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HIF-1 α Is an Essential Mediator of IFN- γ -Dependent Immunity to *Mycobacterium tuberculosis*

Jonathan Braverman,* Kimberly M. Sogi,*[†] Daniel Benjamin,[‡] Daniel K. Nomura,[‡] and Sarah A. Stanley*[†]

The cytokine IFN- γ coordinates macrophage activation and is essential for control of pathogens, including *Mycobacterium tuberculosis*. However, the mechanisms by which IFN- γ controls *M. tuberculosis* infection are only partially understood. In this study, we show that the transcription factor hypoxia-inducible factor-1 α (HIF-1 α) is an essential mediator of IFN- γ -dependent control of *M. tuberculosis* infection both in vitro and in vivo. *M. tuberculosis* infection of IFN- γ -activated macrophages results in a synergistic increase in HIF-1 α protein levels. This increase in HIF-1 α levels is functionally important, as macrophages lacking HIF-1 α are defective for IFN- γ -dependent control of infection. RNA-sequencing demonstrates that HIF-1 α regulates nearly one-half of all IFN- γ -inducible genes during infection of macrophages. In particular, HIF-1 α regulates production of important immune effectors, including inflammatory cytokines and chemokines, eicosanoids, and NO. In addition, we find that during infection HIF-1 α coordinates a metabolic shift to aerobic glycolysis in IFN- γ -activated macrophages. We find that this enhanced glycolytic flux is crucial for IFN- γ -dependent control of infection in macrophages. Furthermore, we identify a positive feedback loop between HIF-1 α and aerobic glycolysis that amplifies macrophage activation. Finally, we demonstrate that HIF-1 α is crucial for control of infection in vivo as mice lacking HIF-1 α in the myeloid lineage are strikingly susceptible to infection and exhibit defective production of inflammatory cytokines and microbicidal effectors. In conclusion, we have identified HIF-1 α as a novel regulator of IFN- γ -dependent immunity that coordinates an immunometabolic program essential for control of *M. tuberculosis* infection in vitro and in vivo. *The Journal of Immunology*, 2016, 197: 000–000.

Mycobacterium tuberculosis, the causative agent of tuberculosis, infects 2 billion people worldwide and is responsible for more deaths annually than any other single bacterial pathogen (1). IFN- γ activation of macrophages leads to restriction of *M. tuberculosis* growth and is crucial for successful immunity. Patients lacking components of the IFN- γ signaling pathway are highly susceptible to mycobacterial infection (2). Similarly, mice lacking IFN- γ rapidly succumb to infection with *M. tuberculosis* (3, 4). Some of the proposed antibacterial responses induced by IFN- γ include nutrient restriction (5), enhanced production of antimicrobial peptides (6, 7), autophagy (8), expression of cell-intrinsic restriction factors, including

IFN-inducible GTPases (9, 10), and production of NO by inducible NO synthase (iNOS). NO has bactericidal activity against *M. tuberculosis* (11) and is essential for host defense against *M. tuberculosis* infection in mice (12), accounting for a substantial portion of the susceptibility of IFN- γ -deficient mice.

The transcription factor hypoxia-inducible factor-1 α (HIF-1 α) canonically functions to induce glycolytic gene expression under conditions of hypoxia. More recently, HIF-1 α has been implicated in macrophage function. HIF-1 α contributes to the transition to aerobic glycolysis and expression of genes associated with M1 polarization in response to LPS stimulation (13). In the context of sepsis, HIF-1 α was demonstrated to mediate a transition from a proinflammatory to an immunosuppressive phenotype while maintaining antimicrobial and protective functions (14). Furthermore, HIF-1 α has been identified as important for control of group A *Streptococcus*, *Pseudomonas aeruginosa*, and uropathogenic *Escherichia coli* (15–17). The susceptibility of HIF-1 α -deficient mice to infection has been attributed to an inability to produce the ATP required for migration to sites of inflammation (15, 18) and to decreased production of iNOS and antimicrobial peptides (15–17). Intriguingly, recent studies suggest that HIF-1 α may play a role in host defense against mycobacteria. Exogenously increasing the levels of active HIF-1 α using pharmacological or genetic tools in zebrafish embryos enhances bactericidal activity against *Mycobacterium marinum* (19). Mice lacking HIF-1 α in the myeloid lineage exhibit more rapid progression of hypoxic granulomatous lesions in the liver following i.v. infection with *Mycobacterium avium* (20). These data suggest the intriguing possibility that HIF-1 α may be an important mediator of resistance to *M. tuberculosis* infection. Importantly, HIF-1 α is thought to be important in the context of hypoxia or during innate immune responses to infection, and has not previously been shown to be involved in IFN- γ -dependent immunity.

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Abbreviations used in this article: BMDM, bone marrow-derived macrophage; COX2, cyclooxygenase 2; 2-DG, 2-deoxyglucose; DMOG, dimethylxylglycine; HIF-1 α , hypoxia-inducible factor-1 α ; iNOS, inducible NO synthase; qPCR, quantitative PCR; qRT-PCR, quantitative RT-PCR; RNA-seq, RNA-sequencing; WT, wild-type.

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In this study, we demonstrate that HIF-1 α is required for host defense against infection with virulent *M. tuberculosis*. Mice lacking HIF-1 α in the myeloid lineage are strikingly susceptible to infection. Surprisingly, we do not find evidence that HIF-1 α is important for innate defense of macrophages against *M. tuberculosis*. In contrast, we find that HIF-1 α is critical for IFN- γ -dependent control of *M. tuberculosis* infection. RNA-sequencing (RNA-seq) reveals that approximately one-half of the transcriptional response to IFN- γ during *M. tuberculosis* infection requires HIF-1 α . Furthermore, HIF-1 α -deficient macrophages are impaired for important effector functions, including production of NO, PGE₂, as well as inflammatory cytokines and chemokines. In addition to regulating these key immune effector functions, we find that HIF-1 α regulates a metabolic transition to aerobic glycolysis in IFN- γ -activated macrophages during *M. tuberculosis* infection. We show that this transition to aerobic glycolysis is required for IFN- γ -dependent control of *M. tuberculosis* infection of macrophages. In addition, we identify a positive feedback loop between HIF-1 α and glycolytic flux that reinforces IFN- γ -mediated activation of macrophages and control of *M. tuberculosis* infection.

Materials and Methods

Ethics statement

All procedures involving the use of mice were approved by the University of California, Berkeley, Institutional Animal Care and Use Committee (Protocol R353-1113B). All protocols conform to federal regulations, the National Research Council's *Guide for the Care and Use of Laboratory Animals*, and the Public Health Service's *Policy on Humane Care and Use of Laboratory Animals*.

Reagents

Mouse rIFN- γ (485 MI/CF) was obtained from R&D Systems (Minneapolis, MN) and was used at indicated concentrations. The 2-deoxyglucose (2-DG) and D-glucose were obtained from Sigma-Aldrich (St. Louis, MO) and were used at indicated concentrations. U-[¹³C]-glucose was obtained from Cambridge Isotope Laboratories (Andover, MA). Dimethylallylglycine (DMOG) was obtained from Cayman Chemical (Ann Arbor, MI) and was used at indicated concentrations.

Mice and cell culture

Wild-type (WT) mice were C57BL/6 and were obtained from The Jackson Laboratory (Bar Harbor, ME). All knockout mice were backcrossed to C57BL/6. B6.129-Hif1a^{tm3Rsjd/J} mice were obtained from The Jackson Laboratory and were crossed with B6.129P2-Lyz2^{tm1(cre)lfo/J} to generate mice that had Hif1a deletion targeted to the myeloid lineage. B6.129P2-Nos2^{tm1Lat/J} mice were obtained from The Jackson Laboratory and were bred in house. Bone marrow-derived macrophages (BMDM) were obtained by flushing cells from the femurs and tibias of mice and culturing in DMEM with 10% FBS and 10% supernatant from 3T3-M-CSF cells (BMDM media) for 6 d with feeding on day 3. After differentiation, BMDM continued to be cultured in BMDM media containing M-CSF.

Bacterial culture

The *M. tuberculosis* strain Erdman was used for all experiments. *M. tuberculosis* was grown in Middlebrook 7H9 liquid media supplemented with 10% albumin-dextrose-saline, 0.4% glycerol, and 0.05% Tween 80 or on solid 7H10 agar plates supplemented with 10% Middlebrook OADC (BD Biosciences) and 0.4% glycerol. The TB-lux strain used for measuring bacterial growth was derived from an Erdman strain, and was cultured as described above.

