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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

BRD4 Regulates Glycolysis-Dependent *Nos2* Expression in Macrophages Upon *H pylori* Infection

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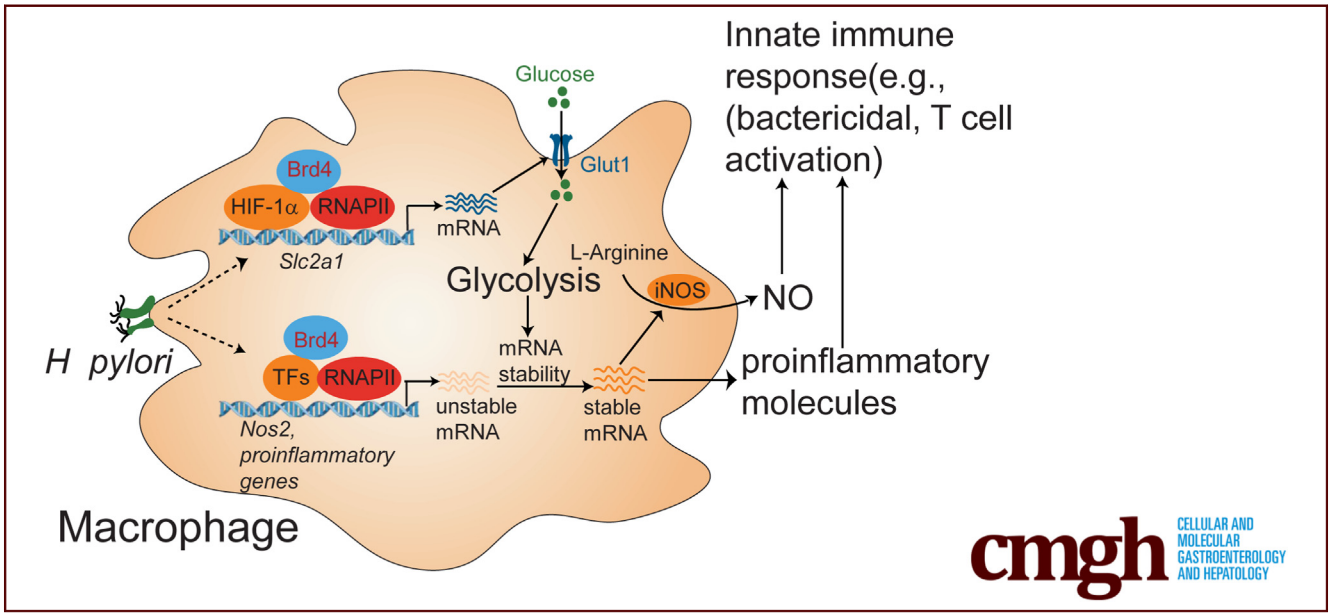

SUMMARY

This study showed that bromodomain-containing protein 4 is a critical regulator of hypoxia-inducible factor-1α–dependent glycolysis and M1 macrophage activation in response to *Helicobacter pylori* infection. Bromodomain-containing protein 4–mediated glycolysis stabilized *Nos2* messenger RNA for inducible nitric oxide synthase expression and nitric oxide production, which is essential for bacterial killing and *H pylori* colonization in mice.

BACKGROUND & AIMS: Metabolic reprogramming is essential for the activation and functions of macrophages, including bacterial killing and cytokine production. Bromodomain-containing protein 4 (BRD4) has emerged as a critical regulator of innate immune response. However, the potential role of BRD4 in the metabolic reprogramming of macrophage activation upon *Helicobacter pylori* infection remains unclear.

METHODS: Bone marrow–derived macrophages (BMDMs) from wild-type (WT) and *Brd4*-myeloid deletion conditional knockout (*Brd4*-CKO) mice were infected with *H pylori*. RNA sequencing was performed to evaluate the differential gene expression between WT and *Brd4*-deficient BMDMs upon infection. An in vivo model of *H pylori* infection using WT and *Brd4*-CKO mice was used to confirm the role of BRD4 in innate immune response to infection.

RESULTS: Depletion of *Brd4* in BMDMs showed impaired *H pylori*–induced glycolysis. In addition, *H pylori*–induced expression of glycolytic genes, including *Slc2a1* and *Hk2*, was decreased in *Brd4*-deficient BMDMs. BRD4 was recruited to the promoters of *Slc2a1* and *Hk2* via hypoxia-inducible factor-1α, facilitating their expression. BRD4-mediated glycolysis stabilized *H pylori*–induced nitric oxide synthase (*Nos2*) messenger RNA to produce nitric oxide. The NO-mediated killing of *H pylori* decreased in *Brd4*-deficient BMDMs, which was rescued by pyruvate. Furthermore, *Brd4*-CKO mice infected with *H pylori* showed reduced gastric inflammation and increased *H pylori* colonization with reduced inducible NO synthase expression in gastric macrophages.

CONCLUSIONS: Our study identified BRD4 as a key regulator of hypoxia-inducible factor-1α–dependent glycolysis and macrophage activation. Furthermore, we show a novel regulatory role of BRD4 in innate immunity through glycolysis to stabilize *Nos2* messenger

RNA for NO production to eliminate *H pylori* infection. *(Cell Mol Gastroenterol Hepatol 2024;17:292–308; https://doi.org/10.1016/j.jcmgh.2023.10.001)*

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See editorial on page 313.

