene expression affecting a substantial part of the cellular genome (Kellam and Weiss, 2006). The transcriptional modules that drive this response fashion the phagocyte with a multipronged armamentarium against invading microorganisms that includes the elaboration of numerous antimicrobial peptides, cytokines, chemokines, and reactive nitrogen and oxygen species (Kolls et al., 2008). Successful containment of the pathogen typically leads to resolution followed by tissue repair. However, if these initial efforts are unsuccessful, endotoxins produced by the pathogen can lead to overexuberant activation of phagocytes that can rapidly become deleterious to the host (Gordon and Martinez, 2010; Martinez et al., 2009). This scenario is seen clinically in the context of the host response to bacterial infection (Munford, 2006). If successful containment is not achieved, bacterial products such as lipopolysaccharides (LPS) can lead to uncontrolled myeloid cell activation and culminate in a cytokine storm that leads to tissue damage, vascular collapse, multi-organ failure and death. These observations suggest that while a robust myeloid response is necessary for pathogen clearance, it can be detrimental to the host if left unchecked. Thus, endogenous mechanisms must exist to strictly maintain cell quiescence yet allow for rapid cellular activation with precise spatiotemporal control.

Because sites of bacterial infection are characterized by both hypoxia and high amounts of bacterial products, there has been intense interest in understanding how this microenvironment induces myeloid cell activation. Elegant studies have identified a synergistic and interdependent relationship between key transcriptional pathways of the hypoxic and innate immune response in governing myeloid cell activation - namely hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) and Nuclear Factor-KappaB (NF $\kappa$ B) (Rius et al., 2008). HIF-1 is a heterodimeric helix-loop-helix transcription factor whose expression is tightly regulated at both the mRNA and protein expression. The importance of HIF-1 $\alpha$  in myeloid cell biology is best highlighted by loss-of-function studies which show that HIF-1 $\alpha$  is necessary for myeloid activation. Cramer and colleagues demonstrated that, HIF-1 $\alpha$  deficient myeloid cells exhibited reduced glycolysis and ATP production, and a profound impairment of cellular motility, invasiveness, and bacterial killing (Cramer et al., 2003). Subsequent work from the same group and others has verified the importance of HIF-1 $\alpha$  in myeloid cell bactericidal capacity *in vivo* (Bayele et al., 2007; Peyssonnaud et al., 2005). These studies also revealed that bacteria are a potent stimulus for HIF-1 $\alpha$  accumulation even under normoxic conditions. Efforts to understand the molecular basis for this observation led to an appreciation of an intimate and synergistic relationship between HIF-1 $\alpha$  and the NF $\kappa$ B pathway (Nizet and Johnson, 2009). HIF-1 $\alpha$  has been shown to mediate NF $\kappa$ B activation in neutrophils and promote the expression of NF $\kappa$ B regulated proinflammatory cytokines (Walmsley et al., 2005). Conversely, both hypoxia and bacterial products (e.g. LPS) induce HIF-1 $\alpha$  mRNA accumulation in an NF $\kappa$ B dependent manner (Rius et al., 2008). This induction of HIF-1 $\alpha$  mRNA is a crucial precursor to the post-transcriptional stabilization and accumulation of HIF-1 $\alpha$  protein that occurs in the hypoxic microenvironment. Thus, the combination of hypoxia and bacterial products (e.g. LPS), as seen at sites of infection, can lead to robust induction of the NF $\kappa$ B-HIF-1 $\alpha$  module, thereby resulting in myeloid cell activation (Nizet and Johnson, 2009). As the intersection of NF $\kappa$ B-HIF-1 $\alpha$  signaling represents a key nodal point in myeloid activation, factors that inhibit this module might be critical for maintenance of the quiescent state. In the current study, we identify Kruppel-like factor 2 (KLF2) as such a factor.

Emerging evidence implicates the Kruppel-like factor (KLF) family of zinc-finger transcription factors as endogenous regulators of cellular activation (Cao et al., 2010). Kruppel-like factor 2 (KLF2) was initially identified and termed Lung Kruppel-like factor owing to its high expression in lung tissues (Anderson et al., 1995). However, the *in vivo* role for this factor in the context of myeloid cell biology has not been elucidated. Herein, we identify myeloid KLF2 as an essential tonic repressor of myeloid cell activation.

Furthermore, we provide evidence that KLF2-mediated regulation of the NF $\kappa$ B-HIF-1 $\alpha$  axis titrates cellular activation and is a critical determinant of the organism's response to infection and endotoxic shock.

## RESULTS

### Myeloid-specific KLF2 Deficiency Results in Spontaneous Pro-inflammatory Activation

Myeloid specific deletion of KLF2 was achieved through crossing the *Klf2<sup>fl/fl</sup>* mouse to the *Lyz2cre* line. Extensive deletion of KLF2 was observed in myeloid cells and no substantial effect was seen on other major hematopoietic lineages, tissues, or monocytic subsets (Figure S1). Previous work from our lab and others implicated KLF2 as a critical transcription factor that maintains cellular quiescence (Bista et al., 2008; Buckley et al., 2001; Dekker et al., 2006; SenBanerjee et al., 2004). Therefore we hypothesized that deficiency of KLF2 may promote derepression of inflammatory cytokine gene expression under non-inflammatory conditions. Intriguingly, basal expression of several pro-inflammatory cytokines was induced significantly ( $p < 0.05$ ) while anti-inflammatory cytokines were unaltered (Figure 1A). These data suggest that KLF2 deficiency results in spontaneous myeloid cell activation.

### Myeloid KLF2 Deficiency is Protective Against Polymicrobial Infection

Activation of myeloid cells is requisite for optimal bactericidal activity (Serbina and Pamer, 2008). As such, we hypothesized that the heightened basal activation observed in *Lyz2creKlf2<sup>fl/fl</sup>* mice may confer a greater capacity to withstand bacterial infection. To address this consideration, *Lyz2cre* and *Lyz2creKlf2<sup>fl/fl</sup>* mice were subjected to a polymicrobial infection by cecal ligation and puncture (CLP) (Buras et al., 2005). As shown in Figure 1B, survival of the *Lyz2creKlf2<sup>fl/fl</sup>* mice was significantly ( $p < 0.01$ ) higher than that of *Lyz2cre* mice. *Lyz2creKlf2<sup>fl/fl</sup>* mice were also protected from polymicrobial infection induced hypotension and hypothermia (Figure 1C and D). Enumeration of bacterial colonies from blood and homogenates of several major organs revealed significantly lower ( $p < 0.05$ ) colony forming units in *Lyz2creKlf2<sup>fl/fl</sup>* mice (Figure 1E–H). Consistent with this reduction in bacterial colonization, *Lyz2creKlf2<sup>fl/fl</sup>* mice demonstrated moderately elevated expression of several proinflammatory cytokines (Figure 1I–L). Collectively, these findings indicate that myeloid specific deficiency of KLF2 leads to reduced bacterial burden and increased host survival in the setting of polymicrobial infection.

### KLF2 Deficiency Enhances Myeloid Cell Bactericidal Activity

The reduced bacterial burden observed in *Lyz2creKlf2<sup>fl/fl</sup>* mice suggested that myeloid cells from these animals may exhibit enhanced bactericidal capability. To test this directly, we performed antibiotic protection assays (APA) in primary macrophages and neutrophils from *Lyz2cre* and *Lyz2creKlf2<sup>fl/fl</sup>* mice. As shown in Figure 2 A and B, *Lyz2creKlf2<sup>fl/fl</sup>* myeloid cells exhibited significantly enhanced ( $p < 0.002$ ) bactericidal capacity. This effect was not due to an alteration in the rate of bacterial uptake or phagocytosis as assessed by deconvolution microscopy (Figure S2A) and Fluorescent Activated Cell Sorting (FACS) analysis (Figure S2B and C). In agreement with these loss-of-function observations, adenoviral overexpression of KLF2 in the RAW 264.7 macrophage cell line significantly attenuated ( $p < 0.00216$ ) bactericidal capacity (Figure S2 D and E).

As KLF2 expression did not directly affect phagocytic capacity, we focused attention on mechanisms of intracellular pathogen killing. As shown in Figure 2 C and D, KLF2 deficient macrophages and neutrophils express significantly higher mRNA encoding both CRAMP and iNOS, two agents with potent antimicrobial activity ( $p < 0.003$ ). Consistent with these regulatory effects on iNOS mRNA, parallel effects were observed for iNOS protein accumulation and NO production (Figure 2E and F). Conversely, overexpression of KLF2

in RAW264.7 cells strongly attenuated the LPS-induced upregulation of CRAMP and iNOS mRNA as well as iNOS protein expression and activity (Figure. S2F–I).