In vitro infections

BMDM were plated into 96-well or 24-well plates with 5×10^4 and 3×10^5 macrophages/well, respectively, and were allowed to adhere and rest for 24 h. BMDM were then treated with vehicle or IFN- γ (1.25 ng/ml) overnight and then infected in DMEM supplemented with 5% horse serum and 5% FBS at a multiplicity of infection of 5, unless otherwise noted. After a 4-h phagocytosis period, infected BMDM were washed with PBS before replacing with BMDM media. For experiments with DMOG, 2-DG, or galactose, these reagents were added to the BMDM media after the 4-h

phagocytosis. For 2-DG and galactose treatment, this was done to minimize the amount of time that the BMDM experienced glycolytic inhibition. For IFN- γ -pretreated wells, IFN- γ was also added postinfection at the same concentration. To measure intracellular growth of *M. tuberculosis*, cells were infected with TB-lux (Erdman) and luminescence was measured at 32°C immediately following the 4-h phagocytosis, PBS wash, and media replacement. Luminescence was then read again at the noted time points. All growth was normalized to day 0 luminescence readings for each infected well and is presented as fold change in luminescence compared with day 0. For enumeration of CFU, *M. tuberculosis* Erdman strain was used; infected BMDM were washed with PBS and lysed in water with 0.1% Triton X-100 for 10 min; and serial dilutions were prepared in PBS with 0.05% Tween 80 and were plated onto 7H10 plates.

In vivo infections

Cohorts of age- and sex-matched WT, Nos2^{-/-}, and Hif1a^{tm3Rsjd}LysMcre^{+/+} mice were infected by aerosol route with *M. tuberculosis* strain Erdman. All mice were on the C57BL/6 background, and were 7–10 wk of age when infected, with cohorts of 10–12 mice for each genotype for time to death experiments. Aerosol infection was done using a Nebulizer and Full Body Inhalation Exposure System (Glas-Col, Terre Haute, IN). A total of 9 ml OD₆₀₀ = 0.01 culture was loaded into the nebulizer. This resulted in ~400 CFU per mouse 1 d postinfection. Mice were weighed the day of infection, and weights were followed until a humane 15% weight loss cutoff was reached, at which point the mice were euthanized. For CFU, one lung lobe (the largest) was homogenized in PBS plus 0.05% Tween 80, and serial dilutions were plated on 7H10 plates. For CFU experiments, cohorts of four to five mice were used for each genotype at each time point. For quantitative RT-PCR (qRT-PCR) experiments, lung lobes from three to five mice per genotype were pooled, and a cell suspension was obtained by pressing the lungs through a 40- μ m filter. The cells were then washed, and CD11b⁺ cells were purified by MACS magnetic bead separation using CD11b MicroBeads (130-049-601; Miltenyi Biotec, San Diego, CA), as described in the manufacturer's protocol. Cells were purified to ~95% CD11b⁺. For quantitative PCR (qPCR), purified CD11b⁺ cells were lysed in TRIzol, and qRT-PCR was performed, as described below. For NO measurements from CD11b⁺ cells ex vivo, the same procedure described above was performed; CD11b⁺ cells were replated in 96-well plates with 3×10^5 cells/well; and Griess assays were performed on the supernatants at the indicated time points.

Griess assays

The Griess reaction was used to detect nitrite in the supernatants of BMDM or ex vivo CD11b⁺ cells as a proxy for NO production. Briefly, a solution of 0.2% naphthylethylenediamine dihydrochloride was mixed 1:1 with a 2% sulfanilamide, 4% phosphoric acid solution. A total of 50 μ l supernatant from cultured cells was added to 50 μ l this mixture, and absorbance was measured at 546 nm. Concentrations were determined by comparing to a standard curve of nitrite in BMDM media.

Western blots

Infected BMDM were washed with PBS, lysed in 1 \times SDS-PAGE buffer on ice, and heat sterilized for 30 min at 100°C. Total protein lysates were analyzed by SDS-PAGE using precast Tris-HCl criterion gels (Bio-Rad, Hercules, CA). The following primary Abs were used: rabbit Ab against HIF-1 α (NB100-479, Novus Biologicals, Littleton, CO and also D2U3T, Cell Signaling Technology, Danvers, MA) and goat Ab against mouse IL-1 β (AF-401-NA; R&D Systems). HRP-conjugated secondary Abs were used. Western Lightning Plus-ECL chemiluminescence substrate (Perkin-Elmer, Waltham, MA) was used, and blots were developed on film or using a ChemiDoc MP System (Bio-Rad). Blots were stripped using 0.2 M NaOH and then washed in ddH₂O and TBST before blocking and reprobing for actin as a loading control, using a HRP-conjugated rabbit Ab against β -actin (13E5; Cell Signaling Technology).

qRT-PCR and RNA-seq

For q-RT-PCR, 3×10^5 BMDM were seeded in 24-well dishes and infected, as described. At 24 h postinfection, cells were washed with room temperature PBS and lysed in 500 μ l TRIzol (Invitrogen Life Technologies, Carlsbad, CA). Total RNA was extracted using chloroform (100 μ l), and the aqueous layer was further purified using RNeasy spin columns (Qiagen, Limburg, Germany). For qPCR, cDNA was generated from 1 μ g RNA using Superscript III (Invitrogen Life Technologies, Carlsbad, CA) and oligo(dT) primers. Select genes were analyzed using Maxima SYBR Green qPCR master mix (Thermo Scientific, Waltham, MA). Each sample was analyzed in triplicate on a CFX96 real-time PCR detection system

(Bio-Rad). C_Q values were normalized to values obtained for actin, and relative changes in gene expression were calculated using the $\Delta\Delta C_Q$ method.

For RNA-seq, three independent experiments were performed. For each experiment, BMDM were seeded in 24-well dishes, and infection and RNA preparation were performed, as described. For each sample in each experiment, two duplicate wells were pooled. RNA-seq was performed at the Genome Center and Bioinformatics Core Facility at the University of California Davis (Davis, CA). SR50 reads were run on an Illumina HiSeq, with ~30 million reads per sample. Data analysis was performed by the University of California Davis bioinformatics group using FastQC for read quality assessment, Sythe and Sickle for Illumina adapter and quality trimming, and Tophat2 for read alignment. Raw counts were derived from alignments using a STSeq-count python script (21). Tests of differential expression were conducted using a multifactorial model in edgeR/limma (voom). Data was uploaded to National Center for Biotechnology Information: <http://www.ncbi.nlm.nih.gov/sra/SRP075696>.

Glucose assays and lactate assays

Lactate accumulation in supernatants of BMDM was measured using the lactate assay kit (MAK064; Sigma-Aldrich), as described in the manufacturer's protocol. Glucose depletion from the media was measured using the glucose (HK) assay kit (GAHK20; Sigma-Aldrich). The protocol was modified to perform the assays in 96-well plates with 100 μ l reactions instead of 1 ml reactions in cuvettes, as described in the manufacturer's protocol. Glucose consumption was calculated by measuring glucose levels in the media postinfection and subtracting from glucose measured from cell-free media.

ELISAs

For IL-1 β ELISAs, supernatants from BMDM in 24 well plates were used. A mouse IL-1 β ELISA kit (DY401; R&D Systems) was used, as described in the manufacturer's protocol. For PGE₂ ELISAs, the PGE₂ enzyme immunoassay kit (EA02; Oxford Biomedical Research, Rochester Hills, MI) was used, as described in the manufacturer's protocol.

Metabolomic profiling by liquid chromatography–tandem mass spectrometry

Preparation of lysates for metabolomics profiling followed published protocols (22). In brief, 4×10^6 BMDM were plated in 6-cm dishes in DMEM containing 10% FBS, 10 mM glucose, 4 mM L-glutamine, and 20 ng/ml rGM-CSF (Cell Signaling Technologies, Danvers, MA). BMDM were infected at a multiplicity of infection of 1, as described above. At 24 h postinfection, cells were washed with ice-cold PBS, immediately lysed with ice-cold 40:40:20 MeCN/MeOH/H₂O, and immediately placed on ice. D₃-Serine (1 nmol) was added to each sample as an external standard. Samples were vigorously vortexed and sonicated, and insoluble material was removed by centrifugation. An aliquot of the supernatant (20 μ l) was analyzed by selected reaction monitoring–based liquid chromatography–mass spectrometry. Polar metabolite separation was achieved with a Luna normal-phase NH₂ column (50 \times 4.6 mm, with 5- μ m-diameter particles; Phenomenex, Torrance, CA). Mobile phase A was composed of 100% acetonitrile, and mobile phase B consisted of water and acetonitrile in a 95:5 ratio. Solvent modifier 0.2% ammonium hydroxide with 50 mM ammonium acetate was used to assist ion formation and to improve the LC resolution in negative ionization mode. The gradient started at 0% B and increased linearly to 100% B over the course of 30 min with a flow rate of 0.7 ml/min. Mass spectrometry analysis was performed with an electrospray ionization source on an Agilent 6430 QQQ liquid chromatography–tandem mass spectrometry (Agilent Technologies, Santa Clara, CA). The capillary voltage was set to 3.0 kV, and the fragmentor voltage was set to 100 V. The drying gas temperature was 350°C, the drying gas flow rate was 10 l/min, and the nebulizer pressure was 35 ψ . Representative metabolites were quantified by selected reaction monitoring of the transition from precursor to product ions at associated optimized collision energies.

