Received: 30 August 2019 | Revised: 26 November 2019 | Accepted: 23 December 2019

DOI: 10.1096/fj.201902221R

RESEARCH ARTICLE

THE FASEB JOURNAL

Kruppel-like factor 6 promotes macrophage inflammatory and hypoxia response

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Funding information
HHS | NIH | National Heart, Lung, and Blood Institute (NHLBI), Grant/Award Number: HL126626; HHS | NIH | National Heart, Lung, and Blood Institute (NHLBI), Grant/Award Number: HL141423; Crohn's and Colitis Foundation of America (CCFA), Grant/Award Number: 421904

Abstract

Macrophages are the professional phagocytes that protect the host from infection or injury. Tissue microenvironment at the site of injury and inflammation is characterized by low oxygen concentration and poor supply of nutrients. The responding macrophages have to advance against oxygen and nutrient gradients to reach the site of inflammation to perform host protection, and tissue repair functions. Thus, evolution has fashioned macrophages to orchestrate a coordinated inflammatory and hypoxic gene program to mount an effective immune response. Here, we discovered that Kruppel-like factor 6 (KLF6) governs macrophage functions by promoting inflammatory and hypoxic response gene programming. Our in vivo studies revealed that myeloid-KLF6-deficient mice were highly resistant to endotoxin-induced systemic inflammatory response syndrome symptomatology and mortality. Using complementary gain- and loss-of-function studies, we observed that KLF6 overexpression elevate and KLF6 deficiency attenuate inducible HIF1α expression in macrophages. Our integrated transcriptomics and gene set enrichment analysis studies uncovered that KLF6 deficiency attenuates broad inflammatory and glycolytic gene expression in macrophages. More importantly, overexpression of oxygen stable HIF1α reversed attenuated proinflammatory and glycolytic gene expression in KLF6-deficient macrophages. Collectively, our studies uncovered that KLF6 govern inflammatory and hypoxic response by regulating HIF1α expression in macrophage.

KEYWORDS

glycolysis, inflammation, KLF6, macrophage, metabolism

1 | INTRODUCTION

Macrophages are principal phagocytes that populate every organs and play an essential role in development, host defense, tissue homeostasis, and repair.[1] Studies over the decades have established that environmental milieu at the site of injury and/or inflammation is characterized by low oxygen concentration, and a diminished supply of nutrients.[2] Therefore, responding myeloid cells has to advance against oxygen and nutrient gradients to reach the site of inflammation to

Abbreviations: BMDMs, Bone marrow-derived macrophages; ChIP, Chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle medium; FBS, Fetal bovine serum; HIF1α, Hypoxia-inducible factor 1-alpha; HKCA, Heat-killed *Candida albicans*; KLF6, Kruppel like factor 6; LPS, Lipopolysaccharides; IFNγ, Interferon gamma; IL1β, Interleukin 1-beta; TNF, Tumor necrosis factor.

perform host protection, and tissue repair functions.[3] The effective macrophage activation, cellular migration, and host protective functions are highly energy-demanding biological processes. Macrophages typically derive most of the energy through glycolysis likely due to their operational microenvironment low in oxygen concentration.[4,5] Studies by Semenza et al discovered that genes encoding for glycolytic enzymes are transcriptionally regulated by hypoxia-inducible factor 1-alpha (HIF1α).[6] HIF1α is a heterodimeric helix-loop-helix transcription factor whose expression is tightly controlled at the mRNA and protein levels.[7] In addition to hypoxia, bacteria and bacterial endotoxins are the potent stimulators of HIF1α mRNA expression and protein accumulation even under normoxic conditions.[8,9] Interestingly, HIF1α-deficient macrophages exhibited a significant reduction in glycolysis, ATP production, cellular motility, and invasiveness.[10] Concordant with these observations, HIF1α deficiency dramatically attenuated bacterial endotoxin-induced pro-inflammatory gene expression in macrophages and neutrophils.[11] More importantly, myeloid-HIF1α-deficient mice are protected from LPS-induced clinical symptoms of sepsis such as cytokine storm, hypothermia, hypotension, and host mortality.[12] At the molecular level, a number of inflammatory signaling pathways converge on HIF1α linking innate immune cell inflammatory and hypoxic response in macrophages.[13,14] In this study, we identify Kruppel-like factor 6 (KLF6) as a novel regulator of HIF1α expression and functions in macrophages.

KLF6 is a member of zinc-finger family transcription factors that regulate critical cellular processes including development, differentiation, proliferation, and programmed cell death.[15] Based on the genomic organization, intron/exon structure, sequence differences, distinct DNA-binding domains, the position of nuclear localization signals and unique DNA-binding consensus sequences distinguish KLF6 from other members of the KLF family.[16,17] Alterations in KLF6 expression or function has been associated with the pathogenesis of numerous human ailments, including inflammatory bowel diseases, cancer, hepatic steatosis, and hepatic fibrosis.[15] A recent study by Syafruddin et al demonstrated that HIF2α directly elevates KLF6 expression to promote SREBF1/SREBF2-dependent lipid metabolism, growth and survival of clear cell renal cell carcinomas.[18] Studies from our laboratory have uncovered that KLF6 is predominantly expressed in cells of myeloid origin and KLF6 deficiency significantly attenuated inducible pro-inflammatory gene expression in macrophages.[19] At the molecular level, KLF6 robustly elevate pro-inflammatory gene expression by promoting NFκB functions as well as repressing critical anti-inflammatory genes such as PPARγ, BCL6, and miR-223 expression in macrophages.[19-21] More importantly, myeloid-KLF6-deficient mice are protected from chronic inflammatory disorders such as cutaneous inflammation, experimental models of colitis, high-fat diet-induced obesity, insulin resistance, and glucose intolerance.[19-22] In this context, whether myeloid-KLF6 regulate inflammatory and metabolic gene program has not been investigated. In this study, we provide the evidence that KLF6 promotes broad innate immune cell inflammatory and hypoxic response gene programming by directly elevating HIF1α expression in macrophages.

2 | MATERIALS AND METHODS

2.1 | Cell culture

RAW264.7 were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 10 μg/mL streptomycin, and 2 mM glutamine in a humidified incubator (5% CO_2 and 37°C). Human monocytes were isolated from unfractionated peripheral blood mononuclear cells (PBMC) by negative selection using magnetic beads and then were cultured with recombinant human M-CSF ex vivo for 7 days to generate primary macrophages. All the studies involving human samples were approved by the Case Western Reserve University Institutional Review Board. To evaluate inflammatory gene expression under in vivo condition, $Lyz2^{cre}$ and $Klf6^{fl/fl}$:$Lyz2^{cre}$ mice were intraperitoneally injected with 1 mL of thioglycollate with or without LPS (10 mg/kg body weight). These mice were euthanized on day 3 and the macrophages were purified from peritoneal lavage for further analyses. Mice bone marrow-derived macrophages (BMDMs) were generated by ex vivo differentiation of bone marrow cells. Briefly, bone marrow cells from 8-week-old wild-type, $Lyz2^{cre}$, and $Klf6^{fl/fl}$:$Lyz2^{cre}$ mice were harvested from the femur and tibia. These bone marrow cells were cultured in DMEM supplemented with recombinant mouse M-CSF for 7 days. These BMDMs were collected and utilized for the indicated experiments. For hypoxia treatment, the cell culture plates were incubated in a modular incubator chamber that was subsequently infused with a mixture of 1% O_2, 5% CO_2, and 94% N_2 gases and placed at 37°C for the indicated period.

2.2 | Generation of myeloid-specific KLF6-deficient mice and endotoxic shock studies

All animal procedures were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University and conformed to guidelines established by the American Association for Accreditation of Laboratory Animal Care. All mice were bred and maintained under pathogen-free conditions, fed standard laboratory chow (Harlan Teklad, Indianapolis, IN), and kept on a 12-hour light/dark cycle. Myeloid KLF6-specific null mice were generated as

described previously.[19] Briefly, *Klf6* floxed (*KLF6*$^{fl/fl}$) mice were crossed with *Lyz2*^{cre} mice to generate a mouse line harboring the *Klf6* floxed and *Lyz2*^{cre} alleles. These mice were further cross-bred to generate male and female offspring expressing two *Lyz2*^{cre} and *Klf6* floxed alleles. The mice with two *Klf6* floxed and *Lyz2*^{cre} alleles were used as the KLF6 myeloid-specific null group (C57BL/6 background). Mice with only two *Lyz2*^{cre} alleles were used as the control group.

The *Lyz2*^{cre} and *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice (8-10 weeks old) were challenged with an intraperitoneal injection of 5×10^6 HKCA. Serum cytokines levels were evaluated by a commercially available ELISA kit. Similarly, *Lyz2*^{cre} and *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice were intraperitoneally injected with 24 mg/kg body weight of LPS or saline solution. Mice were monitored for 4 days following LPS injection. Survival data were analyzed by the construction of Kaplan-Meier plots and the use of the log-rank test.

2.3 | RNA extraction, real-time quantitative PCR, and western blot

Total RNA was isolated from indicated samples using the High Pure RNA Isolation Kit. One microgram of total RNA was reverse transcribed using M-MuLV reverse transcriptase in the presence of random hexamers and oligo (dT) primers. Real-time quantitative PCR was performed using Universal SYBR Green PCR Master Mix or TaqMan Universal Master Mix on Applied Biosystems Step One Plus real-time PCR system in presence of gene-specific primers.