In addition to the elaboration of antimicrobial substances, optimal myeloid cell function during inflammatory challenge requires alterations in cellular metabolism (Krauss et al., 2001). As shown in Figure 2G and H, KLF2 deficiency led to a significant increase ( $p < 0.009$ ) in LPS-induced glycolysis as evidenced by higher lactate and intracellular ATP amounts. Furthermore, enhanced expression of Glut1 and PGK were observed (Figure 2 I and J). Conversely, overexpression of KLF2 in RAW 264.7 cells led to a reduction in lactate production, ATP amounts, and Glut1, PGK expression after LPS stimulation (Figure S2J–M). Collectively, these results indicate that myeloid-specific deficiency of KLF2 results in altered expression of anti-microbial and metabolic genes in a manner conducive to enhanced bactericidal activity.

### Myeloid KLF2 Deficiency Renders Animals Susceptible to Endotoxic Shock

Whereas increased inflammation can be advantageous in the context of bacterial killing, unbridled inflammation can lead to septic shock and death (Russell, 2006). We hypothesized that *Lyz2creKlf2<sup>fl/fl</sup>* mice, which are resistant to polymicrobial infection and exhibit a proinflammatory phenotype, might be more susceptible to direct challenge with endotoxin. As shown in Figure 3A, LPS challenge of *Lyz2creKlf2<sup>fl/fl</sup>* mice produced 100% mortality by 72 h, whereas *Lyz2cre* mice experienced only 20% mortality. In addition, *Lyz2creKlf2<sup>fl/fl</sup>* mice exhibited all the cardinal features of endotoxic shock including hypothermia (Figure 3B), hypotension (Figure 3C), and elevated shock index (Figure 3D). By comparison to *Lyz2cre* mice, LPS-treated *Lyz2creKlf2<sup>fl/fl</sup>* mice had significantly elevated ( $p < 0.03$ ) amounts of plasma cytokines (Figure 3 E–K). Of note, the amounts of many of these cytokines were dramatically higher than that observed in the CLP studies and are consistent with the fact that this model of LPS-induced endotoxic shock produces a cytokine storm. Concordant effects were seen following *ex vivo* stimulation of *Lyz2cre* and *Lyz2creKlf2<sup>fl/fl</sup>* primary macrophages with LPS (Figure S3 A – K). Collectively, these results strongly suggest that enhanced inflammation seen in the *Lyz2creKlf2<sup>fl/fl</sup>* mice is protective with respect to bacterial clearance, it is detrimental in the face of direct endotoxin challenge.

### Myeloid KLF2 is regulated by hypoxia and inflammatory stimuli

We next sought to understand the molecular mechanism that rendered *Lyz2creKlf2<sup>fl/fl</sup>* mice resistant to polymicrobial infection and susceptible to LPS-induced endotoxemia. Intriguingly, previous studies in the myeloid-specific gene ablation of HIF-1 $\alpha$  reported observations in contrast to those made in our *Lyz2creKlf2<sup>fl/fl</sup>* line. For example, myeloid deficiency of HIF-1 $\alpha$  has been shown to reduce myeloid cell pro-bactericidal-pro-inflammatory gene expression, glycolysis (Cramer et al., 2003; Peyssonnaud et al., 2005), and mortality in the face of an LPS-challenge (Peyssonnaud et al., 2007). Therefore, we focused our attention on HIF-1 $\alpha$ , a central integrator of both hypoxic and innate immune stimulation in myeloid cells.

As a first step, the expression profile of KLF2 and HIF-1 $\alpha$  in wild-type primary peritoneal macrophages incubated with heat-inactivated gram positive (*S. Aureus*) or gram negative (*E. Coli*) extracts in combination with hypoxia was assessed. As expected, HIF-1 $\alpha$  expression was induced by both hypoxia and heat inactivated extracts of gram positive or negative bacteria (Figure 4A and S4B). A more robust induction of HIF-1 $\alpha$  was seen using dual stimulation of macrophages with hypoxia and bacterial extracts. By contrast, KLF2 expression was reduced by hypoxia and exposure to bacterial products at the protein (Figure 4A) and mRNA expression (Figure S4A). As hypoxia has been shown to alter KLF2 expression in a time-dependent fashion in endothelial cells (Kawanami et al., 2009), we

examined the kinetics of KLF2 expression in macrophages following hypoxia. Our results demonstrate that hypoxia induces KLF2 expression at 1 hour followed by a marked reduction in expression by 2 hours (Figure S4C). At later time points (6, 12, and 24 hours) KLF2 protein is essentially undetectable (Figure S4C). Similar results were observed at the RNA expression (Figure S4D). Finally, we sought to understand the molecular basis for how hypoxia reduced KLF2 expression. We note a previous publication from our lab showed that KLF2 expression is negatively regulated by activation of NF $\kappa$ B in the context of cytokine stimulation (Kumar et al., 2005). In addition, a recent report from Culver et. al. (Culver et al., 2010) demonstrated that hypoxia activates NF $\kappa$ B and is a critical component of transcriptional response to hypoxia. Therefore we hypothesized that NF $\kappa$ B may regulate KLF2 expression under hypoxic conditions. To test this hypothesis, we exposed wild-type primary peritoneal macrophages to hypoxia in the presence and absence of a NF $\kappa$ B specific peptide inhibitor, SN-50. Hypoxia alone significantly reduced ( $p < 0.02$ ) KLF2 expression in macrophages. However, in the presence of SN-50, this reduction is strongly attenuated at both the mRNA and protein expression (Figures S4E and F).

Finally, to determine if these changes in macrophage KLF2 expression were observed in patients who were exposed to bacterial infection and developed sepsis. Whole blood-derived RNA was generated from 98 patients with septic shock and 32 normal pediatric controls (Wong et al., 2009). As shown in Figure 4B, septic patients exhibited a significant reduction in KLF2 and increase in HIF-1 $\alpha$  mRNA expression ( $p < 0.002$ ). These data suggest that the expression pattern of KLF2 and HIF-1 $\alpha$  observed in cultured cells (Figure 4A) is recapitulated in human sepsis.

### KLF2 Inhibits NF $\kappa$ B Mediated HIF-1 $\alpha$ Expression

These observations prompted us to directly examine the role of KLF2 in the molecular regulation of HIF-1 $\alpha$  following LPS stimulation. Consistent with previous reports (Blouin et al., 2004), LPS induced HIF-1 $\alpha$  protein accumulation (Figure 4C). Further, actinomycin D studies confirmed that this induction was at the transcriptional level (data not shown). KLF2 overexpression in RAW264.7 cell line attenuated HIF-1 $\alpha$  mRNA expression (Figure 4D) while *Lyz2creKlf2<sup>fl/fl</sup>* macrophages and neutrophils exhibited enhanced HIF-1 $\alpha$  mRNA expression both at baseline and following LPS stimulation (Figure 4E and F). To gain greater insight into how KLF2 regulates HIF-1 $\alpha$  mRNA, we undertook gene reporter assays using a HIF-1 $\alpha$  promoter-luciferase construct. Recent studies indicate that NF $\kappa$ B is a direct transcriptional regulator of HIF-1 $\alpha$  (Rius et al., 2008). Thus we hypothesized that KLF2 may reduce HIF-1 $\alpha$  transcription via inhibition of NF $\kappa$ B activity. Indeed, KLF2 attenuated the ability of p65 to induce the HIF-1 $\alpha$  promoter under both basal and LPS-stimulated conditions (Figure 4G). However, chromatin immunoprecipitation (ChIP) studies revealed that neither overexpression nor deficiency of KLF2 altered p65 recruitment to the endogenous HIF-1 $\alpha$  promoter (Figure 4H and I). Thus, we reasoned that the recruitment of key co-activators required for optimal NF $\kappa$ B activity may be affected by KLF2. Indeed, KLF2 overexpression attenuated and deficiency augmented recruitment of critical NF $\kappa$ B transcriptional co-activators p300 and PCAF to the HIF-1 $\alpha$  promoter (Figure 4H and I). A similar conclusion was gleaned from studies on the expression of I $\kappa$ B $\alpha$ , a well-established NF $\kappa$ B target (Figure S4G). To gain a more global understanding of KLF2 targets, we performed Affymetrix microarray analyses (accession number GSE26727) using primary peritoneal macrophages from *Lyz2cre* and *Lyz2creKlf2<sup>fl/fl</sup>* mice stimulated with LPS for 6 hours. The full microarray data set has been submitted to Gene Expression Omnibus (GEO), a public functional genomics data repository. However, we note that, in addition to confirming an increase in pro-inflammatory target genes (e.g. increase in NOS2), the microarray revealed a number of novel targets relevant to the inflammatory response [e.g. reduced expression of anti-inflammatory targets such as arginase-1(*Arg-1*) and resistin-like

molecule alpha (*Retnla*)]. A subset of genes relevant to inflammation is shown in Figure S4H.

Finally, to determine whether the effects of KLF2 on HIF-1 $\alpha$  mRNA translated into protein expression, immunoblot analyses were performed. KLF2 deficiency enhanced basal and LPS stimulated increase in HIF-1 $\alpha$  protein expression in primary peritoneal macrophages and neutrophils (Figure 4J–L). Conversely, adenoviral overexpression of KLF2 in RAW264.7 cells strongly attenuated LPS induced HIF-1 $\alpha$  protein expression (Figure S4I). Collectively, these studies indicate that KLF2 negatively regulates LPS induced HIF-1 $\alpha$  expression through modulating recruitment of critical coactivators of NF $\kappa$ B to the promoter of HIF-1 $\alpha$ .