Results

HIF-1 α is required for IFN- γ -mediated control of *M. tuberculosis* infection

To characterize the role of HIF-1 α in macrophages during *M. tuberculosis* infection, HIF-1 α protein levels were first assayed by Western blot following infection with *M. tuberculosis*. Infection of BMDM with *M. tuberculosis* resulted in accumulation of HIF-1 α . However, the induction was weak and transient, with accumulation of HIF-1 α that peaked at 4 h postinfection and was

undetectable by 12 h postinfection (Fig. 1A). This was surprising given that HIF-1 α protein levels increase substantially in macrophages treated with LPS and during infection with several bacterial species (13, 15). However, in the context of IFN- γ stimulation, *M. tuberculosis* infection resulted in a substantially more robust and prolonged increase in HIF-1 α protein levels (Fig. 1A). The increase in HIF-1 α protein levels observed with infection of IFN- γ -activated macrophages is synergistic, as neither infection nor IFN- γ treatment alone induced substantial accumulation of HIF-1 α (Supplemental Fig. 1A).

Next, growth of *M. tuberculosis* in WT and HIF-1 α -deficient BMDM was compared both in resting and IFN- γ -activated macrophages. Bacterial numbers were enumerated by counting CFU at multiple time points postinfection. After the initial phagocytosis period, CFU bacterial numbers were equivalent across all genotypes and conditions (Fig. 1B). Over a 3-d time course, BMDM are a relatively restrictive environment for *M. tuberculosis* replication, with only a 2.5-fold increase in CFU observed, with WT and HIF-1 α -deficient BMDM able to restrict *M. tuberculosis* replication to the same degree in the absence of IFN- γ stimulation (Fig. 1B). However, following IFN- γ stimulation, WT BMDM are able to kill *M. tuberculosis*, whereas bacterial numbers remain constant in the HIF-1 α -deficient BMDM (Fig. 1B). HIF-1 α therefore regulates processes in IFN- γ -activated macrophages that enable bacterial killing.

HIF-1 α is constitutively transcribed and translated, and protein levels are governed by the activity of prolyl hydroxylases that mark HIF-1 α for ubiquitination and degradation. Inhibition of prolyl hydroxylases by low oxygen, metabolic intermediates, or small molecule inhibitors causes HIF-1 α stabilization and accumulation (23). To test whether pharmacological stabilization of HIF-1 α in the absence of IFN- γ activation would impact *M. tuberculosis* replication in macrophages, BMDM were infected with *M. tuberculosis* and treated with the prolyl hydroxylase inhibitor DMOG. DMOG was tested in the presence of IFN- γ at both a standard activating concentration (1.25 ng/ml) and subactivating concentration (0.05 ng/ml). The addition of 200 μ M DMOG enhanced HIF-1 α levels under both conditions, as well as in resting macrophages infected with *M. tuberculosis* (Supplemental Fig. 1B). To assess the impact on bacterial replication, a reporter strain that constitutively expresses the bacterial luciferase encoding *luxCDABE* operon (TB-lux, gift from the Cox Laboratory, University of California, Berkeley) was used. Luminescence from this strain was linear with bacterial number in axenic culture and during infection of macrophages, and expression of the lux genes did not attenuate growth (Supplemental Fig. 1C, 1D). Exogenously stabilizing HIF-1 α during *M. tuberculosis* infection of resting macrophages slightly reduced *M. tuberculosis* growth (Fig. 1C). However, the addition of DMOG to IFN- γ -activated macrophages resulted in a larger decrease in bacterial numbers (Fig. 1C), indicating that pharmacological stabilization of HIF-1 α can activate microbicidal mechanisms effective against *M. tuberculosis* more robustly in the context of IFN- γ . The fact that the enhancement of bacterial restriction occurs both at effective IFN- γ concentrations as well as at lower, subactivating IFN- γ concentrations suggests that artificial HIF-1 α stabilization might be of therapeutic utility in the context of an insufficient IFN- γ response to *M. tuberculosis* infection.

HIF-1 α is a key mediator of IFN- γ -dependent gene expression

HIF-1 α has a large number of potential target genes. A comprehensive analysis of HIF-1 α target genes in the context of infection has not been performed. Therefore, RNA-seq was used to identify HIF-1 α -dependent changes in the macrophage transcriptome during infection with *M. tuberculosis* (data available

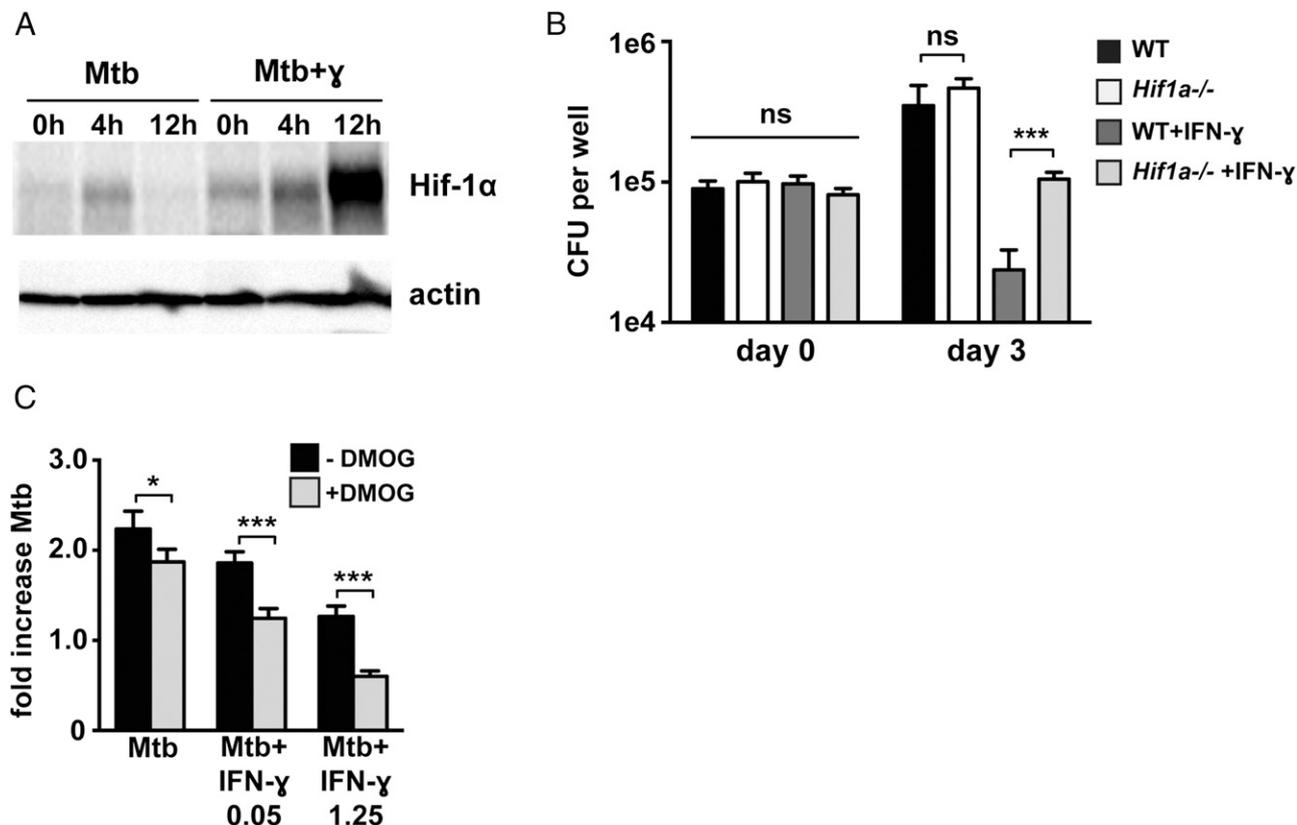


FIGURE 1. HIF-1 α is required for IFN- γ -based control of *M. tuberculosis* replication in macrophages. **(A)** Time course of HIF-1 α protein stabilization by Western blotting postinfection of resting and IFN- γ -activated WT BMDM with *M. tuberculosis* at multiplicity of infection = 5, with the 0-h time point reflecting the end of the 4-h phagocytosis period. **(B)** WT and *Hif1a*^{-/-} BMDM were infected with *M. tuberculosis* at multiplicity of infection = 5, and bacterial replication was monitored with and without IFN- γ treatment by plating for CFU. CFU on days 0 and 3 are shown. **(C)** Resting and IFN- γ -activated WT BMDM were infected with the TB-lux strain of *M. tuberculosis* and treated with 200 μ M DMOG after phagocytosis of bacteria. Bacterial growth was assessed by reading relative light units immediately after phagocytosis and at 72 h postinfection, and fold change is shown. For all experiments, error bars represent the SD of a minimum of quadruplicate wells, and a representative experiment of a minimum of three is shown. The *p* values were determined using an unpaired *t* test. ****p* \leq 0.001, **p* \leq 0.05.

at <http://www.ncbi.nlm.nih.gov/sra/SRP075696>). Infection of macrophages with *M. tuberculosis* resulted in changes in expression in 3330 genes, and the addition of IFN- γ to *M. tuberculosis*-infected macrophages results in differential expression of 2595 genes relative to *M. tuberculosis* infection alone. In resting *M. tuberculosis*-infected macrophages, HIF-1 α contributed to altered expression in only 118 genes, or 3.5% of all regulated genes. In IFN- γ -activated macrophages, however, HIF-1 α contributed to regulation of 1191 genes during infection, demonstrating that HIF-1 α is responsible for ~45% of IFN- γ -induced alterations in the macrophage transcriptome 24 h after *M. tuberculosis* infection.