Indicated primary cells and cell lines were lysed in ice-cold RIPA buffer containing protease and phosphatase inhibitors. Protein concentration was measured by the BCA protein assay. An equal amount of protein samples was electrophoresed using 8% or 4%-15% Mini-PROTEAN TGXTM precast gels (Bio-Rad) and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk or 5% bovine serum albumin in TBS-T for 1 hour at room temperature. These blots were further incubated with primary antibodies diluted in 5% BSA in TBS-T. After overnight incubation, primary antibodies were removed by washing with TBS-T. These blots were incubated for 1 hour at room temperature in horseradish peroxidase-conjugated secondary antibody. Blots were visualized using enhanced chemiluminescence western blotting substrate. The primary antibodies were used at following dilutions. HIF1α (1:1000), KLF6 (1:2000), and β-actin (1:5000).

2.4 | Polar metabolite profiling by LC-MS

Targeted polar metabolite profiling was performed as described before.[23] Briefly, macrophages after indicated treatments were washed with filter-sterilized 0.9% NaCl and incubated with extraction buffer (80% methanol, 20% H_2O plus isotopically labeled internal standards (MSK-A2-1.2, Cambridge Isotope Laboratories). Cells were harvested by scraping, were subjected to vortexing for 10 minutes at 4°C and the debris was pelleted by a 10 minutes spin at 18 000 *g*. The supernatant was then transferred to a new tube and dried under nitrogen. Dried polar samples were resuspended in 100-μL water and 2 μL was injected into a ZIC-pHILIC 150 × 2.1 mm (5 μm particle size) column (EMD Millipore). The analysis was conducted on a QExactive benchtop Orbitrap mass spectrometer equipped with an Ion Max source and a HESI II probe, which was coupled to a Dionex UltiMate 3000 UPLC system (Thermo Fisher Scientific). External mass calibration was performed using the standard calibration mixture every 7 days. Chromatographic separation was achieved using the following conditions: buffer A was 20 mM ammonium carbonate, 0.1% ammonium hydroxide; buffer B was acetonitrile. The column oven and autosampler tray were held at 25°C and 4°C, respectively. The chromatographic gradient was run at a flow rate of 0.150 mL minutes^{-1} as follows: 0-20 minutes: linear gradient from 80% to 20% B; 20-20.5 minutes: linear gradient from 20% to 80% B; 20.5-28 minutes: hold at 80% B. The mass spectrometer was operated in full-scan, polarity switching mode with the spray voltage set to 3.0 kV, the heated capillary held at 275°C, and the HESI probe held at 350°C. The sheath gas flow was set to 40 units, the auxiliary gas flow was set to 15 units, and the sweep gas flow was set to 1 unit. The data acquisition was performed over a range of 70-1000 m/z, with the resolution set at 70 000, the automatic gain control target at 10e6, and the maximum injection time at 20 ms Relative quantitation of polar metabolites was performed with XCalibur QuanBrowser 2.2 (Thermo Fisher Scientific) using a 5 p.p.m. mass tolerance and referencing an in-house library of chemical standards. Metabolite levels were normalized to the total protein amount for each condition.

2.5 | Transient transfection, luciferase assay, and ChIP analysis

RAW264.7 cells were transiently transfected with the indicated nucleotide with Lipofectamine transfection reagents according to the manufacturer's instructions. Luciferase reporter plasmids driven by HIF1α (HRE-Luc) were transfected alone or together with plasmids encoding *Klf6* or *siKlf6*. These cells were treated with 100 ng/mL 18 hours. Luciferase reporter activity was measured and normalized according to the manufacturer's instructions. Results are presented as relative luciferase activity over the control group. Chromatin immunoprecipitation (ChIP) analyses were performed using the EZ-Magna ChIP G kit (17-409, EMD Millipore, Billerica, MA, USA) according to the manufacturer's instruction. Briefly, wild-type mice BMDMs

were stimulated with 100 ng/mL LPS for 4 hours and chromatin immunoprecipitations were performed using an anti-KLF6 antibody. Primer pairs (FW: 5′-gggtgatctcaatgcggtag-3′ REV: 5′-cacacgcaaacacacacg-3′) flanking the KLF6-binding site on mouse *Hif1α* promoter region (−974 to −848) were utilized for amplification by real-time quantitative RT-PCR. Chromatin immunoprecipitation performed using isotype IgG was used as a negative control. DNA levels were first normalized to the internal control region in the first intron of the mouse *Actb* gene (FW: 5′-cgtattaggtccatcttgagagtac-3′, REV: 5′-gccattgaggcgtgatcgtagc-3′). Relative enrichment was calculated by dividing the normalized levels of ChIP DNA to that of input DNA at the corresponding locus.

2.6 | RNAseq analysis

Quality control of total RNA samples were assessed using Qubit (Invitrogen) for quantification and Agilent 2100 BioAnalyzer analysis to assess quality using a cutoff of RIN > 7.0 to select specimens for further analysis. cDNA library for RNAseq was generated from 150 ng of total RNA using the Illumina TruSeq Stranded Total RNA kit with Ribo Zero Gold for rRNA removal according to the manufacturer's protocol. The resulting purified mRNA was used as input for the Illumina TruSeq kit in which libraries are tagged with unique adapter indexes. Final libraries were validated on the Agilent 2100 BioAnalyzer, quantified via qPCR and pooled at equimolar ratios. Pooled libraries were diluted, denatured, and loaded onto the Illumina NextSeq 550 System using a high-output flowcell. STAR Aligner was used for mapping the sequencing reads to the mm10 mouse reference genome. The aligned reads were then analyzed with Cuffdiff to obtain gene-level expression data using the GENCODE gene annotation for mm10 and reported as fragments per kilobase per million reads mapped (FPKM). Differential expression analysis was also performed using the Cuffdiff package and significantly differentially expressed genes were defined using an adjusted P-value < .05 (FDR corrected). Gene expression tables for relevant pairwise comparisons were analyzed for gene set enrichment (GSEA)[24] using GenePattern (Broad Institute). We specifically utilized Hallmark pathways data datasets for current studies. A gene set was considered to be significantly enriched using an FWER cutoff <0.05. Heatmaps were generated using ClustVis.[25] The accession number for the sequencing data reported in this paper is GEO: GSE136476.

2.7 | Quantification and statistical analysis

All data, unless indicated are presented as the mean ± SD. The statistical significance of differences between two groups were analyzed by Student's *t*-test, one-way ANOVA, or two-way AVOVA with Bonferroni multiple comparison tests. Host survival data were analyzed by the construction of Kaplan-Meier plots and the use of the log-rank test. $P < .05$ was considered statistically significant.

2.8 | Supporting information

Supporting Information includes two tables.

3 | RESULTS

3.1 | KLF6 deficiency attenuates broad proinflammatory gene program

Our studies over the years have demonstrated that KLF6-deficient myeloid cells exhibit diminished pro-inflammatory activations and functions.[19-22] However, whether macrophage KLF6 deficiency alters the global proinflammatory gene program following inflammatory agent exposure has not been examined. To identify the broadly deregulated inflammatory gene targets and signaling pathways associated with KLF6 deficiency, we performed unbiased RNAseq analyses. Previous studies have demonstrated that heat-killed candida albicans (HKCA) activates a number of pattern recognition receptors including TLR2, TLR4, TLR6, dectin-1, dectin-2, mannose receptor, galectin-3, CD36, and complement receptor 3 in macrophages.[26-28] Therefore, we utilized HKCA to induce broad inflammatory signaling pathways and gene expression in macrophages. Accordingly, BMDMs from $Lyz2^{cre}$ and $Klf6^{fl/fl}$:$Lyz2^{cre}$ mice were stimulated with HKCA and total RNA samples were obtained to perform gene expression profiling studies. To define signaling pathways that were altered due to KLF6 deficiency, RNAseq data were subjected to Gene Set Enrichment Analysis (GSEA).[24] Remarkably, our analyses discovered that HKCA-induced gene expression profile from $Lyz2^{cre}$ mice BMDMs were positively enriched for broad inflammatory programs such as TNF signaling via NFκB, inflammatory response, IL6-induced JAK-STAT3 signaling, complement, hypoxia, and IFNγ response (Figure 1A-E). As anticipated, HKCA stimulation significantly induced a large number of NFκB-regulated pro-inflammatory gene targets in $Lyz2^{cre}$ mice BMDMs (Figure 1F). However, the induction of these pro-inflammatory gene targets (*Tnc*, *Cxcl3*, *Cd44*, *Cox2*, *Tnf*, *Cxcl1*, *Bmp2*, etc.) were significantly attenuated in $Klf6^{fl/fl}$:$Lyz2^{cre}$ mice BMDMs stimulated with HKCA (Figure 1F). Concordant with these observations, KLF6 deficiency significantly attenuated HKCA-induced inflammatory response (*Tlr1*, *Il1β*, *Il1α*, *Nlrp3*, *Cd14*, etc), IL6-induced JAK-STAT3-mediated gene expression (*Dock4*, *C3*, *Mmp13*, *F10*, etc.), and IFNγ response (*Ccl5*, *Vcam1*, *Icam1*, *Cfb*, etc.) in macrophages (Figure 1G-I). Collectively, our analyses revealed that KLF6