Macrophages are a central component of the innate immune system and the first line of defense against pathogens.[1] Upon infection or stimulation, macrophages can be polarized into 2 distinct phenotypes: M1 and M2 macrophages.[2] M1 macrophages, also called *classically activated macrophages*, are characterized by an increased ability to secrete proinflammatory cytokines, including interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-12, in response to stimuli such as lipopolysaccharide (LPS) and interferon (INF)-γ.[2] Another key marker for M1 polarization is the production of inducible nitric oxide synthase (iNOS), which catalyzes the conversion of L-arginine and O_2 to nitric oxide (NO) and L-citrulline.[3] NO has been shown to eradicate bacteria through oxidative and nitrosative stressors, targeting bacterial lipids, membrane proteins, and DNA.[4] M2 macrophages, also called *alternatively activated macrophages*, are producers of anti-inflammatory cytokines with wound healing capability in response to stimuli such as IL-4 or IL-13.[2]

Recent studies have suggested that metabolic reprogramming plays an important role in macrophage polarization and activation.[5,6] The shift of macrophages to an M1 phenotype is associated with a robust metabolic shift toward glycolysis with increased pentose–phosphate pathway activity and increased iNOS production but reduced oxidative activity,[5,7] whereas IL4-induced M2-like macrophages have moderate glycolytic activity but higher oxidative activity.[3,5] Glycolytic activation in M1 macrophages is coupled with the activation of hypoxia-inducible factor 1α (HIF-1α), a transcription factor essential for sustaining a pseudo-hypoxic state by activating glycolytic gene expression in macrophages.[8,9] HIF-1α–dependent glycolysis also has been implicated in the production of proinflammatory cytokines because inhibition of glycolysis decreased LPS-induced inflammatory gene expression in macrophages.[8,10] In addition, HIF-1α also regulates the expression of iNOS and the release of NO, contributing to the bactericidal activity of macrophages.[11]

Bromodomain-containing protein 4 (BRD4) is an epigenetic transcriptional regulator critical for the expression of inflammatory genes.[12] BRD4 contains 2 bromodomains that facilitate its binding to acetylated histone and nonhistone proteins for the recruitment and activation of the positive transcription elongation factor (P-TEFb)/cyclin-dependent kinase 9 complex and stimulate RNA polymerase II (RNAPII)-mediated transcription elongation in epithelial cells and in macrophages.[13,14] Deletion of *Brd4* in macrophages or inhibition of BRD4 by small molecules suppresses nuclear factor-κB (NF-κB)–dependent inflammatory gene expression.[12,14–17] Our recent studies have identified a critical role of BRD4 in macrophage-mediated inflammatory gene expression by its binding to various transcription factors.[12,18,19] For example, BRD4 binds to interferon regulatory factor 8 (IRF8) and facilitates Nod-like receptor (NLR) family CARD (caspase recruitment) domain-containing 4 inflammasome–mediated processing of the proinflammatory cytokines IL-1β and IL-18.[12,18] These studies highlight the critical role of BRD4 in innate immunity regulating inflammatory gene expression in macrophages. However, the potential role of BRD4 in the metabolic reprograming of macrophage activation remains unclear.

Helicobacter pylori is a gram-negative pathogen that infects half of the world's population and is a major risk factor for gastric cancer.[20] Adherence and colonization of *H pylori* on the gastric mucosa triggers inflammation and, if left untreated, could lead to chronic gastric inflammation, peptic ulceration, and gastric cancer.[20] Upon *H pylori* infection, macrophages are recruited to the gastric mucosa, where they release proinflammatory cytokines and chemokines, contributing to the severity of gastric inflammation and gastric pathology.[21] Both epithelial cells and macrophages are critical for the activation of the innate immune response, and the subsequent adaptive immune response during *H pylori* infection.[21,22] Depletion of macrophages in mice ameliorated *H pylori* infection–induced gastritis, highlighting the importance of macrophages in the innate immune response to *H pylori*–triggered gastritis.[23] We previously showed that BRD4 is essential for inflammatory gene expression in *H pylori*–infected epithelial cells and gastric tissues of mice.[15] However, the contribution of BRD4 to *H pylori*–induced metabolic reprogramming and macrophage activation remains undetermined.

Here, we show that BRD4 is essential for HIF-1α–dependent glycolysis and M1 macrophage activation upon *H pylori* infection. BRD4-mediated glycolysis stabilized *Nos2* messenger RNA (mRNA) and optimized the production of iNOS and NO for the efficient killing of *H pylori*. This study identified a novel regulatory function of BRD4 in the innate immune response through its ability to regulate glycolysis and M1 polarization of macrophages to control bacterial infection.

Abbreviations used in this paper: **AG, aminoguanidine; BMDM, bone marrow-derived macrophage; BRD4, bromodomain-containing protein 4; ChIP, chromatin immunoprecipitation assay; CKO, conditional knockout; DMEM, Dulbecco's modified Eagle medium; ECAR, extracellular acidification rate; FBS, fetal bovine serum; GLUT1, glucose transporter 1; HIF-1α, hypoxia-inducible factor 1α; HK2, hexokinase 2; HRE, hypoxia-responsive element; IL, interleukin; iNOS, inducible nitric oxide synthase; KEGG, Kyoto Encyclopedia of Genes and Genomes; LPS, lipopolysaccharide; MOI, multiplicity of infection; mRNA, messenger RNA; NF-κB, nuclear factor-κB; NO, nitric oxide; PBS, phosphate-buffered saline; RNAPII, RNA polymerase II; RNA-seq, RNA sequencing; RT-PCR, real-time polymerase chain reaction; SS1, Sydney strain 1; TNF, tumor necrosis factor; UIUC, University of Illinois at Urbana-Champaign; WT, wild-type; 2-DG, 2-deoxy-D-glucose, Slc2a1, solute carrier family 2 member 1.**