Prominent among genes that were downregulated in HIF-1 α -deficient macrophages during infection with IFN- γ activation were inflammatory cytokines and chemokines, including *Il1a*, *Il1b*, *Il6*, and the neutrophil chemoattractant *Cxcl1* (Fig. 2A). Loss of HIF-1 α did not result in a global defect in transcription of cytokine and chemokine genes. *Tnf* levels were unaffected by HIF-1 α deficiency, and IL-10 levels were increased (Fig. 2A). The cytokine IL-1 is essential for control of *M. tuberculosis* infection in mice. HIF-1 α contributes to IL-1 transcription downstream of LPS activation (13); however, a role for HIF-1 α in expression of IL-1 during *M. tuberculosis* infection has not been demonstrated. Interestingly, levels of *Il1b* mRNA were dependent on HIF-1 α in both resting and IFN- γ -activated macrophages. This defect translated to a significant defect in pro-IL-1 β protein production, as assayed by Western blotting from cell lysates (Fig. 2C) and by ELISA from cell supernatants (Fig. 2D). Furthermore, enhancing HIF-1 α stabilization with DMOG further increased *Il1b* mRNA

levels in *M. tuberculosis*-infected and IFN- γ -activated macrophages (Fig. 2E). Of note, we observed that IFN- γ increased levels of pro-IL-1 β in cell lysates and had no impact on IL-1 β levels in cell supernatants. This contrasts with several reports that have demonstrated that IFN- γ suppresses IL-1 β production in the context of TLR stimulation or *M. tuberculosis* infection (24, 25). Nevertheless, our data clearly indicate that HIF-1 α is required for production of IL-1 during *M. tuberculosis* infection in both resting and IFN- γ -activated macrophages. Thus, whereas HIF-1 α only contributes to control of *M. tuberculosis* in infected macrophages in the context of IFN- γ activation, the low levels of HIF-1 α observed in the absence of IFN- γ do promote transcription of a small number of immunologically important genes.

IL-6 expression was also found to be dependent on HIF-1 α during *M. tuberculosis* infection (Fig. 2F). To test whether the deficient expression of other cytokines and chemokines in the HIF-1 α -deficient macrophages was downstream of an autocrine effect of IL-1 on macrophage activation, we tested whether IL-6 expression was altered in macrophages lacking IL-1R. However, IL-6 levels were unaffected by the absence of IL-1R (Fig. 2G), supporting the hypothesis that HIF-1 α plays a direct role in regulating the expression of cytokine and chemokine genes.

HIF-1 α regulates production of PGs and NO in M. tuberculosis-infected macrophages

A recent study demonstrated that one important function of IL-1 during *M. tuberculosis* infection is to promote the production of PGE₂ (26), a critical component of host immunity to *M. tuberculosis*

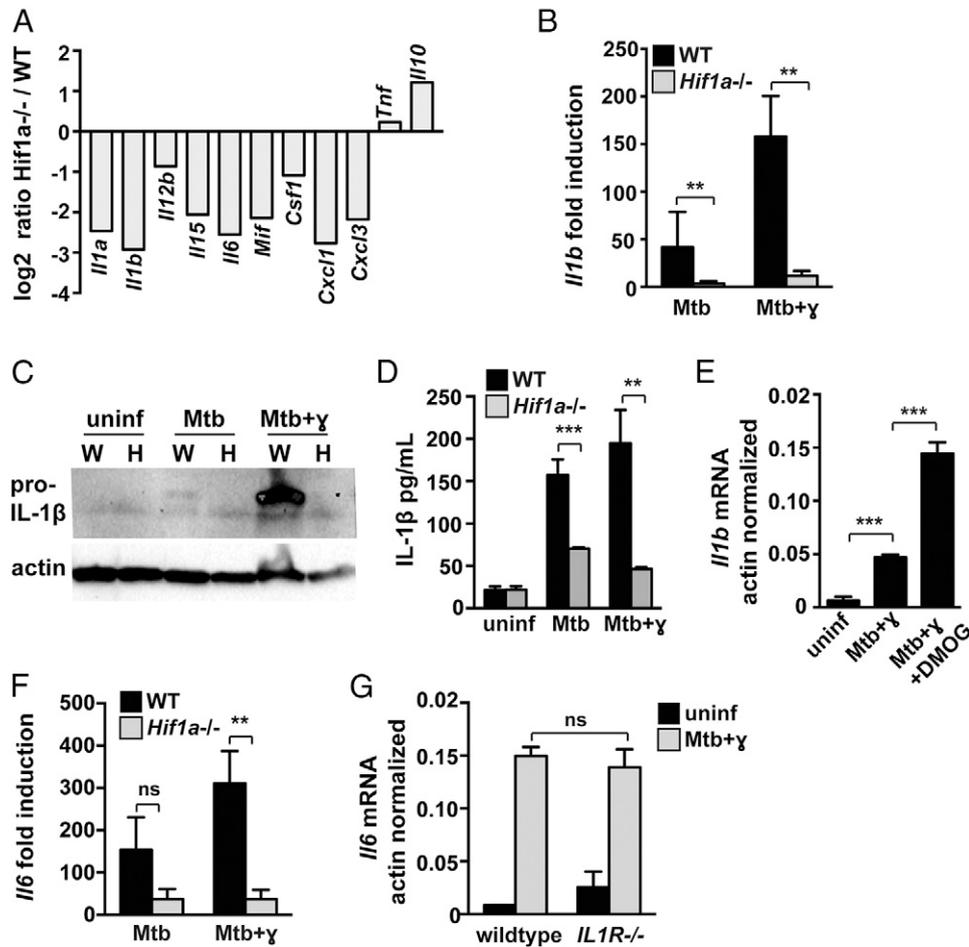


FIGURE 2. HIF-1 α regulates cytokine and chemokine production. Resting and IFN- γ -activated WT and *Hif1a*^{-/-} BMDM were infected with *M. tuberculosis* at multiplicity of infection = 5. RNA was prepared for RNA-seq at 24 h postinfection. (A) RNA-seq data showing transcript levels of cytokines and chemokines in *Hif1a*^{-/-} BMDM relative to WT BMDM on a log₂ scale. Data shown are from macrophages treated with IFN- γ and infected with *M. tuberculosis*. (B) RNA-seq data showing fold induction of *Il1b* transcript over untreated macrophages in WT and *Hif1a*^{-/-} BMDM. (C) Western blotting for pro-IL-1 β from cell lysates at 12 h postinfection in WT (W) and *Hif1a*^{-/-} (H) macrophages. (D) ELISA for IL-1 β from cell supernatants at 36 h postinfection. (E) qPCR data showing actin normalized *Il1b* transcript in WT BMDM with DMOG treatment. (F) RNA-seq data showing fold induction of *Il6* transcript over untreated macrophages in WT and *Hif1a*^{-/-} BMDM. (G) qPCR data showing actin normalized *Il6* transcript levels in WT and IL-1R- deficient BMDM. For RNA-seq, RNA was prepared from three independent infections. All other experiments were repeated two to three times, and representative experiments are shown. Error bars represent SD. The *p* values were determined using an unpaired *t* test. ****p* \leq 0.001, ***p* \leq 0.01.

(27–29). PGE₂ is an eicosanoid derived from arachidonic acid via the enzymes cyclooxygenase 2 (COX2) and PGE synthase. The defective IL-1 production in HIF-1 α -deficient macrophages suggests that there might also be a defect in PGE₂ production in HIF-1 α -deficient macrophages. Eicosanoids have been shown to be important in macrophages for cell-intrinsic control of *M. tuberculosis* replication and for productive and balanced inflammatory responses (28, 30, 31). However, eicosanoid production during *M. tuberculosis* infection of macrophages has only previously been characterized in the absence of IFN- γ . Interestingly, *Cox2* expression levels and PGE₂ production in *M. tuberculosis*-infected BMDM were dramatically enhanced by IFN- γ stimulation (Fig. 3A, 3B). In addition, *Cox2* expression was partially dependent on HIF-1 α in IFN- γ -activated *M. tuberculosis*-infected macrophages (Fig. 3A). This decrease in COX2 expression led to a significant defect in PGE₂ production in HIF-1 α -deficient macrophages (Fig. 3B). These data identify the production of enhanced levels of PGE₂ as a potential mechanism of IFN- γ -dependent control of *M. tuberculosis*, and demonstrate that HIF-1 α is essential for PGE₂ production.

Following IFN- γ activation and *M. tuberculosis* infection, HIF-1 α -deficient BMDM had lower levels of iNOS transcript (*Nos2*)

than WT macrophages (Fig. 3C). This observation is consistent with the observation that HIF-1 α can bind at the *Nos2* promoter (32). This defect at the transcript level corresponded to a ~50% defect in NO production (Fig. 3D) and was not a result of decreased cell viability of the HIF-1 α -deficient macrophages (Fig. 3E). Finally, the addition of DMOG to resting and IFN- γ -activated macrophages enhanced NO production, further confirming the importance of HIF-1 α for functional responses of macrophages (Fig. 3F). Taken together, our results implicate HIF-1 α as a crucial regulator of IFN- γ -dependent inflammatory responses as well as cell-intrinsic immune responses to *M. tuberculosis*.