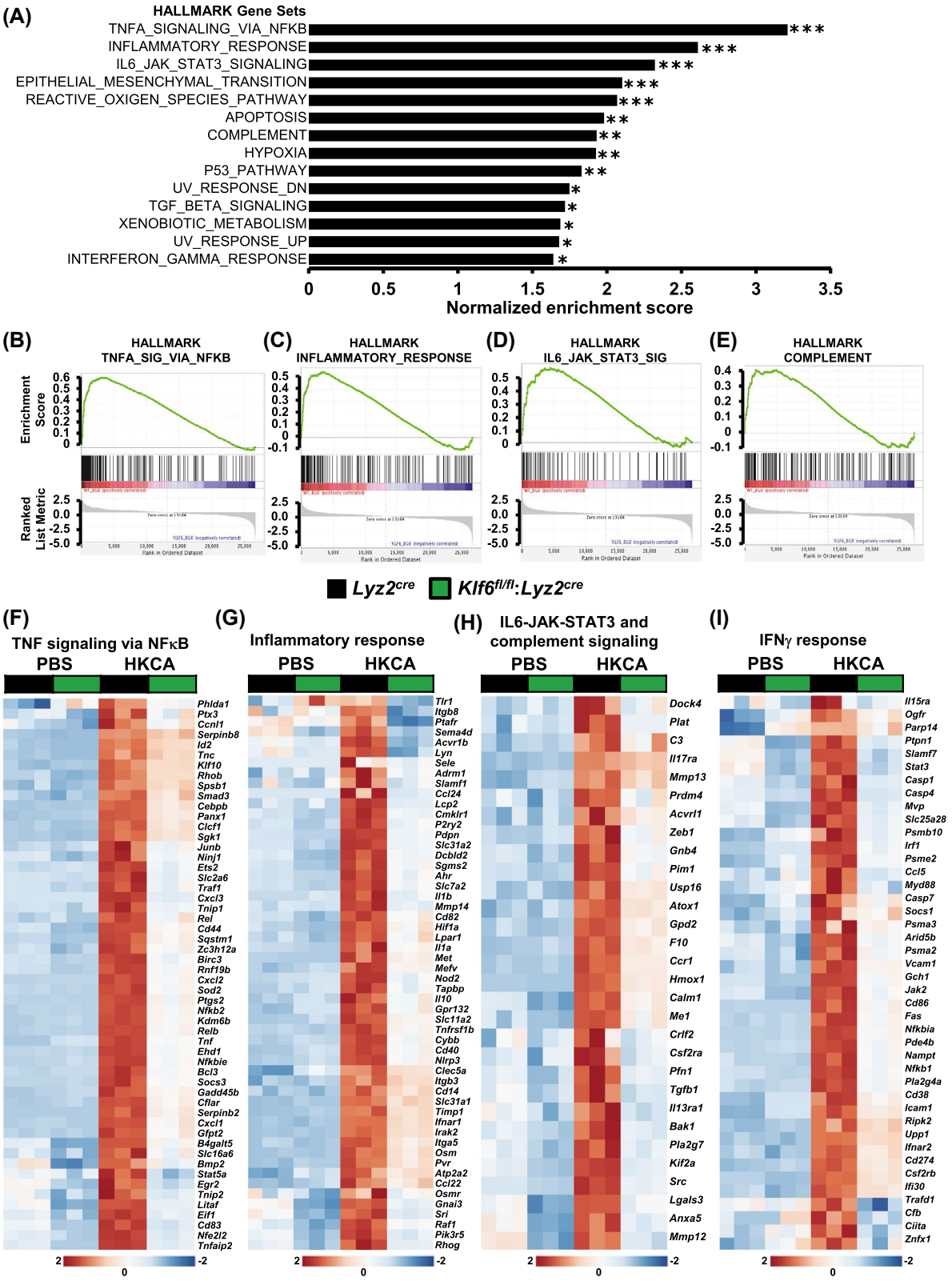

FIGURE 1 KLF6 deficiency attenuates a broad pro-inflammatory gene program. A, GSEA of RNAseq data that are altered in *Lyz2*^{cre} and *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice BMDMs following 5 h of HKCA exposure. FWER *P*-value less than .05 was considered significant. B-E, Enrichment plots of indicated gene set obtained by GSEA comparing *Lyz2*^{cre} and *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice BMDMs RNAseq data following HKCA treatment. F-I, Heatmap of genes involved in TNF signaling via NFκB (F), inflammatory response (G), IL6-JAK-STAT3 and complement signaling (H), and IFNγ response (I) that are altered in *Lyz2*^{cre} and *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice BMDMs following HKCA stimulation. FWER *P*-value *, $P < .05$; **, $P < .01$; ***, $P < .001$

deficiency broadly attenuated inflammatory signaling pathways and gene expression in macrophages. Previous studies have demonstrated that toll-like receptors activation elicit similar inflammatory responses in macrophages.[29] Therefore, we assessed whether KLF6 deficiency altered LPS-induced major pro-inflammatory gene expression in macrophages. As shown in Figure 2A, LPS exposure robustly elevated *Bmp2*, *Cfb*, *Adm*, *Icam1,* and *Vcam1* expression in $Lyz2^{cre}$ mice BMDMs. However, KLF6 deficiency significantly curtailed LPS-induced *Bmp2*, *Cfb*, *Adm*, *Icam1,* and *Vcam1* expression in $Klf6^{fl/fl}$:$Lyz2^{cre}$ mice BMDMs (Figure 2A). Next, we evaluated whether these observations were emulated in vivo. Accordingly, $Lyz2^{cre}$ and $Klf6^{fl/fl}$:$Lyz2^{cre}$ mice peritoneal macrophages were obtained following challenge with thioglycollate with or without LPS. As shown in Figure 2B, LPS challenge greatly elevated *Il1α*, *Il1β*, *Bmp2*, *Adm,* and *Icam1* expression in $Lyz2^{cre}$ primary peritoneal macrophages. Interestingly, KLF6 deficiency significantly attenuated LPS-induced *Il1α*, *Il1β*, *Bmp2*, *Adm,* and *Icam1* expression in $Klf6^{fl/fl}$:$Lyz2^{cre}$ mice peritoneal macrophages in vivo (Figure 2B). Next, we examined whether these observations were recapitulated in human macrophages. As shown in Figure 2C, LPS exposure strongly elevated *IL1α*, *IL1β*, *ADM*, *ICAM1,* and *IL12B* expression in human PBMC-derived macrophages. Interestingly, KLF6 deficiency significantly attenuated LPS-induced these pro-inflammatory (*IL1α*, *IL1β*, *ADM*, *ICAM1,* and *IL12B*) gene expression in human primary macrophages (Figure 2C). Taken together, our results demonstrate that