Most current article

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Results

BRD4 Regulates H pylori*–Induced M1 Macrophage Polarization*

To investigate the role of BRD4 in the innate immune response to *H pylori* infection, we performed RNA sequencing (RNA-seq) analysis of *H pylori*–infected bone marrow–derived macrophages (BMDMs) isolated from wild type (WT) and *Brd4*–conditional knockout (CKO) mice. We analyzed the genes altered in the absence and presence of *H pylori* in WT and *Brd4*-deficient macrophages and found a total of 827 overlapping genes (fold change, $\geq$1.8; false discovery rate, $\leq$ 0.1%) (Figure 1*A*). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed that many inflammation-related pathways were altered by the deficiency of BRD4, including TNF signaling, NF-κB signaling, and cytokine–cytokine receptor interaction pathways (Figure 1*B*). Similarly, Gene Ontology enrichment indicated that several of the overlapping genes were involved in the regulation of cytokine production and inflammatory response, supporting the critical role of BRD4 in the innate immune response in macrophages (Figure 1*C*).

Infection of macrophages with various microorganisms or bacterial endotoxins may polarize the macrophages into either the proinflammatory M1 or anti-inflammatory and pro-healing M2 phenotype.[24] We found that many M1 macrophage markers, including *Nos2*, *Il6*, *Tnfa*, and *Il12*, were up-regulated upon *H pylori* infection in WT BMDMs, but the induction was decreased in *H pylori*–infected *Brd4*-deficient macrophages (Figure 1*D*). These RNA-seq results indicate that BRD4 might be critical for *H pylori*–induced M1 macrophage polarization. Further supporting this notion, real-time polymerase chain reaction (RT-PCR) showed that proinflammatory genes, including *Il6*, *Il1b*, *Nos2*, *Tnfa*, *Il12b*, and *Cxcl10*, were up-regulated in *H pylori*–infected WT macrophages (Figure 1*E*). However, the expression of these genes was significantly lower in *Brd4*-deficient macrophages (Figure 1*E*). A similar decrease in protein levels was observed for TNF-α, IL-6, and IL-1β in *Brd4*-deficient BMDMs (Figure 1*F*). Collectively, these data support the role of BRD4 as a positive regulator of *H pylori*–induced proinflammatory M1 macrophage polarization.

BRD4 Is Critical for H pylori*–Induced Glycolysis in Macrophages*

HIF-1α signaling plays a key role in the metabolic switch of macrophages in response to infection. Metabolism in M1 macrophages is characterized by increased HIF-1α–mediated glycolysis.[25] Interestingly, KEGG analysis also showed that HIF-1α signaling and the glycolysis pathways were affected by *Brd4* deletion in *H pylori*–infected macrophages (Figure 1*B*), raising a possibility that BRD4 also might regulate the innate immune response via HIF-1α–dependent glycolysis. Therefore, we plotted the heat map for genes altered in the HIF-1α and glycolysis pathways and found that several glycolytic genes showed diminished up-regulation in *H pylori*–infected *Brd4*-deficient BMDMs (Figure 2*A*). These genes include glucose transporter 1 (*Slc2a1*), the rate-limiting enzyme hexokinase 2 (*Hk2*), and *Pfkl* (Figure 2*A*). Thus, RNA-seq data support a potential role of BRD4 in the transcriptional regulation of key glycolytic enzymes.

In agreement with the RNA-seq data, the expression of glycolytic genes measured by RT-PCR, including *Slc2a1*, *Hk2*, *Ldha*, *Eno1*, and *Pfk1*, were up-regulated by *H pylori* infection in WT BMDMs, but at lower levels in *Brd4*-deficient BMDMs (Figure 2*B*). In addition, we did not observe any difference in the levels of *Hif1a* expression between WT and *Brd4*-deficient BMDMs (Figure 2*B*), indicating that BRD4 was not involved in the transcriptional regulation of *Hif1a*. Consistent with RT-PCR data, Western blot analysis showed an up-regulation of glucose transporter 1 (GLUT1) and hexokinase 2 (HK2) in *H pylori*–infected WT BMDMs, but not in the *Brd4*-deficient BMDMs (Figure 2*C*). Together, these results show that BRD4 is essential for the expression of key glycolytic genes and likely glycolysis in *H pylori*–infected macrophages.

Aerobic glycolysis is a marker for macrophages to switch to a proinflammatory phenotype with increased lactate production.[26] The increased expression of GLUT1 and HK2 in *H pylori*–infected macrophages suggests a potential increased glucose uptake and aerobic glycolysis. Indeed, glucose uptake was enhanced in WT BMDMs upon *H pylori* infection, however, much less up-regulated glucose uptake was found in *Brd4*-deficient BMDMs (Figure 2*D*). Under aerobic conditions in activated macrophages, glucose is metabolized into pyruvate, which in turn is converted into lactic acid via the induction of the Warburg effect.[25,26] Consistently, lactate, the conjugate base of lactic acid, also was increased in *H pylori*–infected WT BMDMs, but the increase was significantly lower in *H pylori*–infected *Brd4*-deficient BMDMs (Figure 2*E*).

To determine if the defect in lactate production was a direct consequence of reduced glycolytic activation of *H pylori*–infected *Brd4*-deficient macrophages, we compared the extracellular acidification rate (ECAR) in WT and *Brd4*-deficient macrophages using the Seahorse XF extracellular flux analyzer. We found a significant increase in extracellular acidification upon glucose injection in WT BMDMs after infection, and a subsequent decrease after glycolytic inhibitor 2-deoxy-D-glucose (2-DG) injection, indicating increased glycolysis in *H pylori*–infected macrophages (Figure 2*F*). *Brd4*-deficient BMDMs, although showing a similar trend, showed a defect in up-regulation of ECAR after glucose addition (Figure 2*F*). Overall, *H pylori* infection increased glycolysis and the glycolytic capacity in WT BMDMs, but at much diminished levels in *Brd4*-deficient macrophages (Figure 2*G*). Together, these data show that *H pylori* infection triggers BRD4-regulated metabolic reprogramming of macrophages with increased glycolysis.