### Alterations in *Lyz2creKlf2<sup>fl/fl</sup>* Myeloid Cell Gene Expression and Function are HIF-1 $\alpha$ Dependent

We next sought to determine whether the pro-inflammatory phenotype observed in *Lyz2creKlf2<sup>fl/fl</sup>* mice was HIF-1 $\alpha$  dependent using both pharmacologic and genetic approaches. Echinomycin is a well-established inhibitor of HIF-1 $\alpha$  (Kong et al., 2005). As shown in Figure S5A and B, echinomycin treatment strongly attenuated LPS-stimulated cell migration and invasion in *Lyz2creKlf2<sup>fl/fl</sup>* cells. Further, echinomycin treatment also attenuated the LPS-induced hyperinduction of numerous inflammatory gene products (Figure S5C– S5F), glycolytic targets (Glut-1 and PGK) and metabolite markers of glycolysis (lactate and ATP concentration) (Figure S5 G–J).

To substantiate the role of HIF-1 $\alpha$  inhibition *in vivo*, we generated compound mutant mice in which both KLF2 and HIF-1 $\alpha$  (*Lyz2cre Setd2fl/fl*) were deleted in a myeloid-specific fashion (termed *Lyz2creKlf2fl/flSetd2fl/fl*). Dual deficiency of KLF2 and HIF-1 $\alpha$  was confirmed at the mRNA and protein expression (Figure S5K and S5L) and did not alter any hematologic parameters (Figure S1 D , E and K). Consistent with our observations using pharmacologic HIF-1 $\alpha$  inhibition, genetic ablation of HIF-1 $\alpha$  completely rescued LPS induced effects on cellular migration and invasion observed in *Lyz2creKlf2fl/fl* macrophages (Figure 5A and B). Further, the heightened expression of numerous gene products that are critically involved in various aspects of macrophage biology such as COX2, ADM-1, MMP2, TNF- $\alpha$ , IL-6 and IL-1 $\beta$  expression was attenuated by HIF-1 $\alpha$  deficiency in *Lyz2creKlf2fl/fl* macrophages (Figure 5C, E, G, I, K and L). Concordant effects were seen in neutrophils (Figure 5D, F, H and J). Finally, analysis of glycolytic function following LPS stimulation indicated that deficiency of HIF-1 $\alpha$  in *Lyz2creKlf2fl/fl* macrophages also rescued enhanced glycolytic gene expression and activity as determined by Glut-1,PGK expression and lactate,intracellular ATP concentration, respectively (Figure 5M–P). Together, these results indicate that critical components of the pro-inflammatory phenotype observed in KLF2 deficient macrophages are HIF-1 $\alpha$  dependent.

### Response of KLF2-deficient Mice to Infection and Endotoxic Shock are HIF-1 $\alpha$ Dependent

We next sought to determine if the HIF-1 $\alpha$  dependency observed in KLF2-deficient myeloid cells in *ex vivo* assays (Figure 5) was maintained in the intact organism. Examination of basal plasma cytokine concentration revealed that the increased expression of several pro-inflammatory cytokines seen in the *Lyz2creKlf2fl/fl* mice was abrogated in *Lyz2creKlf2fl/flSetd2fl/fl* mice (Figure 6A). Next, we subjected *Lyz2cre*, *Lyz2creKlf2fl/fl*, *Lyz2creSetd2fl/fl* and *Lyz2creKlf2fl/flSetd2fl/fl* mice to both bacterial infection and endotoxic shock models. As observed previously, *Lyz2creKlf2fl/fl* mice were resistant to polymicrobial infection and exhibited increased rates of survival compared to control mice. Strikingly, dual deficiency of myeloid KLF2 and HIF-1 $\alpha$  (*Lyz2creKlf2fl/flSetd2fl/fl*) rendered animals highly susceptible to polymicrobial infection reaching 100% mortality within 48 hours of CLP (Figure 6B).

Enumeration of bacterial culture from blood and tissue homogenates revealed that myeloid deficiency of KLF2 significantly reduced the number of live aerobic bacteria while the double deficient KLF2 and HIF-1 $\alpha$  line (*Lyz2creKlf2fl/flSetd2fl/fl*) had a significantly higher ( $p<0.03$ ) number of live bacteria in the blood and peripheral organs (Figure 6C and D, Figure S6 A – C). Consistent with this observation, deficiency of HIF-1 $\alpha$  in *Lyz2creKlf2fl/fl* macrophages significantly reduced ( $p<0.002$ ) bactericidal properties observed in *Lyz2creKlf2fl/fl* macrophages and was associated with a five-fold increase in survival of intracellular bacteria in *Lyz2creKlf2fl/flSetd2fl/fl* macrophages (Figure 6E). Analysis of myeloid antimicrobial genes indicated that deficiency of HIF-1 $\alpha$  in *Lyz2creKlf2fl/fl* macrophages and neutrophils resulted in significantly attenuated ( $p<0.02$ ) expression of iNOS and CRAMP following LPS stimulation (Figure 6 F–I).

Next, these same lines of mice were subjected to LPS-induced endotoxemia. Following LPS administration, *Lyz2creKlf2fl/fl* mice exhibited 100% mortality within 60 hours while the control line exhibited only 50% mortality at 96 hours. By contrast, genetic deficiency of HIF-1 $\alpha$  in *Lyz2creKlf2fl/fl* mice provided a potent protective effect in the face of LPS-induced endotoxic shock (as assessed by blood pressure and body temperature; Figure 6J and K) and mortality (Figure 6L). Analysis of circulating plasma cytokines following LPS induced endotoxemia indicated that compound deficiency of HIF-1 $\alpha$  and KLF2 significantly ( $p<0.002$ ) attenuated plasma concentration of several key cytokines (Figure 6 M–P). These data are congruent with our *ex vivo* studies (Figure 5) and demonstrate that the response of *Lyz2creKlf2fl/fl* mice to both polymicrobial infection and endotoxic shock are HIF-1 $\alpha$ -dependent.

## DISCUSSION

The central finding of this study is that KLF2 inhibits HIF-1 $\alpha$  dependent myeloid cell activation. Specifically, we find that: (1) KLF2 expression is regulated by hypoxia and bacterial products in a manner that is anti-parallel to that of HIF-1 $\alpha$ , (2) this anti-parallel pattern of expression is recapitulated in human subjects with sepsis, (3) KLF2 transcriptionally inhibits the NF $\kappa$ B-HIF-1 axis and attendant myeloid cell functions, (4) myeloid deficiency of KLF2 confers a pro-inflammatory milieu that offers protection in the context of polymicrobial infection but is deleterious in the setting of LPS-induced endotoxic shock, and (5) the phenotype of *Lyz2creKlf2fl/fl* mice is rescued by inhibition or ablation of HIF-1 $\alpha$ . Collectively, these observations identify KLF2 expression and function as a critical component of the innate immune response to bacterial infection and endotoxic shock.

Our studies provide important insights regarding the role of KLF2 as a transcriptional regulator of both myeloid cell quiescence and activation. On the basis of these observations, we propose the model outlined in the Graphical Abstract, available online. Circulating myeloid cells, which are in the quiescent state, express robust levels of KLF2 and low expression of HIF-1 $\alpha$ . As a consequence, circulating concentration of pro-inflammatory cytokines are held in check at vanishingly low. The importance of this basal KLF2 expression is underscored by the fact that its deficiency leads to spontaneous activation of myeloid cells – which is manifest as elevated serum concentration of several pro-inflammatory cytokines in *Lyz2creKlf2fl/fl* mice. Our studies suggest that this modest increase in basal cytokines is secondary to derepression of HIF-1 $\alpha$ . In support, a modest but appreciable increase in basal HIF-1 $\alpha$  mRNA and protein are observed in KLF2-deficient macrophages. Furthermore, the increase in basal pro-inflammatory cytokines is completely abolished by compound deficiency of KLF2 and HIF-1 $\alpha$ .