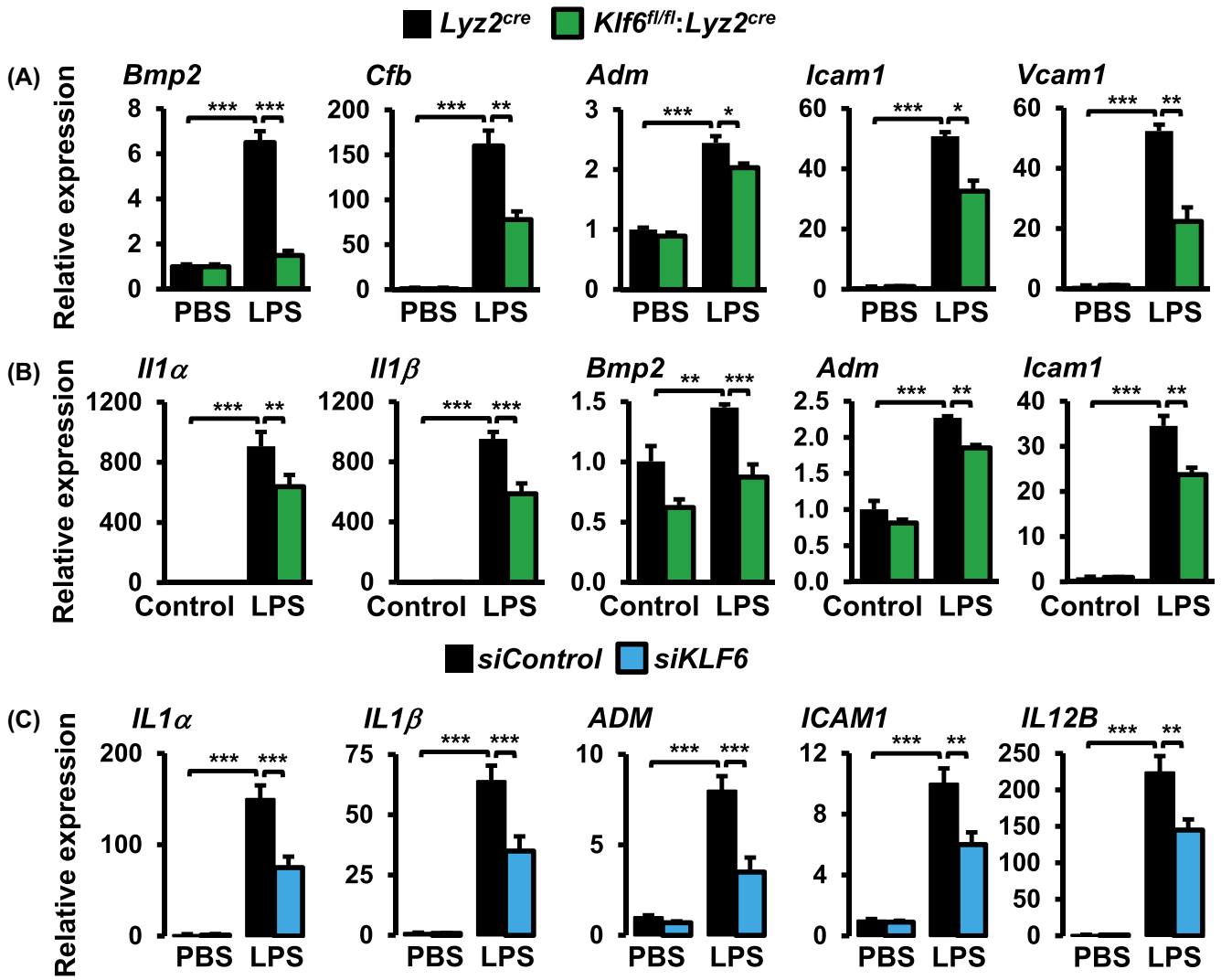

FIGURE 2 KLF6 deficiency curtails pro-inflammatory gene expression in macrophages. A, Total RNA from $Lyz2^{cre}$ and $Klf6^{fl/fl}$:$Lyz2^{cre}$ mice BMDMs treated with 100 ng/mL LPS were evaluated for expression of *Bmp2*, *Cfb*, *Adm*, *Icam1*, and *Vcam1* by RT-qPCR (n = 4). B, $Lyz2^{cre}$ and $Klf6^{fl/fl}$:$Lyz2^{cre}$ mice were intraperitoneally injected with thioglycollate with or without LPS. Purified macrophages from peritoneal lavage were evaluated for *Il1α*, *Il1β*, *Bmp2*, *Adm,* and *Icam1* mRNA expression by RT-qPCR analysis (n = 5). C, Human PBMC-derived macrophages were transfected with control or *siKLF6* siRNA. These cells were stimulated with LPS and expression of *IL1α*, *IL1β*, *ADM*, *ICAM1,* and *IL12B* analyzed by RT-qPCR (n = 4). All values are reported as mean ± SD. Data were analyzed by ANOVA followed by Bonferroni post-testing. *, $P < .05$; **, $P < .01$; ***, $P < .001$

KLF6 deficiency significantly attenuates broad pro-inflammatory gene expression in murine and human macrophages.

3.2 | Myeloid-KLF6 deficiency is protective against LPS-induced sepsis symptomatology and mortality

Our studies demonstrated that KLF6 deficiency significantly attenuates macrophage response to pro-inflammatory agents (Figures 1 and 2). Therefore, we hypothesized that myeloid-KLF6 is required for the establishment of systemic inflammation in vivo. To test this hypothesis, we utilized the lipopolysaccharide-induced systemic inflammatory response syndrome model. Accordingly, *Lyz2*^{cre} and *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice were challenged with LPS by intraperitoneal injection. As shown in Figure 3A, *Lyz2*^{cre} mice challenged to LPS experienced 100% mortality within 60 hours. Interestingly, *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice displayed only 20% mortality by the end of 96 hours and were significantly protected from LPS-induced sepsis mortality. As anticipated, *Lyz2*^{cre} mice exhibited significantly elevated cardinal features of sepsis, including hypothermia (Figure 3B) and hypotension (Figure 3C). Interestingly, these salient features of sepsis symptomatology were significantly abrogated in *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice (Figure 3B,C). More importantly, LPS challenge robustly

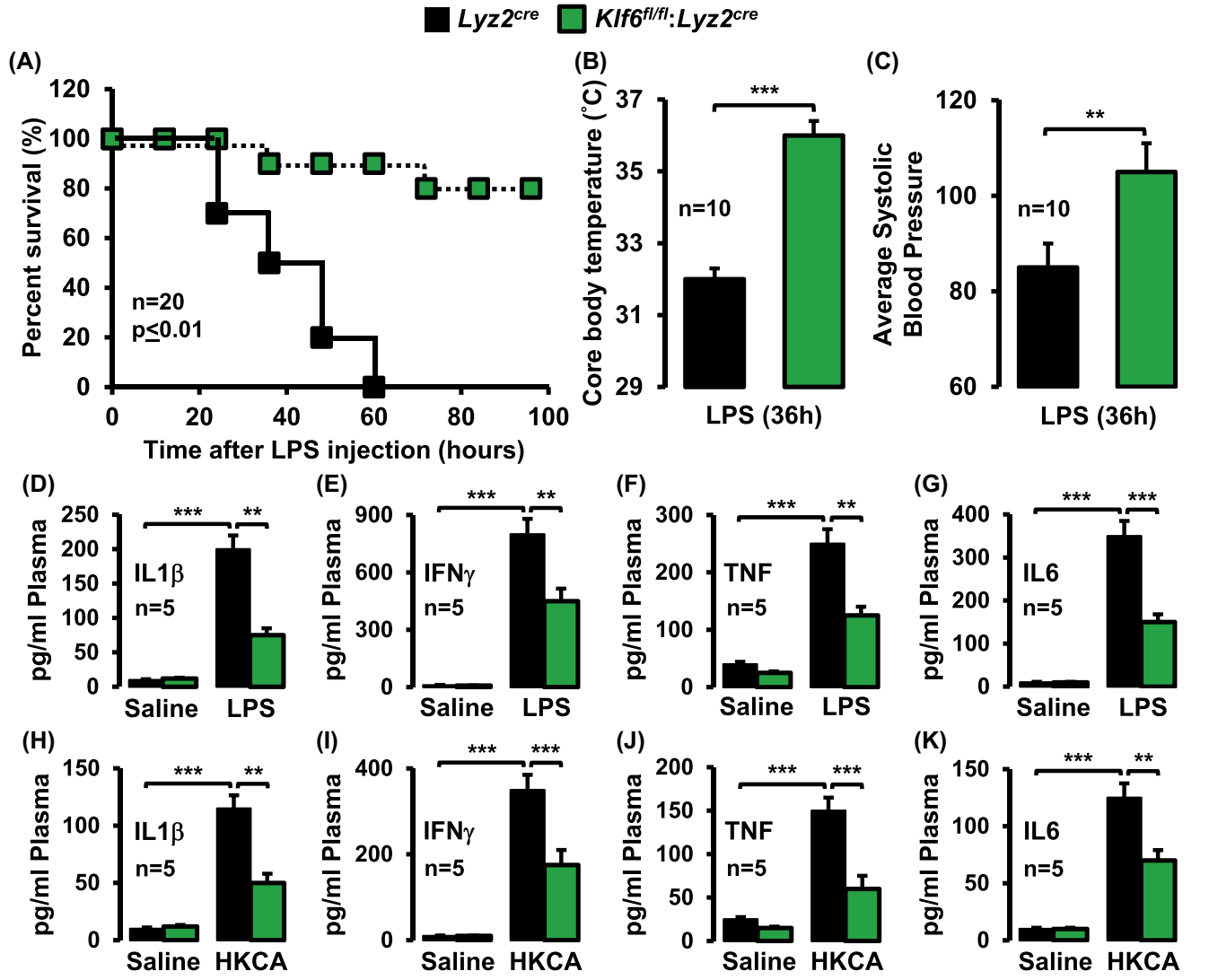

FIGURE 3 Myeloid-KLF6 deficiency is protective against LPS-induced sepsis symptomatology and mortality. A, Age- and sex-matched *Lyz2*^{cre} and *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice were subjected to LPS-induced sepsis. Host survival data were analyzed by the construction of Kaplan-Meier plots and the use of the log-rank test (n = 20). B and C, Age- and sex-matched *Lyz2*^{cre} and *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice were challenged with LPS and changes in core body temperature (B) and systolic blood pressure (C) were recorded (n = 10). D-G, Blood plasma obtained 5 h after LPS or saline administration were quantified for IL1β (D), IFNγ (E), TNF (F), and IL6 (G) by ELISA (n = 5). H-K, Age- and sex-matched *Lyz2*^{cre} and *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice were challenged with HKCA by intraperitoneal administration of HKCA or saline. The plasma level of IL1β (H), IFNγ (I), TNF (J), and IL6 (K) were quantified by ELISA (n = 5). All values are reported as mean ± SD. Data were analyzed by two-way ANOVA (B-G). *, $P < .05$; **, $P < .01$; ***, $P < .001$

and significantly elevated proinflammatory plasma cytokines such as IL1β, IFNγ, TNF, and IL6 levels in *Lyz2*^{cre} mice (Figure 3D-G). However, myeloid-KLF6 deficiency significantly attenuated the production of these pro-inflammatory plasma cytokines following the LPS challenge in vivo (Figure 3D-G). Next, we intended to recapitulate these observations utilizing HKCA. Surprisingly, the HKCA challenge did not elicit sepsis-like symptoms in *Lyz2*^{cre} or *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice. However, HKCA challenge significantly elevated proinflammatory plasma cytokines such as IL1β, IFNγ, TNF, and IL6 levels in *Lyz2*^{cre} mice (Figure 3H-K). However, myeloid-KLF6 deficiency significantly attenuated the production of these pro-inflammatory plasma cytokines following the HKCA challenge in vivo (Figure 3H-K). Collectively, our observations unveil that myeloid-KLF6 deficiency attenuates HKCA or LPS-induced pro-inflammatory plasma cytokines production as well as protective against LPS-induced sepsis symptomatology and mortality in vivo.