BRD4 Cooperates With HIF-1α to Regulate the Expression of Glycolytic Genes and Glycolysis in H pylori*–Infected Macrophages*

HIF-1α has been shown to be the master regulator of glycolysis by binding to the hypoxia-responsive element

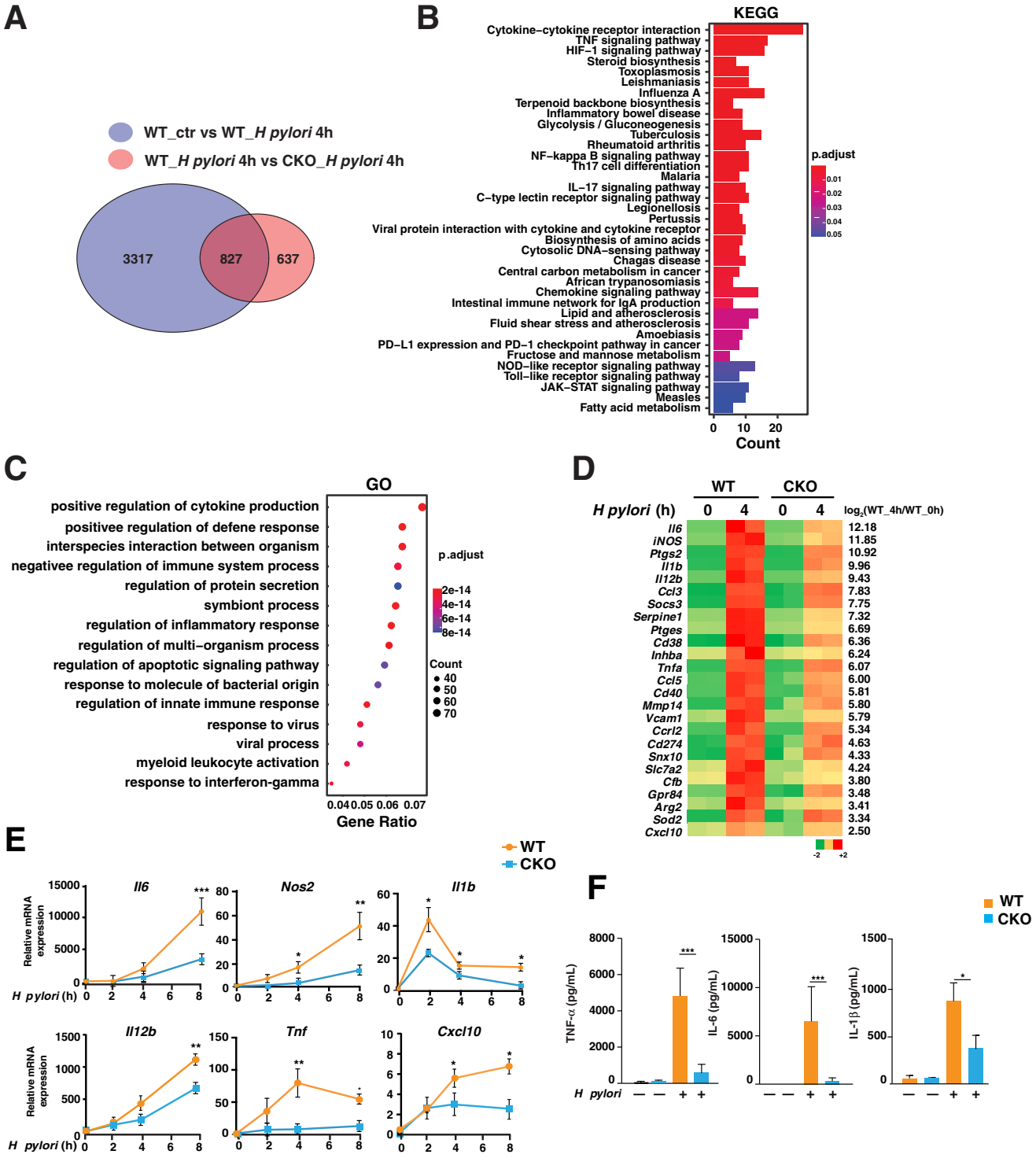


Figure 1. Deletion of BRD4 reduces inflammatory gene expression in *H pylori*–infected macrophages. (*A*) Venn diagram indicates the number of genes with significantly altered gene expression (fold change, ≥1.5; FDR, ≤0.1%) in WT and *Brd4*-deficient macrophages with or without *H pylori* infection (4 h; MOI, 50). A list of 827 overlapped genes from the Venn diagram in panel *A* was classified with (*B*) KEGG pathway enrichment and (*C*) Gene Ontology (GO) analysis. (*D*) Heat map representation of various genes involved in M1 macrophage polarization, color coded by Z-score. (*E*) WT and *Brd4*-deficient BMDMs infected with *H pylori* SS1 for the indicated time points (MOI, 50). Expression of the indicated genes was measured by quantitative RT-PCR. Results are presented as means ± SD in 3 independent experiments. (*F*) The levels of TNF-α, IL-6, and IL-1β in culture supernatant of WT and *Brd4*-deficient BMDMs with or without *H pylori* infection (24 h; MOI, 50) were measured by enzyme-linked immunosorbent assay. Results are presented as means ± SD in 3 independent experiments. *$P < .05$, **$P < .01$, and ***$P < .001$.