Metabolic profiling during *M. tuberculosis* infection reveals increased levels of aerobic glycolysis in IFN- γ -activated macrophages

RNA-seq revealed that infection of IFN- γ -activated macrophages with *M. tuberculosis* causes a dramatic increase in expression of numerous glycolytic genes relative to the increase in gene expression observed with infection alone (Supplemental Fig. 2). We confirmed the increased expression of four of these genes, *Glut1*, *Hk2*, *Pfkfb3*, and *Mct4*, by qPCR and found that the combination of IFN- γ and *M. tuberculosis* infection dramatically

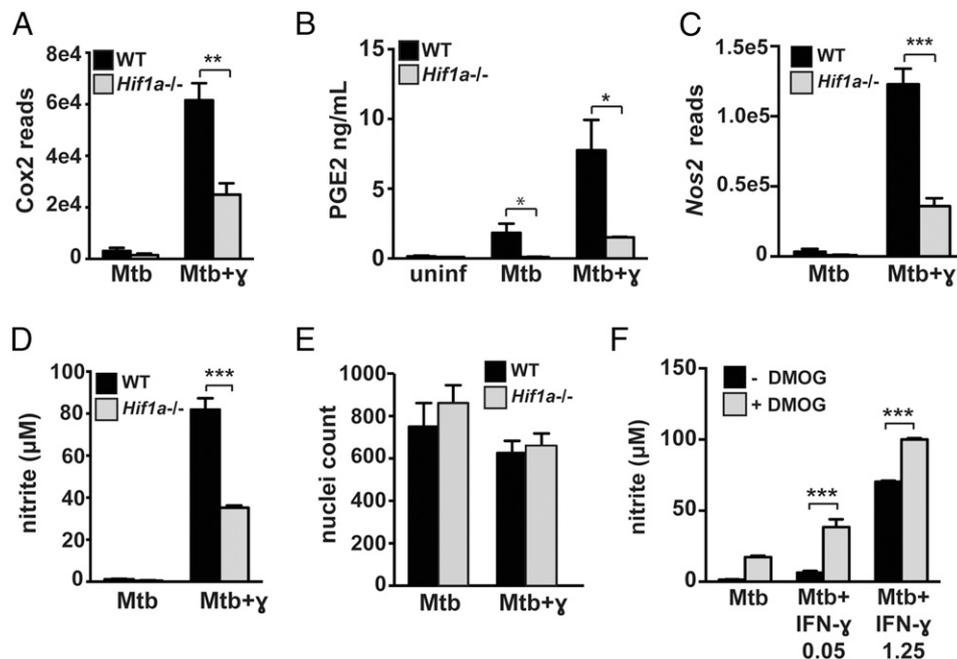


FIGURE 3. HIF-1 α is required for full activation of IFN- γ -dependent cell-intrinsic immune responses. Resting and IFN- γ -activated WT and *Hif1a*^{-/-} BMDM were infected with *M. tuberculosis* at multiplicity of infection = 5. RNA was prepared for RNA-seq at 24 h postinfection. (A) RNA-seq data showing expression levels of *Cox2* (official name *Ptgs2*) in WT and *Hif1a*^{-/-} BMDM. (B) PGE₂ ELISA from macrophage supernatants at 36 h postinfection. (C) RNA-seq data showing expression levels of *Nos2* in WT and *Hif1a*^{-/-} BMDM. (D) Griess assay for NO production in WT and *Hif1a*^{-/-} BMDM. (E) Nuclei counts after DAPI staining in WT and *Hif1a*^{-/-} BMDM 24 h postinfection. (F) Resting and IFN- γ -activated BMDM were infected with *M. tuberculosis* and treated with 200 μ M DMOG after phagocytosis of bacteria, and a Griess assay was performed 72 h postinfection. For RNA-seq, RNA was prepared from three independent infections. All other experiments are representative of three or more. Error bars represent SD. The *p* values were determined using an unpaired *t* test. ****p* \leq 0.001, ***p* \leq 0.01, **p* \leq 0.05.

and synergistically increased expression of these glycolytic genes (Fig. 4A). Aerobic glycolysis in resting macrophages infected with *M. tuberculosis* is thought to promote bacterial replication. Thus, the observation that glycolysis was further increased in IFN- γ -activated macrophages, a bactericidal environment for *M. tuberculosis*, is unexpected. To confirm these results, high-resolution tandem mass spectrometry was used to examine steady state levels of glycolytic intermediates in infected cells (33). *M. tuberculosis* infection of BMDM that had been activated with IFN- γ prior to infection resulted in a substantial increase in steady state levels of glycolytic intermediates compared with *M. tuberculosis* infection alone (Supplemental Fig. 3). Metabolic flux analysis using ¹³C-labeled glucose (U-[¹³C]-glucose) demonstrated a modest increase in levels of ¹³C pyruvate and lactate upon infection of resting macrophages with *M. tuberculosis* (Fig. 4B, 4C). In agreement with previous work, this increase was partially dependent on the ESX-1 alternative secretion system (Fig. 4B, 4C). However, analysis of ¹³C-labeled pyruvate and ¹³C lactate levels confirmed that IFN- γ preactivation resulted in greatly enhanced glycolytic flux relative to infection of resting macrophages (Fig. 4B, 4C). Taken together, these results demonstrate that increased glycolytic flux is associated with macrophages that are able to control infection with *M. tuberculosis*.

*HIF-1 α regulates metabolic transitions in IFN- γ -activated and *M. tuberculosis*-infected macrophages*

In keeping with the known role of HIF-1 α in regulating aerobic glycolysis, the majority of glycolytic genes had decreased expression in HIF-1 α -deficient BMDM relative to WT BMDM after *M. tuberculosis* infection of IFN- γ -activated BMDM (Fig. 4D). HIF-1 α -deficient BMDM also had significantly decreased glucose

utilization during *M. tuberculosis* infection of IFN- γ -activated macrophages (Fig. 4E). Furthermore, HIF-1 α -deficient macrophages produced significantly less lactate upon infection (Fig. 4F). The observation that HIF-1 α -deficient macrophages are not able to engage in aerobic glycolysis upon activation has previously been linked to a defect in ATP production in HIF-1 α -deficient peritoneal macrophages (18), which results in a defect in macrophage trafficking to sites of inflammation. A similar inability to produce ATP during infection could explain the defect in IFN- γ -dependent control of *M. tuberculosis* infection observed in HIF-1 α -deficient BMDM. To test whether ATP production is compromised in HIF-1 α -deficient BMDM, ATP levels during *M. tuberculosis* infection in WT and HIF-1 α -deficient BMDM were measured. No difference in ATP levels between WT and HIF-1 α -deficient BMDM was observed in *M. tuberculosis*-infected BMDM either in the presence or absence of IFN- γ (Fig. 4G). This demonstrates that the observed defects in *M. tuberculosis* control in these macrophages do not result from major perturbations in cellular energetics.

HIF-1 α is sensitive to changes in oxygen levels as well as changes in metabolite levels. HIF-1 α stabilization is promoted by metabolites associated with aerobic glycolysis, including succinate and lactate (13, 34). The 2-DG is a glucose analog that is commonly used to inhibit or block flux through glycolysis. To test whether HIF-1 α stabilization in *M. tuberculosis*-infected and IFN- γ -activated macrophages is promoted by flux through glycolysis, we treated macrophages with 2-DG and measured HIF-1 α levels by Western blotting. Limiting glycolytic flux dramatically reduced HIF-1 α protein levels (Fig. 4H). Thus, there is a positive feedback loop between HIF-1 α and aerobic glycolysis that links aerobic glycolysis to IFN- γ -dependent activation of macrophages. As expected, treatment of IFN- γ -activated and *M. tuberculosis*-infected