Our studies also indicate that release from KLF2-mediated repression is critical for optimal myeloid cell activation. Upon egress into tissue, myeloid cells encounter hypoxic tissues as

well as foreign pathogens. Importantly, we find that both hypoxia and bacterial products reduce KLF2 expression while robustly activating HIF-1 $\alpha$  expression and/or activity. These findings may be clinically relevant as a reduction in KLF2 mRNA and enhanced HIF-1 $\alpha$  mRNA expression were seen in circulating human myeloid cells from patients with sepsis. This reduction in KLF2 expression appears to be required for optimal HIF-1 $\alpha$  activation as sustained expression of KLF2 strongly attenuates the NF $\kappa$ B mediated induction of HIF-1 $\alpha$  mRNA and protein. The coordinated reduction in KLF2 and induction of HIF-1 $\alpha$  allows for optimal bactericidal activity. Consistent with this idea, KLF2 deficient myeloid cells are “primed” for activation in the basal state and exhibit robust bacterial killing in antibiotic protection assays and confer improved survival following CLP with reduced bacterial burden in blood and peripheral tissues. The enhanced expression of HIF-1 $\alpha$  observed in KLF2-null cells is clearly an important mediator of the observed phenotype as myeloid cells deficient in both KLF2 and HIF-1 $\alpha$  lose bactericidal activity and succumb more readily following CLP challenge. Our observations from the LPS- induced endotoxic shock experiments represent an extension of this line of reasoning. In the event that initial host defense efforts fail, bacterial products can leach out into the circulation and induce an overwhelming inflammatory response. In this case, it is anticipated that KLF2 expression will fall both in circulating and non-circulating myeloid cells leading to an exaggerated induction of HIF-1 $\alpha$  and an inflammatory storm. Consistent with this model, *Lyz2creKlf2fl/fl* mice exhibit a profound intolerance to LPS-induced sepsis. Further, exceptionally high concentration of cytokines (especially IL-1 $\beta$ , MCP-1, IL-17 and TNF- $\alpha$ ) was observed after LPS challenge, rendering animals unable to sustain themselves against elevated concentration of bacterial endotoxins. Again, the importance of HIF-1 $\alpha$  in this setting is underscored by the fact that *Lyz2creKlf2fl/flSetd2fl/fl* mice exhibit enhanced survival after LPS challenge. The latter finding is also consistent with the observations of Pessyoneux and colleagues who demonstrated that myeloid HIF-1 $\alpha$  deficiency renders rodents resistant to LPS-induced sepsis (Peyssonnaud et al., 2007). Collectively these studies identify a KLF2-HIF-1 axis as critical in regulating the balance between myeloid quiescence and activation.

Our mechanistic insights suggest that in the context of inflammatory stimuli such as LPS, KLF2’s ability to inhibit HIF-1 $\alpha$  expression occurs primarily through the inhibition of NF $\kappa$ B-dependent induction of HIF-1 $\alpha$  mRNA expression. Our data coupled with previous observations (Das et al., 2006; SenBanerjee et al., 2004) suggest that KLF2 does not affect the NF $\kappa$ B pathway at the cytosolic signaling or the recruitment of NF $\kappa$ B to the endogenous HIF-1 $\alpha$  promoter. Intriguingly, the main effect lies at the level of co-activator recruitment. Sustained expression of KLF2 inhibits while deficiency augments p300 and PCAF recruitment to the HIF-1 $\alpha$  promoter. Previous studies by our group and others reveal that KLF2 can interact directly with both p300 and PCAF and thus, in the setting of sustained KLF2 expression, this likely constitutes the molecular basis for preventing p300-PCAF recruitment to NF $\kappa$ B (Ahmad and Lingrel, 2005; SenBanerjee et al., 2004). This type of control at the target gene transcription is atypical and provides a particularly elegant and important “molecular brake” that titrates the inflammatory transcriptional response. As KLF2 expression fall, unbound p300-PCAF can more freely interact with NF $\kappa$ B and induce target genes. We note, however, that in the setting of an infected tissue, HIF-1 $\alpha$  expression are induced not only through *de novo* transcription but also secondary to protein stabilization. In this regard, a recent report by Kawanami and colleagues showed that in hypoxic endothelial cells KLF2 can also reduce HIF-1 $\alpha$  protein stability by disrupting interaction with its chaperone Hsp90 (Kawanami et al., 2009). Whether a similar mechanism is operative in hypoxic myeloid cells is an important area for future investigation. Collectively, our observations along with previous work, suggest that KLF2 negatively regulates HIF-1 $\alpha$  at both transcriptional and post-transcriptional levels.

We note that although the survival phenotype observed in myeloid KLF2 deficient animals supports a dominant role for HIF-1 $\alpha$ , there is evidence that additional mechanisms may also be operative. For example, because myeloid deficiency of HIF-1 $\alpha$ , KLF2 and HIF-1 $\alpha$  mice display similar rates of survival in experimental models of sepsis one may also expect minimal differences in gene expression or functional responses in cell and/or animals bearing these two genotypes. Whereas this appears to be the case for many key myeloid genes functions, several other parameters demonstrate differential responses between the *Lyz2creSetd2fl/fl* and *Lyz2creKlf2fl/flSetd2fl/fl* genotypes. Additionally, although much of our study highlights the induction of pro-inflammatory targets following KLF2 depletion, our microarrays also show reduced expression of numerous factors with potent anti-inflammatory properties. These factors have diverse cellular functions and include enzymes (e.g. arginase-1 and TIMP3), growth factors (e.g. TGF $\beta$  superfamily members), secreted molecules (e.g. Retnla and Cyr61), and transcription factors (e.g. PPAR $\delta$ ) (Barish et al., 2008; Gill et al., 2010; Nair et al., 2009). We note that because our microarray study was conducted at a single time point, one may be underestimating the full spectrum of KLF2-regulated targets. Indeed, kinetic microarray and/or RNA-seq approaches (Wang et al., 2009) will be helpful towards gaining additional insights regarding KLF2 action in myeloid biology. The importance of these additional regulatory pathways will require additional investigation and serve as the focus of future studies.

Finally, the observations presented in this study coupled with previous studies of KLF2 biology bear important implications for the clinical syndrome of sepsis. We note that the multi-organ failure that typifies end-stage sepsis occurs not only through exuberant myeloid cell activation but also secondary to widespread endothelial damage (Aird, 2003). Characteristically, endothelial injury in sepsis leads to diffuse vascular dysfunction manifest as enhanced permeability, intravascular coagulation, and loss of vascular tone (Schouten et al., 2008). A robust literature indicates that endothelial KLF2 is an essential regulator of endothelial homeostasis (Atkins and Jain, 2007) and confers an anti-inflammatory, anti-thrombotic, and anti-adhesive phenotype to the vessel wall (Dekker et al., 2006; Lin et al., 2006; Lin et al., 2005). Indeed, KLF2<sup>+/-</sup> mice exhibit a pro-inflammatory (Atkins et al., 2008) and pro-permeable vasculature (Lin et al., 2010). Collectively, these observations along with findings presented in the current study, suggest that a reduction in endothelial and myeloid KLF2 expression may be functionally important in regulating the organism's response to sepsis. As such, manipulation of KLF2 expression may offer new opportunities for therapeutic gain. Indeed, agents known to induce KLF2 expression such as statins have been shown to ameliorate experimental sepsis and are being considered for clinical application (Liappis et al., 2001).

## EXPERIMENTAL PROCEDURE

### Generation of Myeloid Specific Deletion of KLF2 and HIF-1 $\alpha$ Mice

Mouse line expressing lysozyme M promoter driven Cre recombinase (*Lyz2cre*) and HIF-1 $\alpha$  floxed (*Setd2fl/fl*) mice were obtained from The Jackson Laboratory. KLF2 floxed (*Klf2fl/fl*) mice were created in Jerry Lingrel's laboratory at the University of Cincinnati as described before (Weinreich et al., 2009). *Klf2fl/fl* mice were crossed with the *Lyz2cre* mice to generate myeloid specific KLF2 deficient mice. Similarly, *Setd2fl/fl* mice were crossed with *Lyz2cre* mice to generate myeloid specific HIF-1 $\alpha$  deficient mice. Finally, *Lyz2creKlf2fl/fl* and *Lyz2creSetd2fl/fl* were crossed to generate KLF2 and HIF1 $\alpha$  myeloid specific double deficient mice. All mice colonies were maintained in a clean animal facility and all animal experimentation was approved by the Case Western Reserve University IACUC committee.

### Bacterial Uptake and Killing Assay

E.Coli was transformed with the GFP-expressing plasmid pTreacer-B. These clones of bacteria were grown to logarithmic phase in Luria-broth medium (at O.D.600, 0.4= 10e8 cfu/ml). These bacterial cultures were pelleted, washed in PBS and diluted with DMEM supplemented with 0.1% BSA to desired concentration. These live or inactivated bacteria were added to monolayer of *Lyz2cre*, *Lyz2creKlf2fl/fl* macrophages and RAW 264.7 cells infected with Ad-GFP or Ad-KLF2 to assess intracellular bacterial killing and cellular bacterial uptake respectively as described before (Cramer et al., 2003).

### Cecal Ligation and Puncture

CLP was performed as described before (Rittirsch et al., 2009). Briefly, mice were anesthetized and a midline incision was performed on abdominal side. The cecum was exteriorized and ligature was placed at center of the cecum. The ligated cecal stump was punctured twice with a 21-gauge needle and small amount of stool was extruded. Next, the cecum was placed back into its intra-abdominal position and closed abdomen with combination of running suture and stainless steel wound clips. All animals were subjected to postoperative fluid resuscitation with 1ml pre-warmed normal saline. Following 12 hours of CLP, rectal temperature, blood pressure were recorded. These mice were anesthetized blood collected by inferior vena cava injection and lung, liver, heart, kidney were isolated and homogenized. Serial dilutions of this homogenate were plated on tryptic soya agar plates for enumerations of bacterial colony forming units. For survival studies, a separate set of mice were observed for 6 days following CLP. Survival data were analyzed by the construction of Kaplan-Meier plots and use of the log-rank test.