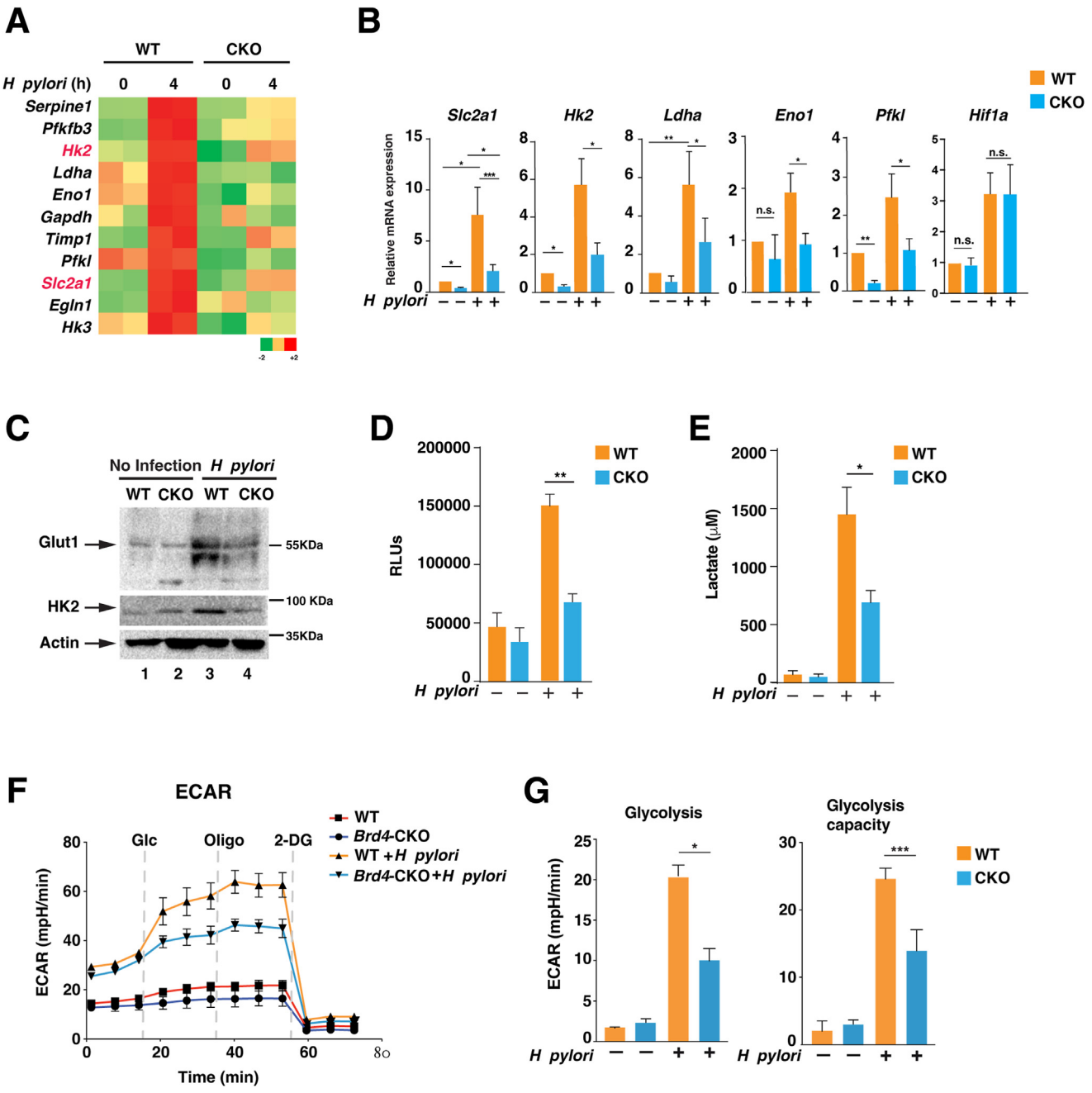


Figure 2. BRD4 deletion leads to reduced glycolysis in macrophages. (*A*) Heat map representation of relative genes in the HIF-1α pathway from Figure 1*B*, color coded by Z-score. (*B*) WT and *Brd4*-deficient BMDMs infected with *H pylori* SS1 (4 h; MOI, 50). Expression of the indicated genes was measured by quantitative RT-PCR. (*C*) WT and *Brd4*-deficient BMDMs were infected with *H pylori* SS1 (8 h; MOI, 50). Protein levels of GLUT1 and HK2 were immunoblotted as indicated. (*D*) Glucose uptake was measured by luminescence in WT and *Brd4*-deficient BMDMs with and without *H pylori* SS1 infection (5 h; MOI, 50) using the Glucose uptake-Glo assay kit. (*E*) Levels of lactate in culture medium of WT and *Brd4*-deficient BMDMs with and without *H pylori* SS1 infection (24 h; MOI, 50) was measured by luminescence using the Lactate-Glo assay kit. (*F*) ECAR was measured in WT and *Brd4*-deficient BMDMs with and without *H pylori* SS1 infection (4 h; MOI, 50) by the Seahorse xFe96 system. (*G*) Glycolysis rate and glycolytic capacity of WT and *Brd4*-deficient BMDMs after *H pylori* infection were calculated from panel *F*. All results are presented as means ± SD of 3 independent experiments. **P* < .05, ***P* < .01, and ****P* < .001. RLU, Relative luminescence units.