3.3 | KLF6 deficiency attenuates HIF1α expression in macrophages

Previous studies have established that HIF1α is a critical regulator of cellular response to hypoxia and macrophage-mediated inflammation.[9,10] Concordant with our in vivo observations (Figure 3), myeloid-HIF1α-deficient mice were protected from LPS-induced sepsis symptomatology and mortality.[12] Therefore, we critically reviewed our GSEA of RNAseq data for changes in hypoxia response pathways. In this direction, our analyses revealed that HKCA exposure significantly elevated hypoxia response gene expression in *Lyz2*^{cre} mice BMDMs (Figures 1A and 4A). However, induction of these hypoxia response genes were significantly attenuated in *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice BMDMs stimulated with HKCA (Figure 4B). Further, our RNAseq studies discovered that HKCA-induced *Hif1α* expression was significantly attenuated in KLF6-deficient macrophages (Figure 1G). Thus, we meticulously corroborated these observations at mRNA and protein levels. As shown in Figure 4C,D, HKCA exposure significantly elevated HIF1α mRNA and protein levels in *Lyz2*^{cre} mice BMDMs. However, HKCA-induced HIF1α mRNA and protein expression were significantly attenuated in *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice BMDMs (Figure 4C,D). Previous studies have shown that bacterial endotoxins such as LPS elevate HIF1α expression in macrophages [8,11] and myeloid-HIF1α deficiency significantly attenuated LPS-induced sepsis symptomatology, and mortality.[11,12] Therefore, we examined whether altered KLF6 levels modulated LPS-induced HIF1α mRNA and protein expression in macrophages. As shown in Figure 4E,F, LPS exposure significantly elevated *Hif1α* mRNA expression in *Lyz2*^{cre} mice BMDMs as well as in control vector-transfected RAW264.7 macrophage cell line. Compellingly, KLF6 deficiency attenuated (Figure 4E) and overexpression of KLF6 (Figure 4F) elevated LPS-induced *Hif1α* mRNA expression in macrophages. Next, we examined whether the altering KLF6 levels modulate HIF1α transcriptional activity in macrophages. Accordingly, RAW264.7 cells were cotransfected with HRE (hypoxia response element)-driven luciferase reporter plasmid in presence of *pCI-neo-Klf6* or *siKlf6*. These cells were stimulated with LPS and luciferase activities were recorded. As shown in Figure 4G,H, over-expression of *Klf6* enhanced and deficiency of *Klf6* diminished LPS-induced HIF1α luciferase reporter activity in macrophages. Subsequently, we assessed whether LPS exposure altered KLF6 enrichment on the *Hif1α* promoter. Compellingly, our chromatin immunoprecipitation (ChIP) analysis illustrates that LPS exposure significantly elevated KLF6 enrichment on *Hif1α* promoter (−974 to −848) in wild-type mice BMDMs (Figure 4I). Consistent with these observations, LPS or hypoxia-induced HIF1α protein expression was attenuated in KLF6-deficient BMDMs compared to *Lyz2*^{cre} mice BMDMs (Figure 4J,K). Next, we intend to evaluate whether these observations were replicated in vivo. Accordingly, primary peritoneal macrophages from *Lyz2*^{cre} and *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice were obtained following challenge with thioglycollate with or without LPS. Our analyses uncovered that LPS exposure robustly elevated HIF1α mRNA and protein expression in *Lyz2*^{cre} mice peritoneal macrophages in vivo (Figure 4L,M). Interestingly, LPS-induced HIF1α mRNA and protein expression were greatly diminished in *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice peritoneal macrophages in vivo (Figure 4L,M). Further, we assessed whether these observations were recapitulated in human macrophages. As shown in Figure 4N,O, LPS exposure robustly elevated HIF1α mRNA and protein expression in human PBMC-derived macrophages. However, KLF6 deficiency significantly curtailed LPS-induced HIF1α mRNA and protein expression in human primary macrophages (Figure 4N,O). Collectively, our studies revealed that KLF6 elevates inducible HIF1α expression in murine and human macrophages.

3.4 | KLF6 deficiency curtails LPS-induced glycolytic gene expression

Macrophages are known to utilize the glycolytic pathway as the main source of energy.[4,5] Previous studies have demonstrated that HIF1α as a critical positive regulator of key glycolytic enzymes.[6,10] Our studies thus far demonstrated that KLF6 deficiency attenuates HKCA or LPS-induced HIF1α mRNA and protein expression in macrophages (Figure 4). Our unbiased screening studies revealed that KLF6 deficiency significantly attenuated HKCA-induced central and peripheral glycolytic genes in macrophages (Figure 5B). Therefore, we examined whether KLF6 deficiency altered

LPS-induced glycolytic gene expression in macrophages. Accordingly, BMDMs from *Lyz2*^{cre} and *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice were stimulated with LPS and total RNA samples were evaluated for glycolytic gene expression by RT-qPCR. As shown in Figure 5C, LPS exposure significantly elevated major glycolytic genes (*Glut1*, *Hk2*, *Hk3*, *Gpi1*, *Pfkfb3*, *Pfkl*, *Aldoa*, *Aldoc*, *Tpi1*, *Gapdh*, *Pgk1*, *Pgam1*, *Eno1*, *Pkm*, *Ldha,* and *Mct4*) expression in *Lyz2*^{cre} mice BMDMs. Compellingly, KLF6 deficiency significantly abrogated these LPS-induced glycolytic genes expression in macrophages (Figure 5C). Next, we examined whether these observations were recapitulated in vivo. Accordingly, primary peritoneal macrophages from *Lyz2*^{cre} and *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice were obtained following challenge with thioglycollate with or without LPS. Our