### LPS Induced Endotoxic Shock

Mice (8–10 weeks old) were injected intraperitoneally with 21mg/kg bodyweight of LPS or saline solution. Mice were monitored for 8 days following LPS injection and their rectal temperature, blood pressure and heart beat rates recorded. Blood samples were drawn by retro-orbital bleeding on separate set of mice for cytokine analysis. Shock index was calculated using 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.

### Quantification of Plasma Cytokines

Blood samples were collected after four hours of LPS or saline injection or twelve hours after CLP or sham surgery by retro-orbital bleeding. Indicated mouse plasma were analyzed using the MILLIPLEX MAP mouse cytokine or chemokine panel from Millipore.

### Real-time Quantitative RT-PCR

Total RNA was extracted from primary macrophages, neutrophils or RAW 264.7 cells following indicated treatment using TRIzol® Reagent (Invitrogen) and 2 µg of total RNA was reverse transcribed using M-MuLV reverse transcriptase (New England Biolabs Inc.) and mixture of random and oligo-dT primers. Real-Time PCR was performed using Universal SYBR Green PCR Master Mix on Applied Biosystems Step One Real-Time PCR System (Applied Biosystems) using gene specific primers.

### Statistical Analysis

All data, unless indicated are presented as the mean ± SD. The statistical significance of differences between two groups was analyzed with Student's *t* test. Values of *P* < 0.05 were considered significant.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

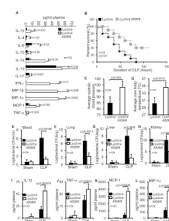
This work was supported, in whole or in part, by National Institutes of Health Grants HL72952, HL75427, HL76754, HL086548, HL084154, and P01 HL048743 (M. K. J.); HL097023 (G.H.M) HL 78806 (J. L.); HL087595 (Z. L.); HL088740 (G. B. A.); HL086614 (S.M.H); HL094660 (D.J); GM064619 and HL100474 (H.R.W.); a Robert Wood Johnson/Harold Amos Medical Faculty Development grant (G. B. A.), a Dominic Visconsi Scholar Award (S.M.H, G.B.A.), American Heart Association Grants 0725297B (D.K); 09POST2060203 (N.S.), Sankyo Foundation of Life Science grants (Y.T.), and a Kanae Foundation for the Promotion of Medical Science grant (Y.T. and D. K.). The authors declare no competing conflict-of financial interests.

## REFERENCES

- Ahmad N, Lingrel JB. Kruppel-like factor 2 transcriptional regulation involves heterogeneous nuclear ribonucleoproteins and acetyltransferases. *Biochemistry*. 2005; 44:6276–6285. [PubMed: 15835916]
- Aird WC. The role of the endothelium in severe sepsis and multiple organ dysfunction syndrome. *Blood*. 2003; 101:3765–3777. [PubMed: 12543869]
- Anderson KP, Kern CB, Crable SC, Lingrel JB. Isolation of a gene encoding a functional zinc finger protein homologous to erythroid Kruppel-like factor: identification of a new multigene family. *Mol Cell Biol*. 1995; 15:5957–5965. [PubMed: 7565748]
- Atkins GB, Jain MK. Role of Kruppel-like transcription factors in endothelial biology. *Circ Res*. 2007; 100:1686–1695. [PubMed: 17585076]
- Atkins GB, Wang Y, Mahabeleshwar GH, Shi H, Gao H, Kawanami D, Natesan V, Lin Z, Simon DI, Jain MK. Hemizygous deficiency of Kruppel-like factor 2 augments experimental atherosclerosis. *Circ Res*. 2008; 103:690–693. [PubMed: 18757824]
- Barish GD, Atkins AR, Downes M, Olson P, Chong LW, Nelson M, Zou Y, Hwang H, Kang H, Curtiss L, et al. PPARdelta regulates multiple proinflammatory pathways to suppress atherosclerosis. *Proc Natl Acad Sci U S A*. 2008; 105:4271–4276. [PubMed: 18337509]
- Bayele HK, Peyssonnaud C, Giatromanolaki A, Arrais-Silva WW, Mohamed HS, Collins H, Giorgio S, Koukourakis M, Johnson RS, Blackwell JM, et al. HIF-1 regulates heritable variation and allele expression phenotypes of the macrophage immune response gene SLC11A1 from a Z-DNA forming microsatellite. *Blood*. 2007; 110:3039–3048. [PubMed: 17606764]
- Bista P, Mele DA, Baez DV, Huber BT. Lymphocyte quiescence factor Dpp2 is transcriptionally activated by KLF2 and TOB1. *Mol Immunol*. 2008; 45:3618–3623. [PubMed: 18555530]
- Blouin CC, Page EL, Soucy GM, Richard DE. Hypoxic gene activation by lipopolysaccharide in macrophages: implication of hypoxia-inducible factor 1alpha. *Blood*. 2004; 103:1124–1130. [PubMed: 14525767]
- Buckley AF, Kuo CT, Leiden JM. Transcription factor LKLF is sufficient to program T cell quiescence via a c-Myc--dependent pathway. *Nat Immunol*. 2001; 2:698–704. [PubMed: 11477405]
- Buras JA, Holzmann B, Sitkovsky M. Animal models of sepsis: setting the stage. *Nat Rev Drug Discov*. 2005; 4:854–865. [PubMed: 16224456]
- Cao Z, Sun X, Icli B, Wara AK, Feinberg MW. Role of Kruppel-like factors in leukocyte development, function, and disease. *Blood*. 2010; 116:4404–4414. [PubMed: 20616217]
- Cramer T, Yamanishi Y, Clausen BE, Forster I, Pawlinski R, Mackman N, Haase VH, Jaenisch R, Corr M, Nizet V, et al. HIF-1alpha is essential for myeloid cell-mediated inflammation. *Cell*. 2003; 112:645–657. [PubMed: 12628185]
- Culver C, Sundqvist A, Mudie S, Melvin A, Xirodimas D, Rocha S. Mechanism of hypoxia-induced NF-kappaB. *Mol Cell Biol*. 2010; 30:4901–4921. [PubMed: 20696840]

- Das H, Kumar A, Lin Z, Patino WD, Hwang PM, Feinberg MW, Majumder PK, Jain MK. Kruppel-like factor 2 (KLF2) regulates proinflammatory activation of monocytes. *Proc Natl Acad Sci U S A*. 2006; 103:6653–6658. [PubMed: 16617118]
- Dekker RJ, Boon RA, Rondaij MG, Kragt A, Volger OL, Elderkamp YW, Meijers JC, Voorberg J, Pannekoek H, Horrevorts AJ. KLF2 provokes a gene expression pattern that establishes functional quiescent differentiation of the endothelium. *Blood*. 2006; 107:4354–4363. [PubMed: 16455954]
- Gill SE, Huizar I, Bench EM, Sussman SW, Wang Y, Khokha R, Parks WC. Tissue inhibitor of metalloproteinases 3 regulates resolution of inflammation following acute lung injury. *Am J Pathol*. 2010; 176:64–73. [PubMed: 20008147]
- Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. *Immunity*. 2010; 32:593–604. [PubMed: 20510870]
- Kawanami D, Mahabeleshwar GH, Lin Z, Atkins GB, Hamik A, Haldar SM, Maemura K, Lamanna JC, Jain MK. Kruppel-like factor 2 inhibits hypoxia-inducible factor 1 $\alpha$  expression and function in the endothelium. *J Biol Chem*. 2009; 284:20522–20530. [PubMed: 19491109]
- Kellam P, Weiss RA. Infectogenomics: insights from the host genome into infectious diseases. *Cell*. 2006; 124:695–697. [PubMed: 16497580]
- Kolls JK, McCray PB Jr, Chan YR. Cytokine-mediated regulation of antimicrobial proteins. *Nat Rev Immunol*. 2008; 8:829–835. [PubMed: 18949018]
- Kong D, Park EJ, Stephen AG, Calvani M, Cardellina JH, Monks A, Fisher RJ, Shoemaker RH, Melillo G. Echinomycin, a small-molecule inhibitor of hypoxia-inducible factor-1 DNA-binding activity. *Cancer Res*. 2005; 65:9047–9055. [PubMed: 16204079]
- Krauss S, Brand MD, Buttgeriet F. Signaling takes a breath--new quantitative perspectives on bioenergetics and signal transduction. *Immunity*. 2001; 15:497–502. [PubMed: 11672532]
- Kumar A, Lin Z, SenBanerjee S, Jain MK. Tumor necrosis factor  $\alpha$ -mediated reduction of KLF2 is due to inhibition of MEF2 by NF- $\kappa$ B and histone deacetylases. *Mol Cell Biol*. 2005; 25:5893–5903. [PubMed: 15988006]
- Liappis AP, Kan VL, Rochester CG, Simon GL. The effect of statins on mortality in patients with bacteremia. *Clin Infect Dis*. 2001; 33:1352–1357. [PubMed: 11565076]
- Lin Z, Hamik A, Jain R, Kumar A, Jain MK. Kruppel-like factor 2 inhibits protease activated receptor-1 expression and thrombin-mediated endothelial activation. *Arterioscler Thromb Vasc Biol*. 2006; 26:1185–1189. [PubMed: 16514085]
- Lin Z, Kumar A, SenBanerjee S, Staniszewski K, Parmar K, Vaughan DE, Gimbrone MA Jr, Balasubramanian V, Garcia-Cardena G, Jain MK. Kruppel-like factor 2 (KLF2) regulates endothelial thrombotic function. *Circ Res*. 2005; 96:e48–e57. [PubMed: 15718498]
- Lin Z, Natesan V, Shi H, Dong F, Kawanami D, Mahabeleshwar GH, Atkins GB, Nayak L, Cui Y, Finigan JH, Jain MK. Kruppel-like factor 2 regulates endothelial barrier function. *Arterioscler Thromb Vasc Biol*. 2010; 30:1952–1959. [PubMed: 20651277]
- Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. *Annu Rev Immunol*. 2009; 27:451–483. [PubMed: 19105661]
- Munford RS. Severe sepsis and septic shock: the role of gram-negative bacteremia. *Annu Rev Pathol*. 2006; 1:467–496. [PubMed: 18039123]
- Nair MG, Du Y, Perrigoue JG, Zaph C, Taylor JJ, Goldschmidt M, Swain GP, Yancopoulos GD, Valenzuela DM, Murphy A, et al. Alternatively activated macrophage-derived RELM- $\alpha$  is a negative regulator of type 2 inflammation in the lung. *J Exp Med*. 2009; 206:937–952. [PubMed: 19349464]
- Nizet V, Johnson RS. Interdependence of hypoxic and innate immune responses. *Nat Rev Immunol*. 2009; 9:609–617. [PubMed: 19704417]
- Peyssonnaud C, Cejudo-Martin P, Doedens A, Zinkernagel AS, Johnson RS, Nizet V. Cutting edge: Essential role of hypoxia inducible factor-1 $\alpha$  in development of lipopolysaccharide-induced sepsis. *J Immunol*. 2007; 178:7516–7519. [PubMed: 17548584]
- Peyssonnaud C, Datta V, Cramer T, Doedens A, Theodorakis EA, Gallo RL, Hurtado-Ziola N, Nizet V, Johnson RS. HIF-1 $\alpha$  expression regulates the bactericidal capacity of phagocytes. *J Clin Invest*. 2005; 115:1806–1815. [PubMed: 16007254]