(HRE) within the promoter or enhancer regions of glycolytic genes, including *Slc2a1*, *Hk2*, *Ldha*, and *Eno1*.[27,28] We hypothesized that BRD4 may cooperate with HIF-1α to regulate glycolysis because BRD4, a known transcriptional coactivator, was found to regulate several glycolytic genes without directly regulating *Hif1a* transcription (Figure 2*A* and *B*). We co-expressed BRD4 and HIF-1α with a luciferase reporter containing 3 copies of HRE (3 × HRE-luc) in HEK

(human embryonic kidney) 293T cells to test this hypothesis. We found that co-expression of BRD4 enhanced HIF-1α–dependent activation of the 3 $\times$ HRE-luc reporter while co-expression of the bromodomain-deletion mutant of BRD4 failed to enhance HIF-1α–dependent activation of the reporter (Figure 3*A*, left). Similarly, we found that BRD4 enhanced HIF-1α–mediated activation of the luciferase reporter containing the promoter of *Slc2a1* (Figure 3*A*, right). These luciferase reporter assays suggest that BRD4 facilitates HIF-1α–mediated glycolytic gene transcription associating with HIF-1α on the HRE.

We next performed chromatin immunoprecipitation (ChIP) assays to examine the binding of HIF-1α and BRD4 on the promoters of *Slc2a1* and *Hk2*, 2 key glycolytic genes whose expression is regulated by BRD4 (Figure 2*B*). Upon *H pylori* infection, HIF-1α, BRD4, and RNAPII were found to be enriched at the HRE sites on the promoters of *Slc2a1* and *Hk2* in macrophages (Figure 3*B*). Furthermore, treatment of macrophages with a HIF-1α inhibitor, echinomycin, reduced infection-induced enrichment of BRD4 on the promoters (Figure 3*B*). This suggests a HIF-1α–dependent recruitment of BRD4 because echinomycin inhibits the DNA binding activity of HIF-1α by selectively preventing the binding of HIF-1α to the HRE.[29] Therefore, these data indicate that BRD4 cooperates with HIF-1α to regulate the transcription of *Slc2a1* and *Hk2*, likely through its binding to HIF-1α on the HRE of their promoters.

Supporting the notion that BRD4 co-activates HIF-1α–mediated expression of *Slc2a1* and *Hk2* and likely glycolysis in *H pylori*–infected macrophages, we found that treatment of macrophages with echinomycin decreased *H pylori*–induced transcription of *Slc2a1* and *Hk2* (Figure 3*C*). The glucose uptake and the lactate levels in *H pylori*–infected macrophages also were inhibited by echinomycin (Figure 3*D* and *E*). Altogether, these results indicate that HIF-1α is important for the up-regulation of glycolysis in *H pylori*–infected macrophages and BRD4 facilitates HIF-1α–mediated glycolysis by co-activating the expression of certain glycolytic genes.

Inhibition of Glycolysis Limits H pylori*–Induced Inflammatory Gene Expression*

The glycolytic shift in macrophages upon LPS treatment or bacterial infection modulates the inflammatory status of cells.[25] We postulated that BRD4-dependent glycolytic activation upon *H pylori* infection was involved in inflammatory cytokine production in macrophages. We next investigated the effect of the inhibition of glycolysis on *H pylori*–induced proinflammatory gene expression. Pretreatment of the BMDMs with 2-DG, a competitive inhibitor of glycolysis, for 2 hours inhibited *H pylori*–induced expression of proinflammatory genes, including *Nos2*, *Il1b*, and *Il6* (Figure 4*A*). However, 2-DG did not affect *H pylori*–induced *Tnfa* expression (Figure 4*A*). We also found that *H pylori*–induced production of IL-1β in culture media was inhibited by 2-DG (Figure 4*B*). In addition, the protein expression of iNOS in BMDMs also was reduced upon 2-DG treatment (Figure 4*C*). However, no significant changes were found in IL-6 and TNF-α production upon 2-DG treatment (Figure 4*B*). These data suggest a selective regulation of proinflammatory gene expression by glycolysis.

Previous reports have indicated that the *Nos2* transcript might be stabilized post-transcriptionally by the RNA binding protein HuR (human antigen R), whose expression was regulated by glycolysis, in LPS-stimulated macrophages.[30,31] The reduced expression of *Nos2* mRNA and the iNOS protein in 2-DG–treated cells might result from the reduced *Nos2* mRNA stability. To test this possibility, *H pylori*–infected BMDMs were treated with the transcription inhibitor actinomycin D in the presence and absence of 2-DG. In the absence of 2-DG, *H pylori*–induced *Nos2* mRNA was quite stable, with a half-life of more than 60 minutes (Figure 4*D*). In contrast, levels of *Nos2* mRNA in *H pylori*–infected macrophages decreased significantly in the presence of 2-DG, with the half-life shortened to approximately 45 minutes (Figure 4*D*). These data suggest that glycolysis regulates the stability of *Nos2* mRNA in *H pylori*–infected macrophages.

Because *H pylori*–induced glycolysis was regulated by BRD4 (Figure 2), we next assessed whether BRD4 might control the stability of *Nos2* mRNA. Compared with WT BMDMs, the level of *Nos2* mRNA was decreased in the presence of actinomycin D, with a significantly reduced half-life (Figure 4*E*), suggesting that BRD4 indeed regulates the stability of *Nos2* mRNA. To confirm that the reduced *Nos2* mRNA stability was owing to the reduced glycolysis in *Brd4*-deficient BMDMs, we supplemented the *Brd4*-deficient cells with pyruvate, the downstream metabolite of glycolysis. Addition of pyruvate extended the half-life of *Nos2* mRNA in *H pylori*–infected *Brd4*-deficient BMDMs (Figure 4*E*). Similarly, pyruvate also increased the protein level of iNOS in *H pylori*–infected *Brd4*-deficient BMDMs (Figure 4*F*). Collectively, these data show that BRD4-regulated glycolysis is critical for maintaining the stability of *Nos2* mRNA and therefore the production of iNOS upon *H pylori* infection.