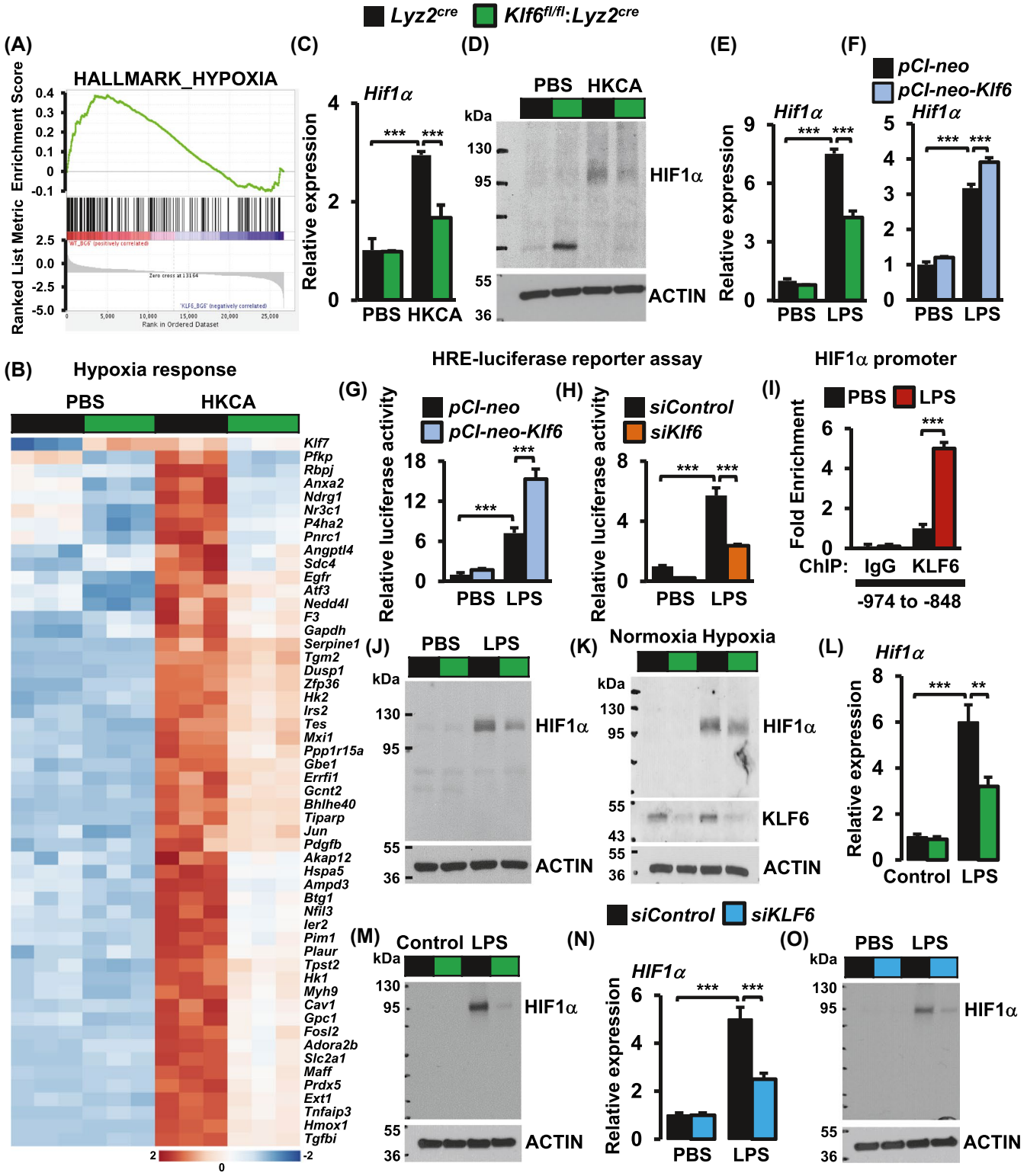

FIGURE 4 KLF6 deficiency attenuates inducible HIF1α expression in macrophages. A, Enrichment plot of the hypoxia gene set obtained by GSEA comparing *Lyz2*^{cre} and *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice BMDMs RNAseq data following HKCA treatment. B, Heatmap showing clustering of hypoxia response genes that are altered in *Lyz2*^{cre} and *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice BMDMs following HKCA stimulation. C and D, *Lyz2*^{cre} and *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice BMDMs were separately stimulated with HKCA for 4 h. Total RNA and protein samples were analyzed for HIF1α mRNA (C) and protein (D) expression by RT-qPCR and western blot, respectively. Some low-molecular weight nonspecific bans are seen in HIF1α blot. E and F, *Lyz2*^{cre} and *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice BMDMs (E) or RAW264.7 cells transfected with pCIneo-*Klf6* plasmid (F) were stimulated with 100 ng/mL LPS for 4 h. Total RNA samples were analyzed for HIF1α expression by RT-qPCR (n = 5). G and H, RAW264.7 cells were transfected with an HRE-luciferase reporter construct in the presence of pCIneo-*Klf6* plasmid (G) or *Klf6*-specific siRNA (H). These cells were stimulated with 100 ng/mL LPS for 18 h and cell lysates were analyzed for luciferase activity (n = 3). I, Wild-type BMDMs were stimulated with 100 ng/mL LPS for 4 h and ChIP analysis was performed on *Hif1α* promoter (−974 to −848) utilizing anti-KLF6 antibody (n = 3). J and K, *Lyz2*^{cre} and *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice BMDMs were stimulated with 100 ng/mL LPS (J) or exposed to hypoxic conditions (K) for 4 h. Total protein extracts were evaluated for the expression of HIF1α by western blot (n = 3). Some low molecular weight nonspecific bans are seen in HIF1α blot. (L and M) *Lyz2*^{cre} and *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice were intraperitoneally injected with thioglycollate with or without LPS. Purified macrophages from peritoneal lavage were evaluated for HIF1α mRNA (L) and protein (M) expression by RT-qPCR (n = 5) and western blot analysis, respectively (n = 3). N and O, Human PBMC-derived macrophages were transfected with control or *siKLF6*-specific siRNA. These cells were stimulated with LPS and expression of HIF1α mRNA (n = 4) was evaluated by RT-qPCR (N). HIF1α protein levels were analyzed by western blot analysis (n = 3). Blots were reprobed for actin as a loading control. All values are reported as mean ± SD. Data were analyzed by ANOVA followed by Bonferroni post-testing. *, $P < .05$; **, $P < .01$; ***, $P < .001$

analyses show that LPS challenge significantly elevated *Hk2*, *Hk3*, *Pfkp,* and *Ldha* expression in *Lyz2*^{cre} mice primary peritoneal macrophages in vivo (Figure 5D). Interestingly, LPS-induced *Hk2*, *Hk3*, *Pfkp,* and *Ldha* expression were significantly attenuated in *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice peritoneal macrophages in vivo (Figure 5D). Further, we assessed whether these observations were reiterated in human primary macrophages. As shown in Figure 5E, LPS exposure significantly elevated *HK2*, *ENO2*, *PFKFB3,* and *PFKP* expression in human PBMC-derived macrophages. Interestingly, diminished KLF6 levels significantly curtailed LPS-induced *HK2*, *ENO2*, *PFKFB3,* and *PFKP* expression in human primary macrophages (Figure 5E). Taken together, our analyses illustrate that KLF6 deficiency attenuates pro-inflammatory agent-induced glycolytic gene expression in murine and human primary macrophages.

3.5 | KLF6 deficiency abrogates LPS-induced glycolysis in macrophages

Next, we examined whether KLF6 deficiency altered the process of glycolysis in macrophages. Our serendipitous observations revealed that KLF6-deficient BMDMs did not change the color of the phenol red pH indicator containing cell culture medium from red to yellow even after prolonged incubation (Figure 6B). This manifestation suggests that *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice BMDMs produce less acidic molecules such as lactate than *Lyz2*^{cre} mice BMDMs. One of the major cellular metabolic pathway that produce lactate is glycolysis. Based on this (Figure 6B) and previous observations (Figure 5), we hypothesized that KLF6 deficiency attenuates the process of glycolysis in macrophages. To test this hypothesis, control and LPS-stimulated *Lyz2*^{cre} and *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice BMDMs polar metabolites cell extracts were quantified by LC-MS-based targeted metabolomics analyses. Our investigation uncovered that LPS stimulation significantly elevated principal glycolytic metabolites in *Lyz2*^{cre} BMDMs (Figure 6C). More importantly, all of these metabolites levels were significantly attenuated in *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice BMDMs at baseline, as well as following LPS exposure (Figure 6C). Next, we examined whether these observations were recapitulated in human primary macrophages. Our analyses revealed that LPS exposure significantly elevated glucose uptake (Figure 6D), ATP generation (Figure 6E), and lactate production (Figure 6F) in human PBMC-derived macrophages. Interestingly, KLF6 deficiency significantly curtailed LPS-induced glucose uptake, ATP generation, and lactate production in human primary macrophages (Figure 6D-F). Taken together, our results demonstrated that KLF6 deficiency significantly attenuates the process of glycolysis in murine and human macrophages.

3.6 | HIF1α overexpression reverse attenuated inflammatory and glycolytic gene expression in KLF6-deficient macrophages

Past studies have established that HIF1α governs LPS-induced inflammatory and glycolytic gene expression in macrophages.[9,10,30] Our studies thus far demonstrated that KLF6 deficiency attenuated LPS-induced HIF1α mRNA/protein expression, inflammatory/glycolytic gene expression, and process of glycolysis in macrophages. Therefore, we intended to examine whether diminished levels of pro-inflammatory and glycolytic gene expression in KLF6-deficient macrophages could be reversed by overexpressing HIF1α. We employed genetic approaches to overexpress oxygen stable form of HIF1α (Δ*Hif1α* -P402A/P564A) in macrophages.[31] Accordingly, RAW264.7 macrophages were