- Rittirsch D, Huber-Lang MS, Flierl MA, Ward PA. Immunodesign of experimental sepsis by cecal ligation and puncture. *Nat Protoc.* 2009; 4:31–36. [PubMed: 19131954]
- Rius J, Guma M, Schachtrup C, Akassoglou K, Zinkernagel AS, Nizet V, Johnson RS, Haddad GG, Karin M. NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1alpha. *Nature.* 2008; 453:807–811. [PubMed: 18432192]
- Russell JA. Management of sepsis. *N Engl J Med.* 2006; 355:1699–1713. [PubMed: 17050894]
- Schouten M, Wiersinga WJ, Levi M, van der Poll T. Inflammation, endothelium, and coagulation in sepsis. *J Leukoc Biol.* 2008; 83:536–545. [PubMed: 18032692]
- SenBanerjee S, Lin Z, Atkins GB, Greif DM, Rao RM, Kumar A, Feinberg MW, Chen Z, Simon DI, Luscinskas FW, et al. KLF2 Is a novel transcriptional regulator of endothelial proinflammatory activation. *J Exp Med.* 2004; 199:1305–1315. [PubMed: 15136591]
- Serbina NV, Jia T, Hohl TM, Pamer EG. Monocyte-mediated defense against microbial pathogens. *Annu Rev Immunol.* 2008; 26:421–452. [PubMed: 18303997]
- Serbina NV, Pamer EG. Coordinating innate immune cells to optimize microbial killing. *Immunity.* 2008; 29:672–674. [PubMed: 19006691]
- Walmsley SR, Print C, Farahi N, Peyssonnaud C, Johnson RS, Cramer T, Sobolewski A, Condliffe AM, Cowburn AS, Johnson N, Chilvers ER. Hypoxia-induced neutrophil survival is mediated by HIF-1alpha-dependent NF-kappaB activity. *J Exp Med.* 2005; 201:105–115. [PubMed: 15630139]
- Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet.* 2009; 10:57–63. [PubMed: 19015660]
- Weinreich MA, Takada K, Skon C, Reiner SL, Jameson SC, Hogquist KA. KLF2 transcription-factor deficiency in T cells results in unrestrained cytokine production and upregulation of bystander chemokine receptors. *Immunity.* 2009; 31:122–130. [PubMed: 19592277]
- Wong HR, Cvijanovich N, Lin R, Allen GL, Thomas NJ, Willson DF, Freishtat RJ, Anas N, Meyer K, Checchia PA, et al. Identification of pediatric septic shock subclasses based on genome-wide expression profiling. *BMC Med.* 2009; 7:34. [PubMed: 19624809]



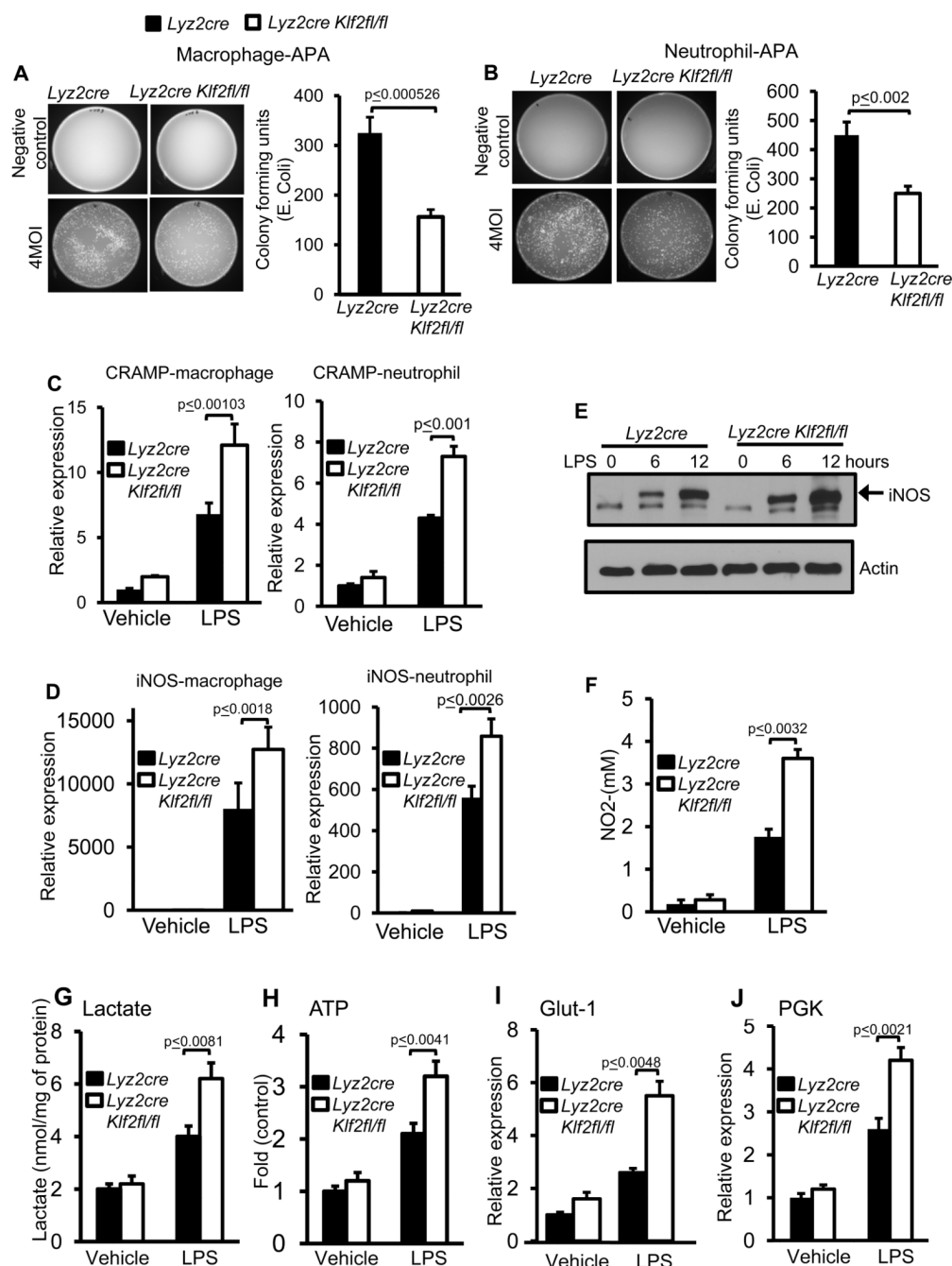
(A). *Lyz2cre* and *Lyz2creKlf2fl/fl* mice plasma were obtained by retro-orbital bleeding and inflammatory cytokines were analyzed using MILLIPLEX MAP mouse cytokine-chemokine panel from Millipore.