BRD4-Regulated Glycolysis Controls NO-Mediated Bactericidal Activity of Macrophages Toward H pylori

The antimicrobial action of iNOS is attributed to its ability to convert L-arginine to L-citrulline and the production of NO, a crucial antimicrobial mechanism of macrophages.[4,32] Macrophages have been shown to kill *H pylori* via NO, which diffuses across the cell membrane and acts as a reactive nitrate species to affect *H pylori* pathogenesis.[33] Therefore, the reduced expression of iNOS in *H pylori*–infected *Brd4*-deficient BMDMs suggests that BRD4 could regulate the ability of macrophages to kill *H pylori*. To test this hypothesis, we performed a bacterial killing assay by co-culturing *H pylori* with WT and *Brd4*-deficient BMDMs for 23 hours and measured the number of extracellular *H pylori.* When WT BMDMs were co-cultured with *H pylori*, extracellular *H pylori* numbers were significantly less than those when *Brd4*-deficient BMDMs were co-cultured with *H pylori* (Figure 5*A*). Macrophages have been shown

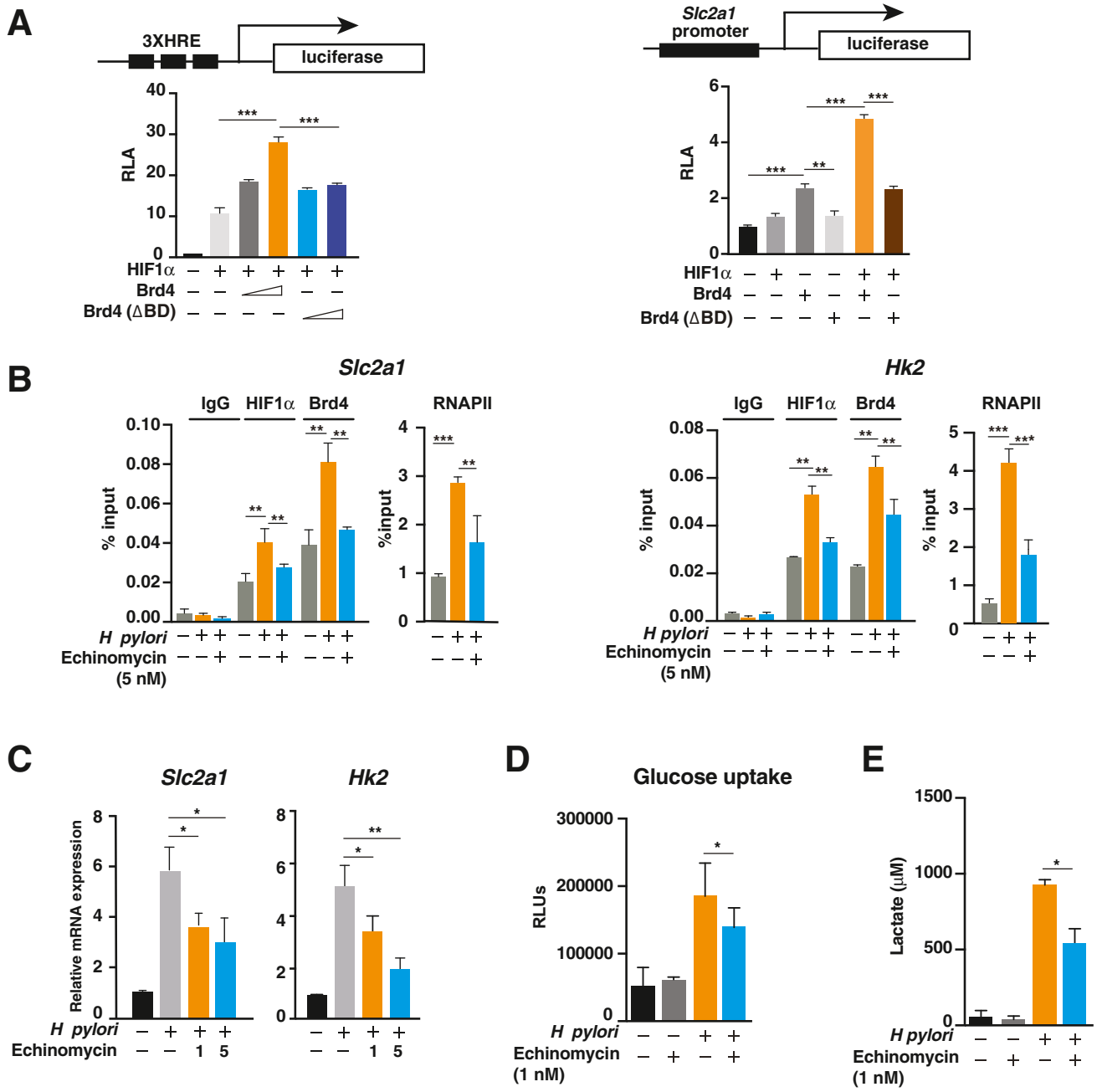


Figure 3. BRD4 and HIF-1α regulate *Slc2a1* and *Hk2* transcription upon *H pylori* infection in macrophages. (*A*) HIF-1α reporter plasmid containing 3 copies of HRE (*left*) or luciferase reporter plasmid with *Slc2a1* promoter containing HRE sites (*right*) was co-transfected with expression vectors for HIF-1α, WT BRD4, or bromodomain deletion mutant BRD4 (ΔBD) into 293T cells as