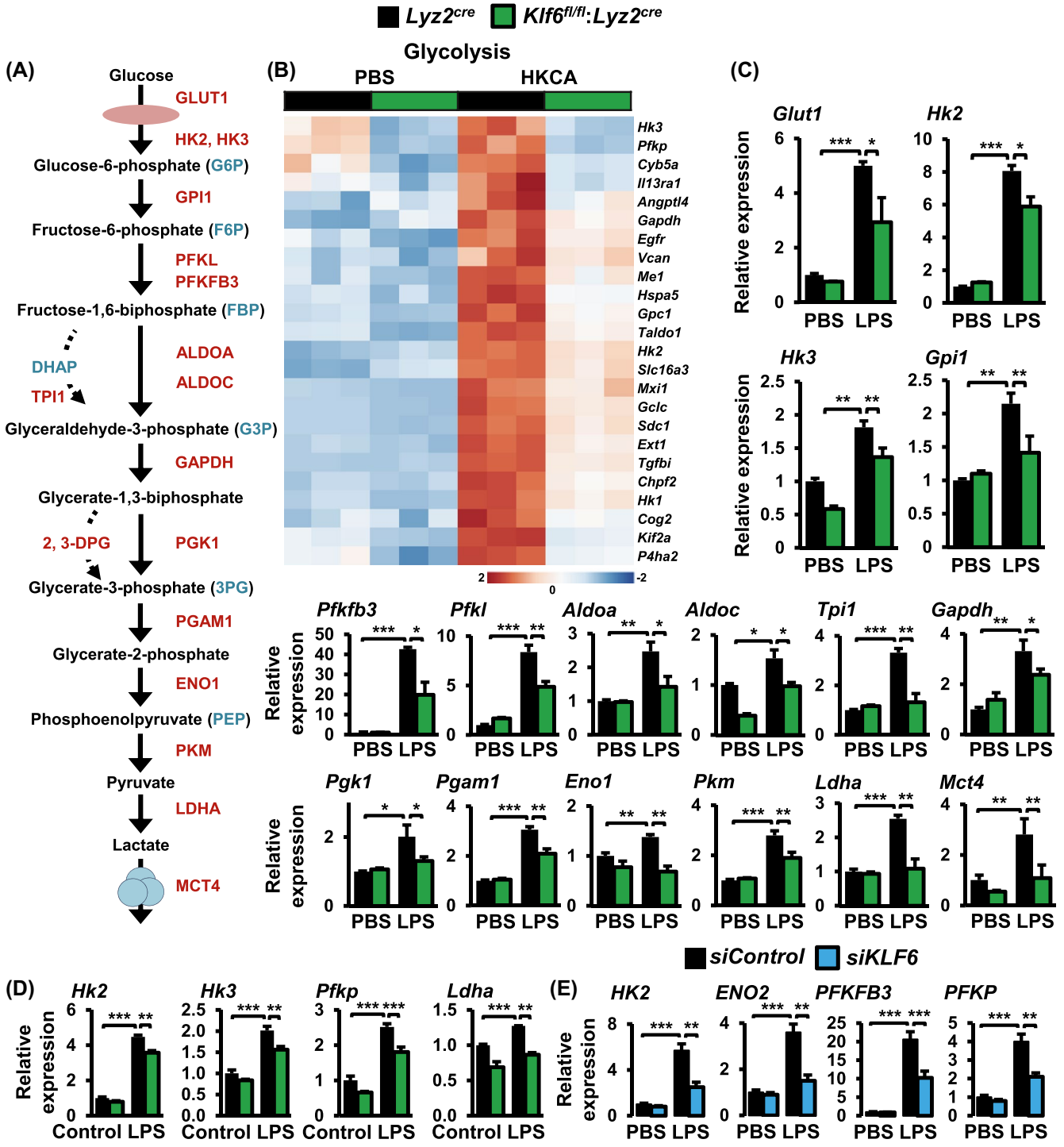

FIGURE 5 KLF6 deficiency attenuates LPS-induced glycolytic gene expression. A, A schematic representation of the glycolysis pathway where abbreviation of glycolytic enzymes are shown in red and metabolites are shown in blue. B, Heatmap of select glycolysis genes that are altered in *Lyz2*^{cre} and *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice BMDMs following HKCA treatment. C, *Lyz2*^{cre} and *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice BMDMs were stimulated with 100 ng/mL LPS for 6 h. Total RNA from these experiments were evaluated for the expression of indicated glycolytic genes by RT-qPCR (n = 4). D, *Lyz2*^{cre} and *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice were intraperitoneally injected with thioglycollate with or without LPS. Purified macrophages from peritoneal lavage were evaluated for *Hk2*, *Hk3*, *Pfkp,* and *Ldha* mRNA expression by RT-qPCR analysis (n = 5). E, Human PBMC-derived macrophages were transfected with control or *siKLF6* siRNA. These cells were stimulated with LPS and expression of *HK2*, *ENO2*, *PFKFB3,* and *PFKP* mRNA (n = 4) was evaluated by RT-qPCR. All values are reported as mean ± SD. Data were analyzed by ANOVA followed by Bonferroni post-testing. *, $P < .05$; **, $P < .01$; ***, $P < .001$ [Color figure can be viewed at wileyonlinelibrary.com]

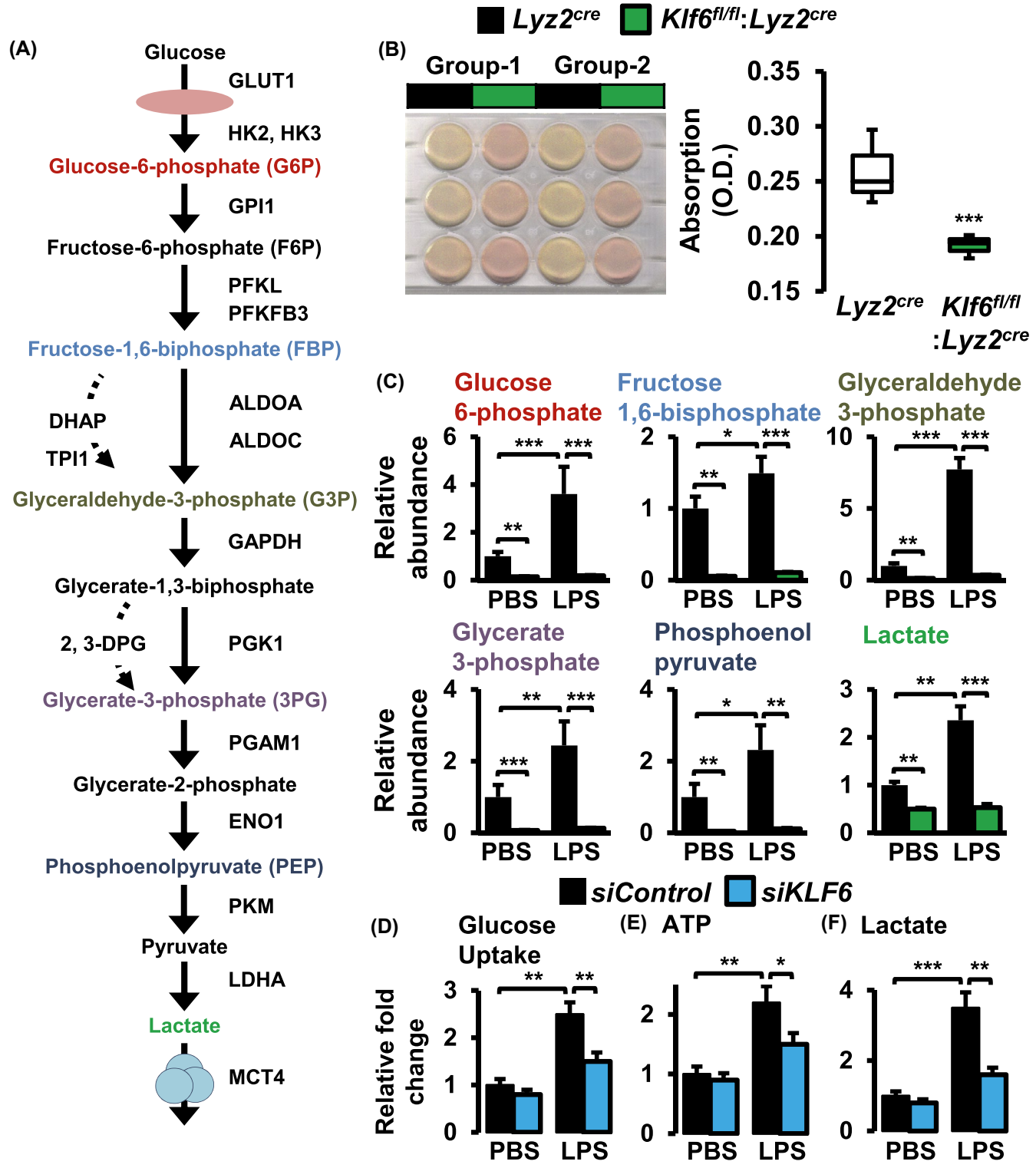

FIGURE 6 KLF6 deficiency attenuates LPS-induced glycolysis in macrophages. A, A schematic representation of the glycolysis pathway where abbreviation of glycolytic enzymes are shown in black and tested metabolites are shown in colors. B, An equal number of *Lyz2*^{cre} and *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice BMDMs were cultured in phenol red-containing media for 72 h. The cell culture supernatants optical density were recorded by spectrophotometer at a wavelength of 600 nm. C, *Lyz2*^{cre} and *Klf6*$^{fl/fl}$:*Lyz2*^{cre} mice BMDMs were unstimulated or stimulated with 100 ng/mL LPS for 6 h. Indicated polar metabolites cell extracts were quantified by LC-MS-based targeted metabolomics analyses (n = 3). D-F, Human PBMC-derived macrophages were transfected with control or *siKLF6* siRNA and stimulated with 100 ng/mL LPS. These cells were evaluated glucose uptake (D), ATP generation (E), and extracellular lactate production (F) by commercial assay kit (n = 4). All values are reported as mean ± SD. Data were analyzed by ANOVA followed by Bonferroni post-testing. *, $P < .05$; **, $P < .01$; ***, $P < .001$

transfected with *siKlf6*, Δ*Hif1α* plasmid or cotransfected with *siKlf6* and Δ*Hif1α* plasmid. These cells were stimulated with LPS for 6 hours. Total RNA derived from these experiments were analyzed for HIF1α inflammatory and glycolytic gene targets by qPCR analysis (Figure 7A-F). As anticipated, LPS exposure significantly elevated HIF1α inflammatory (*Adm*, *Cox2,* and *Mmp12)* and glycolytic (*Glut1*, *Hk2,* and *Pfkfb3*) gene targets in macrophages (Figure 7A-F). However, *Klf6* deficiency significantly attenuated LPS-induced inflammatory (Figure 7A-C) and glycolytic (Figure 7D-F) gene expression in macrophages. Interestingly, over-expression of a stable form of HIF1α completely reversed attenuated inflammatory (Figure 7A-C) and glycolytic (Figure 7D-F) gene expression in KLF6-deficient macrophages. Collectively, our studies demonstrate that KLF6 promotes inflammatory and glycolytic gene expression by elevating HIF1α expression in macrophages.

4 | DISCUSSION

Our findings demonstrate that KLF6 as a novel molecular toggle that promotes pro-inflammatory and glycolytic gene expression by elevating HIF1α expression in macrophages. The central findings of this study are as follows: (a) KLF6 deficiency attenuates broad pro-inflammatory gene program in macrophages; (b) myeloid-KLF6 deficiency is protective against LPS-induced sepsis symptomatology and mortality in vivo; (c) KLF6 deficiency attenuates innate immune inflammatory and hypoxic response gene programming; (d) diminished KLF6 level curtail inducible HIF1α expression in macrophages; (e) KLF6 deficiency constrain LPS-induced glycolytic gene expression; (f) KLF6 deficiency dwindle LPS-induced glycolysis in macrophages; (g) over-expression of HIF1α reverse attenuated inflammatory and glycolytic gene expression in KLF6-deficient macrophages. Collectively, our findings establish that KLF6 promotes inducible inflammatory and glycolytic gene expression by directly enhancing HIF1α expression in macrophages (Figure 8).