(B) Age and sex matched *Lyz2<sup>cre</sup>* and *Lyz2<sup>cre</sup>Klf2<sup>fl/fl</sup>* mice were subjected to CLP. These mice were observed for survival for 5 days.

(C and D) Age and sex *Lyz2<sup>cre</sup>* and *Lyz2<sup>cre</sup>Klf2<sup>fl/fl</sup>* mice were subjected to CLP and their average systolic blood pressure and core body temperature measured following 24 hours of CLP.

(E–H) Blood, lung, liver and kidney were collected from *Lyz2cre* and *Lyz2creKlf2fl/fl* mice following 12 hours of sham or CLP procedure. Serial dilutions of these tissue homogenates were plated on tryptic soya agar plates and enumerations of bacterial colony forming units were indicated.

(I–L) Age and sex matched *Lyz2cre* and *Lyz2creKlf2fl/fl* mice were subjected to sham or CLP procedure. Plasma of these mice was obtained by retro-orbital bleeding and inflammatory cytokines were analyzed using MILLIPLEX MAP mouse cytokine and chemokine panel from Millipore.



**Figure 2. KLF2 Negatively Regulates Bactericidal Activity of Macrophages**

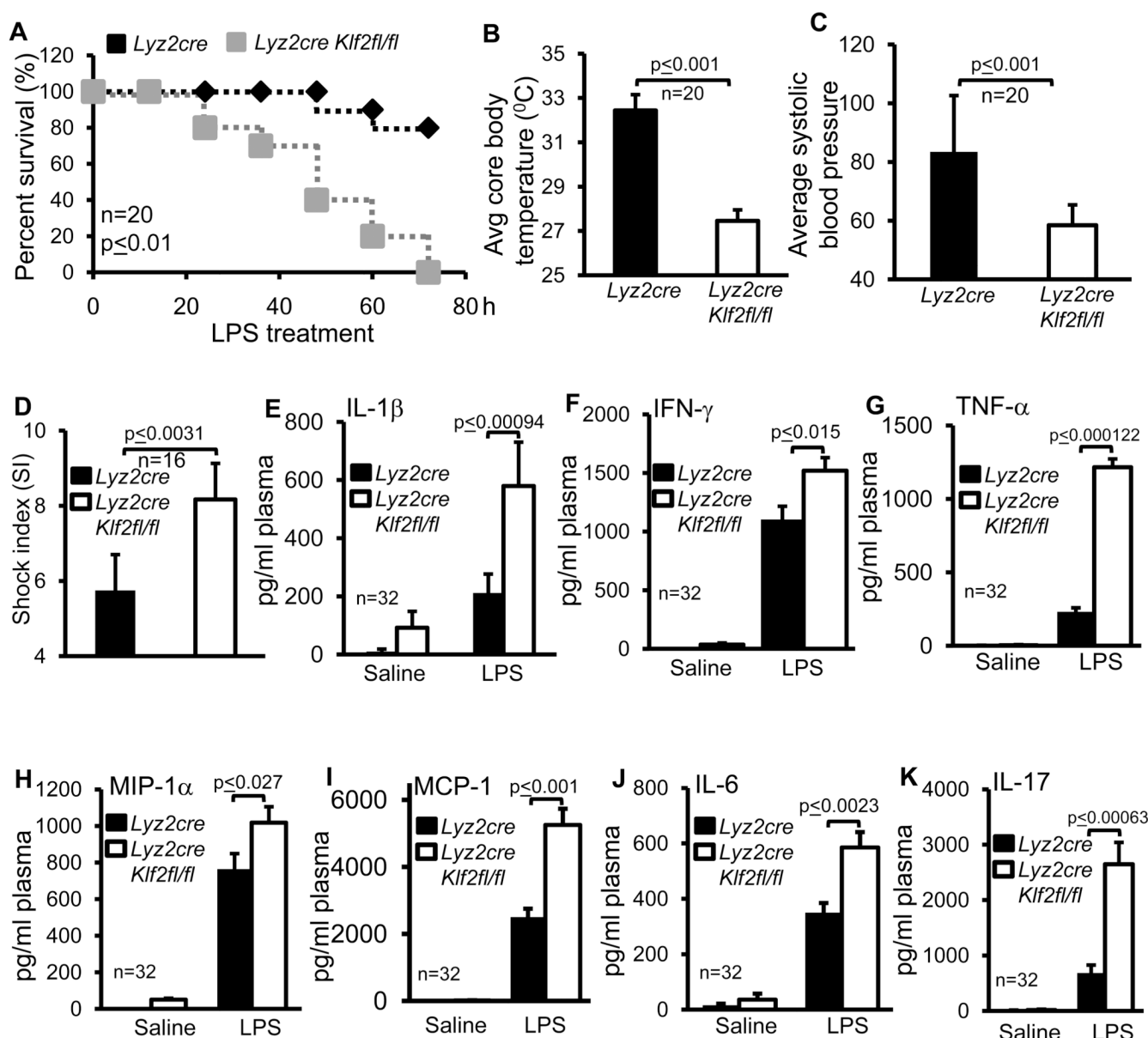
(A and B) Peritoneal macrophages or neutrophils from *Lyz2cre* and *Lyz2creKlf2fl/fl* mice were inoculated with *E. Coli* at a MOI of 4 and intracellular bacterial killing analyzed by antibiotic protection assay as described in “Methods”.

(C and D) Primary peritoneal macrophages or neutrophils from *Lyz2cre* and *Lyz2creKlf2fl/fl* mice were stimulated with 100 ng/ml LPS for 12 hr. CRAMP and iNOS mRNA expression was analyzed by qPCR and normalized to 36B4.

(E and F) Peritoneal macrophages from *Lyz2cre* and *Lyz2creKlf2fl/fl* mice were induced with 100 ng/ml LPS for 0–12 hours. Cell lysates were analyzed for expression of iNOS by immunoblot. In a parallel experiment, cell culture supernatants following 20 hr of 100ng/ml

of LPS treatment were analyzed for nitrite concentration by Griess assay as an index of iNOS activity.

(G and H) *Lyz2cre* and *Lyz2creKlf2fl/fl* mice peritoneal macrophages were stimulated with 100ng/ml LPS. Lactate concentration from these cell culture supernatants and intracellular ATP concentration from cell lysates were quantified and normalized to total protein content. (I and J) *Lyz2cre* and *Lyz2creKlf2fl/fl* mice were stimulated with 100 ng/ml LPS. Total RNA was isolated and mRNA expression of Glut-1 and PGK was analyzed by qPCR.

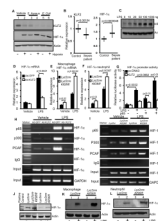


**Figure 3. Myeloid KLF2 is Protective Against Endotoxemic Shock**

(A) Age and sex matched *Lyz2cre* and *Lyz2creKlf2fl/fl* mice were challenged with LPS (21mg/kg). These mice were observed for 72hr for survival.

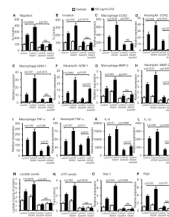
(B–D) Age and sex matched *Lyz2cre* and *Lyz2creKlf2fl/fl* mice were challenged with LPS (21mg/kg) and monitored for changes in core body temperature, systolic blood pressure and shock index (SI=Heart Rate/Systolic Blood Pressure).

(E–J) Age and sex matched *Lyz2cre* and *Lyz2creKlf2fl/fl* mice were challenged with LPS (21mg/kg). Plasma was obtained four hours after LPS or saline administration by retro-orbital bleeding. Inflammatory cytokines for the indicated factors were analyzed using MILLIPLEX MAP mouse cytokine and chemokine panel from Millipore.



#### Figure 4. KLF2 Inhibits LPS Induced NFκB Mediated HIF-1α Expression

- (A) Wild-type primary peritoneal macrophages were exposed to heat inactivated *S. Aureus* or *E. Coli* extracts in normoxic or hypoxic condition. Cell lysates were analyzed for KLF2 and HIF-1α protein expression by immunoblotting.
- (B) Whole blood-derived RNA was obtained from control and patients with septic shock and subjected to microarray analysis. Relative expression of KLF2 and HIF-1α are indicated.
- (C) Wild-type mice peritoneal macrophages were stimulated with increasing dose of LPS for 8h. Cell lysates were subjected to immunoblot using anti-HIF-1α antibody.
- (D–F) RAW264.7 cells infected with Ad-GFP and Ad-KLF2 or primary peritoneal macrophages and neutrophils from *Lyz2cre* and *Lyz2creKlf2fl/fl* mice were stimulated with 100 ng/ml LPS. Total RNA was isolated and HIF-1α mRNA expression was analyzed by qPCR.
- (G) RAW264.7 cells transfected with the HIF-1α promoter luciferase construct were co-transfected with KLF2 or NFκB (p65) plasmid. These cells were stimulated with LPS and cell lysates were analyzed for luciferase activity.
- (H and I) RAW264.7 cells infected with Ad-GFP or Ad-KLF2 or primary peritoneal macrophages from *Lyz2cre* and *Lyz2creKlf2fl/fl* mice were stimulated with LPS for 1hr. Chromatin immunoprecipitation was performed using the indicated antibody on the HIF-1α promoter containing the NFκB binding site at –197/–188 base pairs.
- (J) Basal expression of HIF-1α in *Lyz2cre* and *Lyz2creKlf2fl/fl* mice peritoneal macrophages were analyzed by immunoblot.
- (K and L) Primary peritoneal macrophages and neutrophils from *Lyz2cre* and *Lyz2creKlf2fl/fl* mice were stimulated with 100 ng/ml LPS. Cell lysates were analyzed for HIF-1α protein expression by immunoblot.