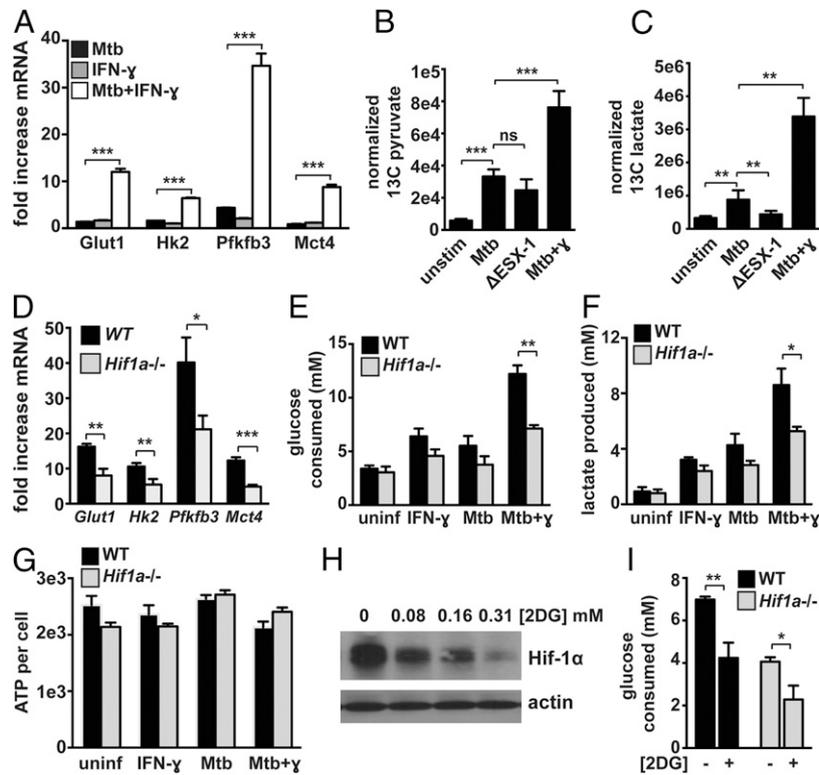


FIGURE 4. HIF-1 α mediates the transition to aerobic glycolysis in *M. tuberculosis*-infected macrophages but is not required for maintenance of TP production. **(A)** Fold increase in transcript levels over resting BMDM for the glycolytic genes *Glut1*, *Hk2*, *Pfkfb3*, and *Mct4* was measured by qPCR in WT BMDM. **(B and C)** BMDM infected with *M. tuberculosis* were cultured in media containing ^{13}C -labeled pyruvate for 24 h, at which time lysates were prepared, and ^{13}C -labeled pyruvate **(B)** and lactate **(C)** were detected by LC-MS/MS. Values represent the average of quintuplicate wells, and error is SD. **(D)** RNAseq data showing fold increase over resting BMDM of glycolytic genes in WT and *Hif1a*^{-/-} BMDM treated with IFN- γ and infected with *M. tuberculosis*. **(E)** Glucose consumption after 36 h by WT and *Hif1a*^{-/-} BMDM. **(F)** Lactate production after 24 h by WT and *Hif1a*^{-/-} BMDM. **(G)** ATP levels were measured using a luciferase-based assay (CellTiter-Glo) and were normalized to cell number measured by DAPI staining and counting of nuclei. **(H)** HIF-1 α Western blot 12 h after *M. tuberculosis* infection of IFN- γ -treated BMDM, treated with increasing dose of 2-DG following 4-h phagocytosis period. **(I)** Glucose consumption 24 h postinfection of WT and *Hif1a*^{-/-} BMDM with and without 2-DG treatment. Data in **(A)**, **(D)**, **(E)**, **(F)**, and **(H)** are representative of three or more experiments; data in **(G)** and **(I)** are representative of two experiments. Error bars represent SD. The *p* values were determined using an unpaired *t* test. ****p* \leq 0.001, ***p* \leq 0.01, **p* \leq 0.05.

macrophages with 2-DG resulted in a significant decrease in glucose uptake from the culture media, confirming that 2-DG limits glycolytic flux in this system (Fig. 4I).

Flux through glycolysis supports IFN- γ -dependent control of *M. tuberculosis* infection in macrophages

Previous reports have suggested that *M. tuberculosis* induces aerobic glycolysis in resting macrophages as a virulence strategy to promote bacterial growth (35, 36). Our data indicate that IFN- γ activation of macrophages, which restricts *M. tuberculosis* growth, also greatly enhances glycolytic flux. We therefore sought to determine whether the induction of aerobic glycolysis altered the ability of *M. tuberculosis* to replicate and/or survive in macrophages. To evaluate the role of aerobic glycolysis, resting and IFN- γ -activated macrophages were infected with *M. tuberculosis* and treated with 2-DG, and bacterial survival was assessed at 24 h postinfection. The 2-DG treatment did not substantially alter *M. tuberculosis* growth or host control in resting BMDM (Fig. 5A). In contrast, we found that the addition of 2-DG to IFN- γ -activated BMDM resulted in enhanced bacterial survival, reversing IFN- γ -dependent killing (Fig. 5B). To confirm that the doses of 2-DG used had no impact on macrophage viability, microscopy was used to enumerate the number of cells surviving under each condition, a measurement of macrophage viability that is not confounded by possible alterations in metabolism (Fig. 5C). To confirm that aerobic glycolysis is necessary for IFN- γ -dependent

killing, the capacity for cells to enhance glycolytic flux was also limited by culturing the macrophages on galactose rather than glucose as the carbon source in the media (37, 38). Macrophages cultured in galactose were unable to restrict the growth of *M. tuberculosis* in an IFN- γ -dependent manner (Fig. 5D). However, culturing macrophages on galactose did not impact infection in resting macrophages (Fig. 5D). Although macrophage viability was not impacted by 24 h of culture with 2-DG or galactose, by 48 h we observed differences in macrophage survival that precluded analysis of bacterial loads at later time points. To circumvent this issue, and validate the importance of flux through glycolysis, we treated macrophages for 24 h with 2-DG, followed by restoration of standard glucose-containing media. We then measured bacterial loads at 3 d postinfection, a time point at which we see a large difference in bacterial numbers between resting and IFN- γ -activated macrophages, compared with the only 2-fold difference at 24 h postinfection. In addition, 3 d postinfection is the time point at which the defect in HIF-1 α -deficient macrophages is most apparent. We found that 2-DG treatment in the first 24 h postinfection resulted in impaired IFN- γ -dependent control at 3 d postinfection. The effect of 2-DG was dose dependent, with the maximal concentration of 2-DG used resulting in a defect of IFN- γ -dependent control that was equivalent to that found in HIF-1 α -deficient macrophages (Fig. 5E). Interestingly, treatment with 2-DG did not further impair the microbicidal activity of HIF-1 α -deficient macrophages (Fig. 5F). Taken together, these findings

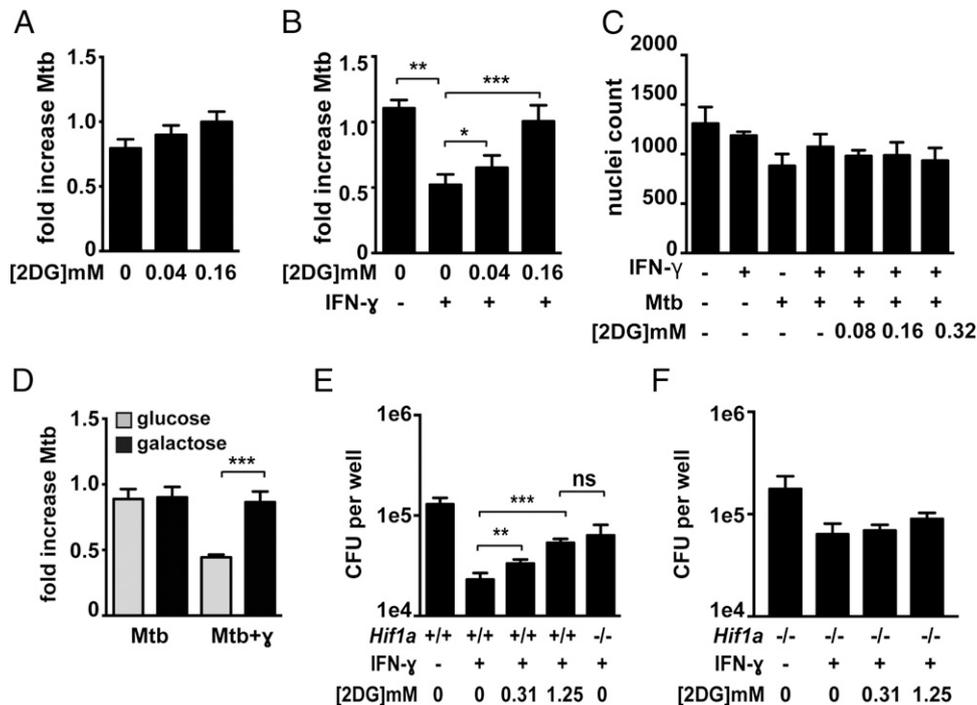


FIGURE 5. Enhanced flux through glycolysis is required for IFN- γ -dependent control of *M. tuberculosis* infection. Resting (A) and IFN- γ -activated (B) BMDM were infected with the TB-lux strain of *M. tuberculosis* and treated with 2-DG immediately after the 4-h phagocytosis period. Bacterial growth was assessed by reading relative light units immediately after phagocytosis and at 24 h postinfection and fold change is shown. (C) Macrophage viability was assessed using DAPI staining of nuclei and microscopy 24 h postinfection. (D) Resting and IFN- γ -activated macrophages were infected with the TB-lux strain of *M. tuberculosis* and were switched to glucose-free media containing galactose immediately after the 4-h phagocytosis period, and fold growth was determined at 24 h postinfection. (E and F) WT and *Hif1a*^{-/-} BMDM were infected with *M. tuberculosis* at multiplicity of infection = 5, and bacterial replication was monitored by plating for CFU. The 2-DG treatment at the indicated concentrations began after the 4-h phagocytosis, and 2-DG was washed out 24 h later. CFU 72 h postinfection is shown. Representative experiments of three or more are shown for (A)–(D) and representative of two experiments for (E) and (F). Error bars are SD from three to six replicate wells for TB-lux data, three replicate wells for nuclei counts, and five replicate wells for CFU. The *p* values were determined using an unpaired *t* test, ****p* \leq 0.001, ***p* \leq 0.01, **p* \leq 0.05.

demonstrate that macrophages infected with *M. tuberculosis* increase rates of glycolysis, that IFN- γ addition enhances this effect, and that aerobic glycolysis is necessary for IFN- γ -dependent restriction of *M. tuberculosis*.