Monocyte-derived macrophages are the professional phagocytes that play a very critical role in the maintenance of human health and the development of diseases.[1] Our prior studies have discovered that KLF6 is most abundantly expressed in human and murine macrophages.[19] More importantly, bacterial endotoxins such as LPS robustly elevated KLF6 mRNA and protein expression in myeloid cells.[19]

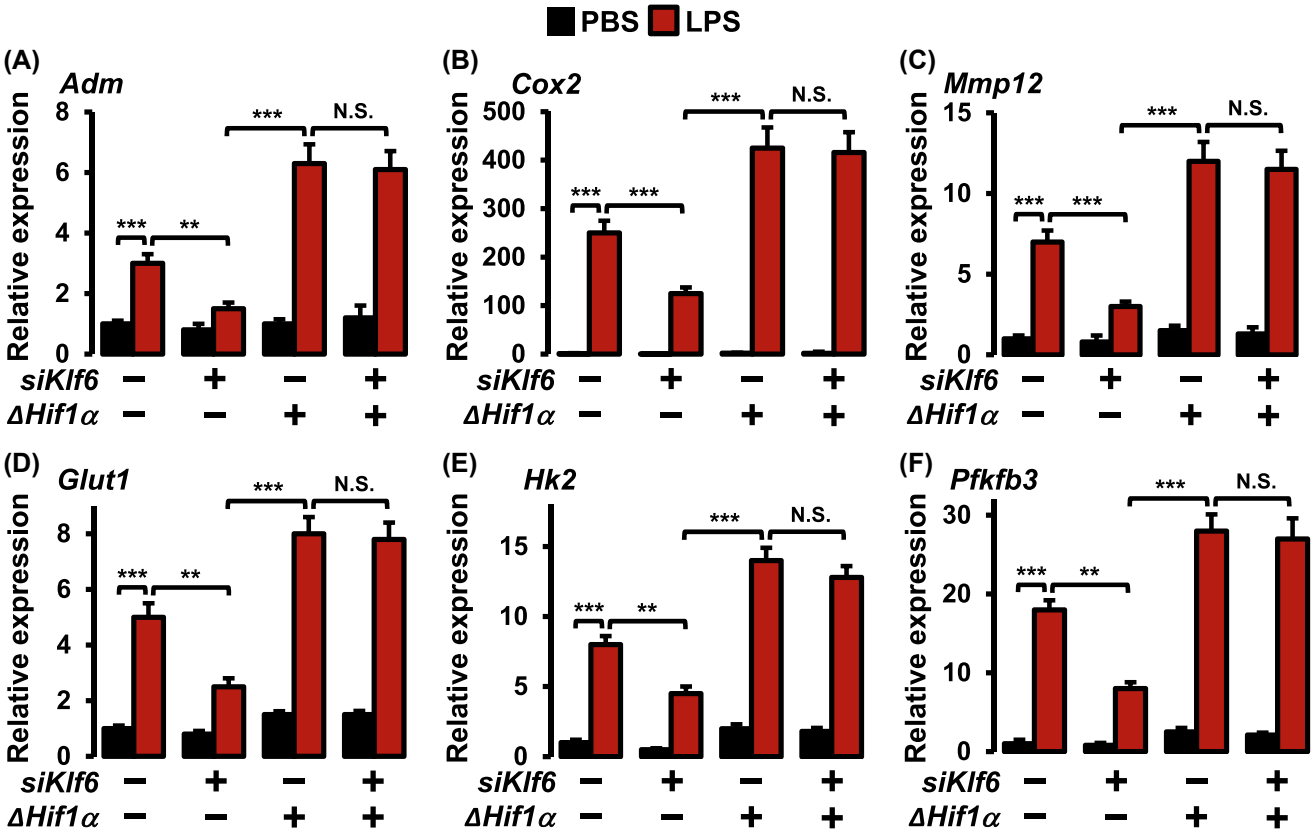

FIGURE 7 KLF6 regulates inflammatory and glycolytic gene expression through HIF1α. A-F, RAW264.7 cells were co-transfected with a combination of *Klf6*-specific siRNA or Δ*Hif1α* plasmid, and stimulated with 100 ng/mL LPS for 6 h. Total RNA from these experiments were evaluated for expression of *Adm*, *Cox2*, *Mmp12*, *Glut1*, *Hk2,* and *Pfkfb3* by RT-qPCR (n = 4). All values are reported as mean ± SD. Data were analyzed by ANOVA followed by Bonferroni post-testing. NS, not significant; *, $P < .05$; **, $P < .01$; ***, $P < .001$

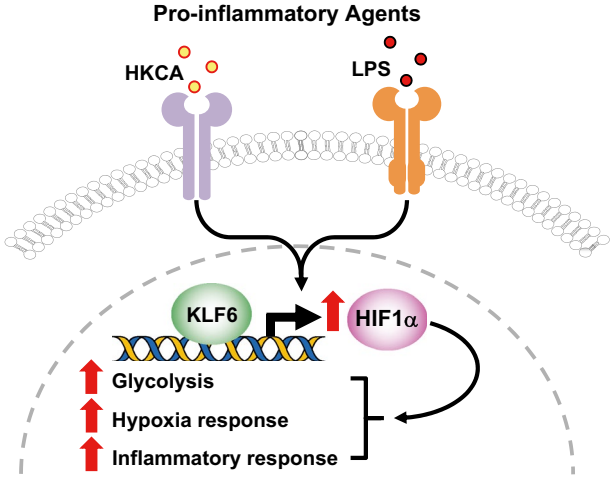

FIGURE 8 KLF6 governs macrophage inflammatory and hypoxic gene program responses by modulating HIF1α expression

Consistent with these observations, studies by Blouin et al reported that LPS exposure elevates HIF1α mRNA and protein expression even under normoxic conditions.[8] In this report, we demonstrate that KLF6 deficiency attenuates LPS-induced HIF1α expression in macrophages. Further, our ChIP analyses revealed that LPS exposure robustly elevated KLF6 enrichment on *Hif1α* promoter and enhanced *Hif1α* mRNA expression. Our transcriptomics studies revealed that KLF6 deficiency broadly attenuates LPS-induced hypoxic response in macrophages. Our current and former [19-21] studies have shown that KLF6 deficiency significantly attenuates inflammatory gene expression ex vivo and in vivo. Concordantly, former studies have reported that HIF1α deficiency significantly abrogates LPS or inflammatory agent-induced pro-inflammatory cytokines and chemokines expression.[10,12] Our analyses of RNAseq studies denote that KLF6 deficiency significantly attenuated innate immune inflammatory gene expression in macrophages. In addition, deficiency of KLF6 largely diminished LPS-induced glycolytic gene expression and the process of glycolysis in macrophages. Studies over the decades have established HIF1α as the guardian of glycolytic gene expression.[6] Intriguingly, our studies uncovered that overexpression of stable form of HIF1α reversed attenuation of inflammatory and glycolytic gene expression in KLF6-deficient macrophages. These observations establish KLF6 as an upstream regulator of HIF1α. The macrophage recruitment to the site of inflammation is greatly determined by its ability to migrate and invade through strenuous tissue microenvironment. Our prior studies have unveiled that KLF6 deficiency vastly diminishes inflammatory agent-induced macrophage motility ex vivo and in vivo.[20] Congruent with these observations, myeloid-HIF1α deficiency significantly diminished the macrophage recruitment site of inflammation and exhibited reduced motility under ex vivo.[10] In this context, one would expect diminished inflammatory responses in myeloid KLF6 as well as HIF1α-deficient mice to a wide array of inflammatory challenges. As shown in our previous report, myeloid-KLF6 deficiency markedly attenuated TPA-induced cutaneous inflammation in vivo.[20] Studies by Cramer et al uncovered that myeloid-HIF1α mice exhibited a diminished response to TPA-induced cutaneous inflammation.[10] Similarly, myeloid-specific deficiency of KLF6 [21,22] or HIF1α [32,33] mice were highly protected from DSS-induced colitis and metabolic complications associated with high-fat diet-induced obesity. More importantly, prior studies have noted that myeloid-HIF1α mice were protected from LPS-induced sepsis symptomatology and mortality.[12] In this context, our current studies illustrate that myeloid-KLF6-deficient mice are highly resistant to LPS-induced sepsis symptoms including, hypothermia, hypotension, inflammatory cytokine storm, and host mortality. Collectively, our studies reveal that KLF6 promote inflammatory and glycolytic gene expression by elevating HIF1α expression in macrophages.

In summary, the in vitro, ex vivo, and in vivo observations presented in this study highlights the importance of KLF6 in generating broad innate immune and hypoxic response to inflammatory challenges (Figure 8). Here, we provide evidence that KLF6 elevates LPS-induced HIF1α expression and attendant functions in macrophages. Further, restoration of functional HIF1α reversed attenuated inflammatory and glycolytic gene expression in KLF6-deficient macrophages. Taken together, our studies uncover a novel KLF6-HIF1α signaling axis that governs inflammatory and hypoxic response gene expression and progression of inflammation, which can be implicated in a broad spectrum of human inflammatory disease conditions.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grant HL126626, HL141423 and Crohn's and Colitis Foundation Senior Research Award 421904 (to G. H. M.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

ACCESSION NUMBER

The accession number for the sequencing data reported in this paper is GEO: GSE136476.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

G.H. Mahabeleshwar conceived and designed the study. G.H. Mahabeleshwar, G.-D. Kim and H.P. Ng performed experiments. G.H. Mahabeleshwar, G.-D. Kim, H.P. Ng, and E.R.

Chan analyzed and interpreted the data. G.H. Mahabeleshwar and G.-D. Kim wrote and edited the manuscript, and that was approved by all authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Kim G-D, Ng HP, Chan ER, Mahabeleshwar GH. Kruppel-like factor 6 promotes macrophage inflammatory and hypoxia response. *The FASEB Journal*. 2020;34:3209–3223. https://doi.org/10.1096/fj.201902221R