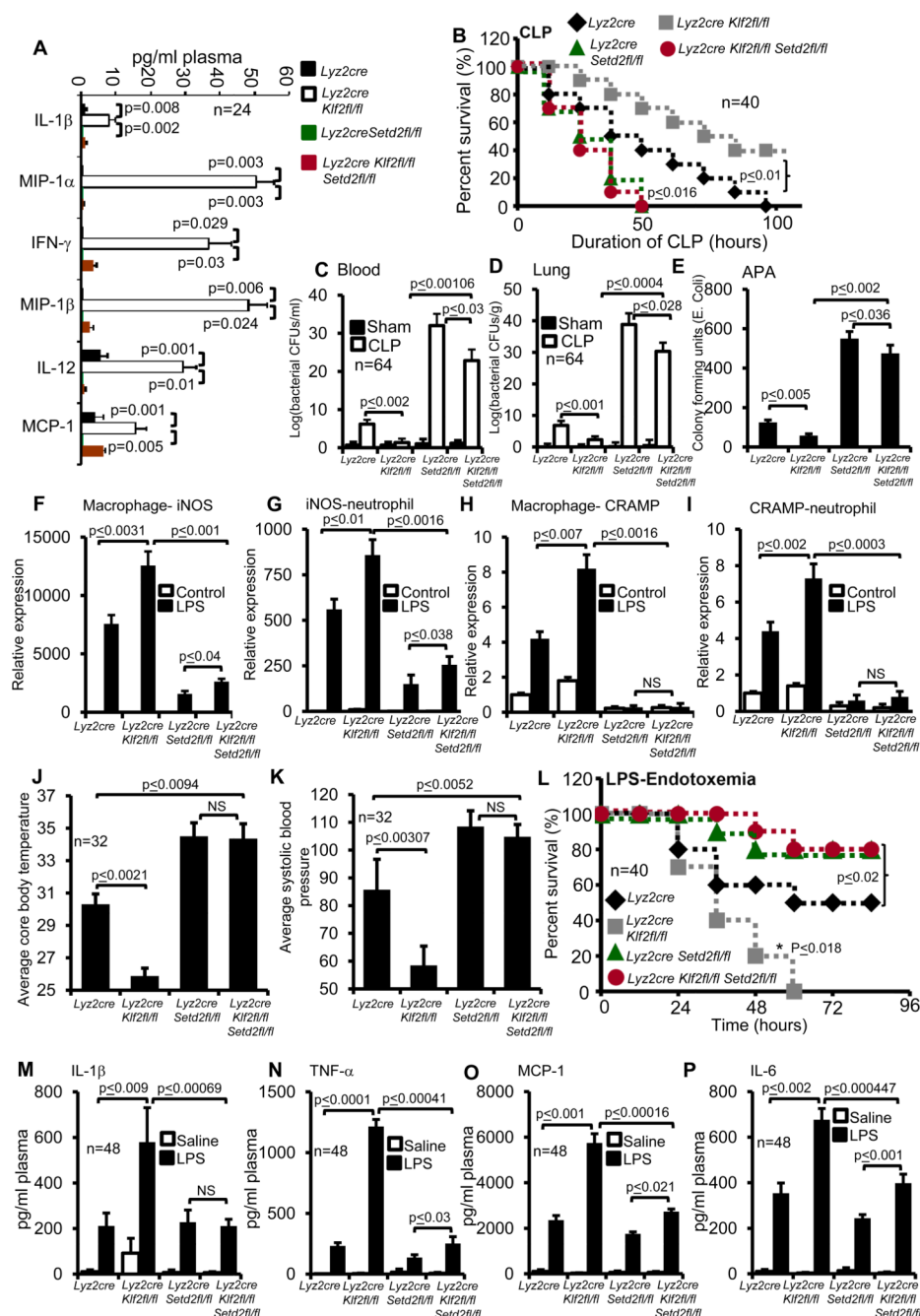
**Figure 5. Impaired Cellular Motility and Metabolism in KLF2 Deficient Macrophages are HIF-1 $\alpha$  Dependent**

(A and B) Primary peritoneal macrophages from *Lyz2cre*, *Lyz2creKlf2fl/fl*, *Lyz2creSetd2fl/fl* and *Lyz2creKlf2fl/flSetd2fl/fl* mice were stimulated with 100ng/ml LPS and added to the upper chamber of a migration or invasion tissue culture insert. Cells were allowed to migrate for 18hr and stained with Giemsa. The number of control, unstimulated cells migrated or invaded across the membrane was assigned as 100% and fold changes over this are indicated.

(C–L) Primary peritoneal macrophages or neutrophils from *Lyz2cre*, *Lyz2creKlf2fl/fl*, *Lyz2creSetd2fl/fl* and *Lyz2creKlf2fl/flSetd2fl/fl* mice were stimulated with 100 ng/ml LPS. Total RNA was isolated and indicated target genes were analyzed by qPCR and normalized to 36B4. Expression of indicated genes in untreated control peritoneal macrophages was set as one.

(M and N) Peritoneal macrophages from *Lyz2cre*, *Lyz2creKlf2fl/fl*, *Lyz2creSetd2fl/fl* and *Lyz2creKlf2fl/flSetd2fl/fl* mice were stimulated with 100ng/ml LPS. Lactate concentration from these cell culture supernatant and intracellular ATP concentration from cell lysates were quantified and normalized to total protein content.

(O and P) Peritoneal macrophages from *Lyz2cre*, *Lyz2creKlf2fl/fl*, *Lyz2creSetd2fl/fl* and *Lyz2creKlf2fl/flSetd2fl/fl* mice were stimulated with 100 ng/ml LPS. Total RNA was isolated, Glut-1 and PGK mRNA expression was analyzed by qPCR and normalized to 36B4.



**Figure 6. Deficiency of HIF-1 $\alpha$  Rescues Polymicrobial Infection and Endotoxic Shock Phenotypes in KLF2 Deficient Mice**

(A) *Lyz2cre*, *Lyz2creKlf2fl/fl*, *Lyz2creSetd2fl/fl* and *Lyz2creKlf2fl/flSetd2fl/fl* mice plasma was obtained by retro-orbital bleeding and inflammatory cytokines analyzed using MILLIPLEX MAP mouse cytokine and chemokine panel from Millipore.

(B) Survival of age and sex matched *Lyz2cre*, *Lyz2creKlf2fl/fl*, *Lyz2creSetd2fl/fl* and *Lyz2creKlf2fl/flSetd2fl/fl* mice subjected to CLP. These mice were observed for 5 days for their survival following CLP.

(C and D) Blood and lungs were collected from *Lyz2cre*, *Lyz2creKlf2fl/fl*, *Lyz2creSetd2fl/fl* and *Lyz2creKlf2fl/flSetd2fl/fl* mice following 12 hours of sham or CLP procedure. Serial

dilutions of blood and lung homogenate were plated on tryptic soya agar plates and enumerations of bacterial colony forming units were indicated.

(E) Peritoneal macrophages from *Lyz2cre*, *Lyz2creKlf2fl/fl*, *Lyz2creSetd2fl/fl* and *Lyz2creKlf2fl/flSetd2fl/fl* mice were inoculated with *E. Coli* at a MOI of 2 and intracellular bacterial killing analyzed by antibiotic protection assay.

(F–I) Primary peritoneal macrophages and neutrophils from *Lyz2cre*, *Lyz2creKlf2fl/fl*, *Lyz2creSetd2fl/fl* and *Lyz2creKlf2fl/flSetd2fl/fl* mice were stimulated with 100 ng/ml LPS. Total RNA was isolated and both iNOS and CRAMP mRNA expression analyzed by qPCR and normalized to 36B4.

(J–L) *Lyz2cre*, *Lyz2creKlf2fl/fl*, *Lyz2creSetd2fl/fl* and *Lyz2creKlf2fl/flSetd2fl/fl* mice were monitored for core body temperature, systolic blood pressure and survival after LPS challenge.

(M–P) Age and sex matched *Lyz2cre*, *Lyz2creKlf2fl/fl*, *Lyz2creSetd2fl/fl* and *Lyz2creKlf2fl/flSetd2fl/fl* mice were challenged with LPS (21mg/kg). Plasma of these mice were obtain four hours after LPS or saline administration by retro-orbital bleeding. Inflammatory cytokines for the indicated factors were analyzed using MILLIPLEX MAP mouse cytokine-chemokine panel from Millipore.