HIF-1 α is crucial for control of M. tuberculosis infection in vivo

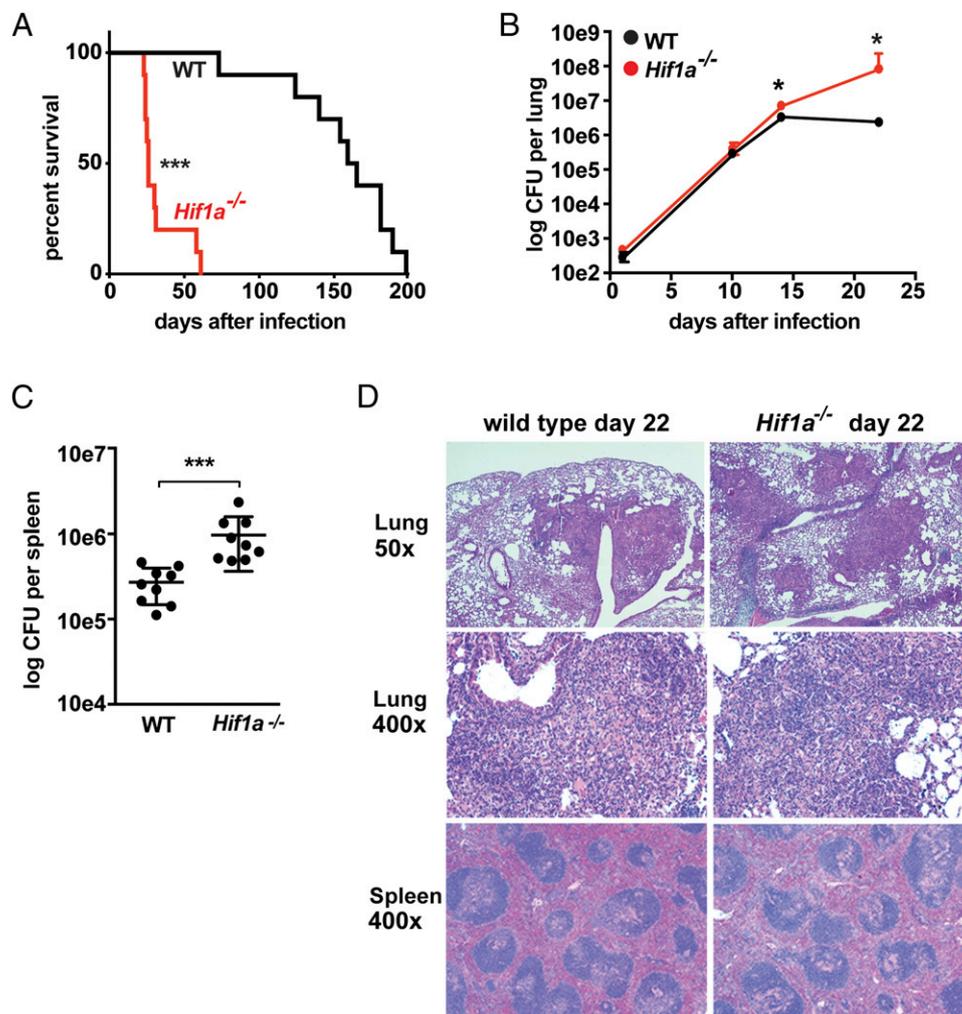
To assess the role of HIF-1 α during *M. tuberculosis* infection in vivo, mice deficient for HIF-1 α in the myeloid lineage (*Hif1a*^{myel} *LysMcre*^{+/+} [hereafter *Hif1a*^{-/-}]) and WT mice were infected with *M. tuberculosis* via the aerosol route. *Hif1a*^{-/-} mice exhibited rapid weight loss, with ~75% of the *Hif1a*^{-/-} mice succumbing to infection within 30 d (Fig. 6A). The increased susceptibility of *Hif1a*^{-/-} mice was also reflected in bacterial burden in the lungs. The earliest time point with a difference in bacterial burden was 14 d postinfection, with a small but statistically significantly higher bacterial burden in the *Hif1a*^{-/-} mice compared with WT. By 22 d postinfection there was >10-fold higher bacterial burden in the *Hif1a*^{-/-} mice (Fig. 6B). Additionally, *Hif1a*^{-/-} mice had higher burdens in spleens at 22 d postinfection (Fig. 6C). Histological analysis was also performed on lungs and spleens from WT and *Hif1a*^{-/-} mice 22 d postinfection. Both WT and HIF-1 α -deficient mice exhibited acute to subacute neutrophilic and histiocytic inflammation, peribronchiolar to perivascular pneumonia, and lymphocytic perivascular cuffing (Fig. 6D). The HIF-1 α -deficient lungs had a more necrotizing character and slightly more area affected (42.6 versus 32.3%); however, the differences were relatively modest despite the ~1 log increased bacterial burden in the lungs at this time point. Interest-

ingly, the HIF-1 α -deficient mice do not have large necrotic lesions, as has been reported for IFN- γ -deficient mice (39). No differences were observed in HIF-1 α -deficient spleens compared with WT. Taken together, these data support the idea that there is not a major defect in immune recruitment to the lungs in *Hif1a*^{-/-} mice, but rather there is a defect in control of bacterial replication in infected macrophages.

HIF-1 α regulates expression of immunologically important genes in vivo

Our in vitro data suggest that HIF-1 α activity is enhanced in the context of IFN- γ stimulation of macrophages. To test whether this is also true in vivo, we isolated CD11b⁺ macrophages from lungs of infected mice and examined the expression of HIF-1 α target genes over time. *Bnip3* is a canonical HIF-1 α target gene. We found that *Bnip3* expression increased between day 11 postinfection and day 22 postinfection, a timing that mirrors the development of the IFN- γ -dependent T cell response (Fig. 7A). As expected, macrophages from *Hif1a*^{-/-} mice had much lower levels of *Bnip3* that did not increase with the onset of IFN- γ signaling (Fig. 7A). Expression of iNOS increased over time in a partially HIF-1 α -dependent manner (Fig. 7B). Furthermore, HIF-1 α -deficient CD11b⁺ cells plated ex vivo were deficient for NO production (Fig. 7C). In addition, we confirmed that inflammatory cytokines and regulators of aerobic glycolysis were dependent on HIF-1 α for expression in vivo. At 21 d postinfection, we found that expression of glycolytic enzymes (*Pfkfb3*, *Hk2*), inflammatory cytokines (*Il1a*, *Il1b*, *Il6*), and *Cox2* were all significantly lower in HIF-1 α -deficient CD11b⁺ cells than WT (Fig. 7D–I). Furthermore,

FIGURE 6. HIF-1 α is required for control of *M. tuberculosis* infection in vivo. WT and *Hif1a*^{-/-} mice on the C57BL/6 background were infected with ~400 CFU of the virulent *M. tuberculosis* strain Erdman via the aerosol route. (A) Survival following aerosol infection is shown for WT and *Hif1a*^{-/-} mice. Experiment shown is representative of three experiments using 10–12 mice per genotype. (B) Bacterial loads in the lungs of WT and *Hif1a*^{-/-} mice were enumerated by plating for CFU. Time course is representative of three experiments with four to five mice used per group at each time point. (C) Bacterial loads in the spleens at 21 d postinfection. Data from two pooled experiments are shown. (D) Lung tissues were fixed, embedded in paraffin, sectioned, and stained with H&E. Images were obtained at original magnification $\times 50$ and original magnification $\times 400$. Error bars represent SD. For survival curves, *p* values were determined using the Mantel–Cox log. For CFU, *p* values were determined using Mann–Whitney *U* test. ****p* < 0.001, **p* \leq 0.05.



we found that expression of these genes was largely independent of HIF-1 α until ~18 d postinfection, coinciding with the onset of IFN- γ -dependent immunity (Supplemental Fig. 4). These results confirm that RNA-seq profiling of HIF-1 α -deficient macrophages in vitro is predictive of HIF-1 α activity in vivo, and that IFN- γ activation enhances HIF-1 α activity both in vitro and in vivo.

Discussion

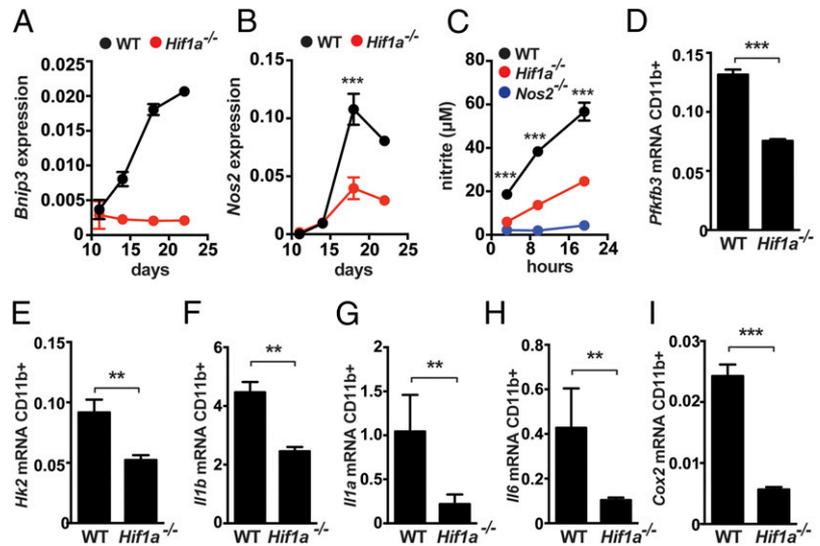
In this work, we identify HIF-1 α as an essential mediator of IFN- γ -dependent immunity to *M. tuberculosis*. We find that *Hif1a*^{-/-} mice are strikingly susceptible to *M. tuberculosis* infection in vivo. This places HIF-1 α among a surprisingly short list of genes essential for control of *M. tuberculosis* infection in vivo. We find that in macrophages HIF-1 α is required for the production of immune effectors, including NO, IL-1, and PGE₂. In addition, we demonstrate that HIF-1 α is required for a transition to aerobic glycolysis in IFN- γ -activated and *M. tuberculosis*-infected macrophages, and that aerobic glycolysis is crucial for IFN- γ -dependent control of *M. tuberculosis* replication. Finally, we find that the immune effectors regulated by HIF-1 α in vitro are also regulated by HIF-1 α in vivo during infection with *M. tuberculosis*.

HIF-1 α is emerging as an important regulator of immune responses to and defense against bacterial infection. In particular, two recent studies using different mycobacterial pathogens raised the possibility that HIF-1 α might be required for defense against *M. tuberculosis* infection. First, it was demonstrated that phar-

macological stabilization of HIF-1 α during infection of zebrafish embryos with *M. marinum* leads to a reduced bacterial burden at early time points (19). Second, HIF-1 α was found to play a role in a mouse model of granuloma caseation in livers of mice infected with *M. avium* (20). This granuloma formation depends upon hypoxia but is independent of effectors of IFN- γ -based immunity, including NO. In this model, the lack of HIF-1 α leads to more rapid necrosis of granulomatous lesions and a modest increase in bacterial numbers in livers and spleens that emerged by ~60 d postinfection. Interestingly, our data indicate that HIF-1 α is much more important for defense against *M. tuberculosis* than might have been predicted from these studies, as we observed a dramatic susceptibility to aerosol infection with most HIF-1 α -deficient mice succumbing to infection within 30 d. As mouse lungs are not hypoxic during infection with *M. tuberculosis* (40–42), our data demonstrate that HIF-1 α is not simply required for defense in areas of hypoxic inflammation, but is a mediator of IFN- γ -dependent immunity regardless of oxygen availability. In addition, our data support the hypothesis that pharmacological stabilization of HIF-1 α might be beneficial in clinical settings in which IFN- γ production is impaired.

HIF-1 α has been identified as important for control of group A *Streptococcus*, *P. aeruginosa*, and uropathogenic *E. coli* (15–17). For these infections, HIF-1 α has been described to regulate innate responses of macrophages, and is crucial for the production of NO and antimicrobial peptides by infected macrophages. Although several antimicrobial peptides have been proposed to contribute to

FIGURE 7. HIF-1 α regulates immune effectors in vivo. WT and *Hif1a*^{-/-} mice on the C57BL/6 background were infected with ~400 CFU of the virulent *M. tuberculosis* strain Erdman via the aerosol route. CD11b⁺ cells were isolated from lungs of infected mice at the indicated time points and analyzed by qPCR for expression of *Bnip3* (A) and *Nos2* (B). (C) Isolated CD11b⁺ cells were plated, and the supernatants were assayed for NO production by Griess assay at the indicated time points. (D–I) qPCR from isolated CD11b⁺ cells at 21 d postinfection in WT and *Hif1a*^{-/-} mice. Data shown in (C)–(I) are representative of two to three experiments. The *p* values were determined using an unpaired *t* test, ****p* \leq 0.001, ***p* \leq 0.01.



host defense against *M. tuberculosis*, we did not observe that expression of these antimicrobial peptides was dependent on HIF-1 α (data not shown). Furthermore, our data indicate that HIF-1 α is primarily important for control of *M. tuberculosis* in IFN- γ -activated macrophages, thus extending the role of HIF-1 α to a mediator of adaptive immunity. In this context, HIF-1 α regulates both cell-intrinsic and inflammatory responses of IFN- γ -activated macrophages. The production of NO is a critical mediator of IFN- γ -dependent control, and we find that maximal production of NO requires HIF-1 α . Interestingly, we also find that the production of PGE₂ is dramatically increased by IFN- γ stimulation of *M. tuberculosis*-infected macrophages. PGE₂ is known to mediate cell-intrinsic control of *M. tuberculosis* infection in the absence of IFN- γ stimulation; however, a possible role for PGE₂ in cell-intrinsic control of infection in IFN- γ -activated macrophages has not been established. We find that HIF-1 α is required for PGE₂ production, potentially due to a defect in *Cox2* expression in HIF-1 α -deficient macrophages. We also find that HIF-1 α is important for expression of numerous cytokines and chemokines in IFN- γ -activated macrophages, including *Il1a*, *Il1b*, and *Il6*. Interestingly, IL-1 is one of the few cytokines that appear to be regulated by HIF-1 α in both resting and IFN- γ -activated macrophages infected with *M. tuberculosis*. In summary, our data suggest that HIF-1 α plays a far more important role in the expression of important immune defenses against bacterial pathogens than has been previously appreciated. Determining the relative importance of each of these factors to the dramatic susceptibility of HIF-1 α -deficient mice will be the subject of future studies.

We also identify aerobic glycolysis as a novel component of IFN- γ -dependent immunity. It was recently published that infection of unstimulated macrophages with *M. tuberculosis* induces aerobic glycolysis, and that this is a pathogenesis strategy employed by *M. tuberculosis* (35, 36). We also find that infection with *M. tuberculosis* induces a modest increase in glycolytic flux and lactate production in macrophages in the absence of IFN- γ , and that this is indeed ESX-1 dependent. However, we observe a much more dramatic shift to aerobic glycolysis in the presence of IFN- γ . In previous studies, treatment of macrophages with glycolytic inhibitors caused a decrease in *M. tuberculosis* replication in resting macrophages (35, 36). In contrast, we observed that glycolytic inhibition with 2-DG reversed control of *M. tuberculosis* replication in the context of IFN- γ activation, but had no impact in the

absence of IFN- γ . One possible explanation for the different observations includes the use of primary murine cells in our study as opposed to transformed human cell lines that have metabolic perturbations at baseline. An additional possibility is our use of significantly lower 2-DG concentrations that limit flux through glycolysis without impacting macrophage viability. It is also possible that, in the context of a permissive growth environment, *M. tuberculosis* actively induces and perhaps slightly benefits from heightened flux through glycolysis and concomitant nutrient availability, but when an infected macrophage is also activated with IFN- γ as part of an adaptive immune response, the immunometabolic program it adopts includes and is dependent upon aerobic glycolysis to differentiate into an effective microbicidal cell.

Our data support a model in which HIF-1 α contributes to a shift to aerobic glycolysis in *M. tuberculosis*-infected and IFN- γ -activated macrophages. However, as yet unidentified mechanisms also promote glycolysis, as the defect in HIF-1 α -deficient macrophages is only partial. Furthermore, the fact that HIF-1 α stabilization requires aerobic glycolysis suggests that a HIF-1 α -independent mechanism initially induces enhanced glycolytic flux, leading to stabilization of HIF-1 α , which further promotes aerobic glycolysis by enhancing expression of glycolytic genes. The importance of aerobic glycolysis for IFN- γ -dependent control of *M. tuberculosis* is most likely at least partially explained by a positive feedback loop between glycolytic flux and HIF-1 α that we identify in which aerobic glycolysis supports the immune effector functions mediated by HIF-1 α . However, it is possible that aerobic glycolysis also mediates HIF-1 α -independent functions in IFN- γ -dependent control of *M. tuberculosis* infection and gene expression in activated macrophages, for example, via regulation of translation (38, 43).

In conclusion, we identify HIF-1 α as an essential component of immunity to *M. tuberculosis* in vitro and in vivo. We further find that HIF-1 α coordinates an immunometabolic program in IFN- γ -activated and *M. tuberculosis*-infected macrophages required for the transition to aerobic glycolysis and the production of numerous immune effectors, including NO, IL-1, and PGE₂. Finally, we demonstrate that aerobic glycolysis also contributes to HIF-1 α stabilization, suggesting that positive feedback amplifies IFN- γ -dependent activation of macrophages during *M. tuberculosis* infection.

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Disclosures

The authors have no financial conflicts of interest.

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