indicated. Luciferase activity was measured 48 hours after transfection. Data represent the means ± SD of 3 independent experiments. ***$P < .001$. (*B*) Immortalized BMDMs were pretreated with or without 5 nmol/L echinomycin for 30 minutes, followed by *H pylori* infection (4 h; MOI, 100). ChIP assays were performed using antibodies against BRD4, HIF-1α, and RNAPII and probed for the promoters of *Slc2a1* (*left panels*) and *Hk2* (*right panels*). (*C*) WT BMDMs were pretreated with or without echinomycin at the indicated concentrations for 30 minutes, followed by *H pylori* infection (4 h; MOI, 100), and the relative expression levels of the indicated genes was measured using quantitative RT-PCR. (*D*) WT and *Brd4*-deficient BMDMs were pretreated with or without echinomycin (1 nmol/L; 30 min), followed by *H pylori* infection (5 h; MOI, 50). The level of glucose uptake were measured using the Glucose-uptake Glo assay kit. PBS was used as control. (*E*) WT and *Brd4*-deficient BMDMs were pretreated with or without echinomycin (1 nmol/L; 30 min), followed by *H pylori* infection (24 h; MOI, 50). Levels of lactate in culture medium were measured by luminescence using Lactate-Glo assay. All results are presented as means ± SD of 3 independent experiments. *$P < .05$, **$P < .01$, and ***$P < .001$. RLA, relative luminescence activity.

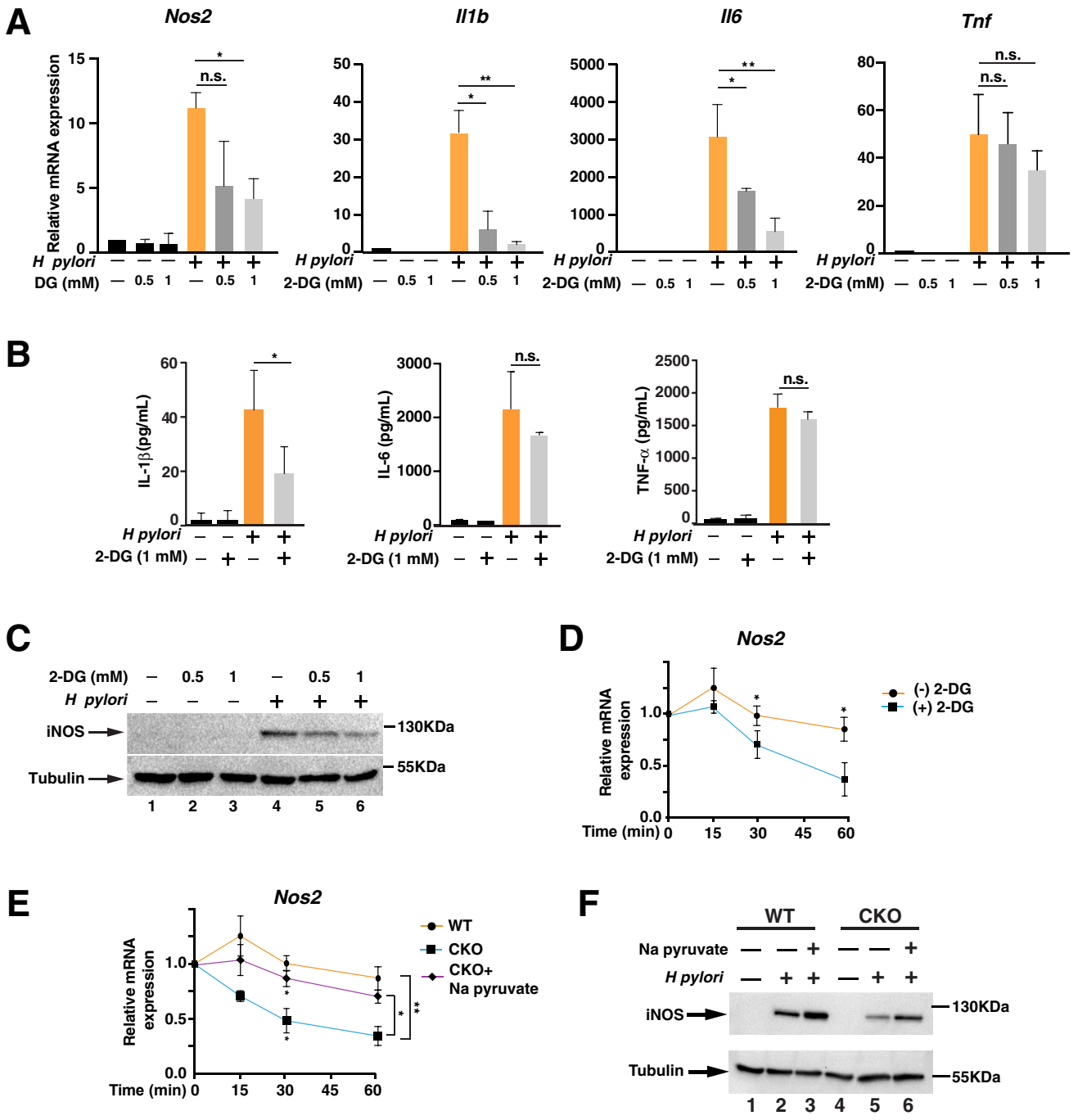


Figure 4. Glycolysis regulates *H pylori*–induced iNOS expression in macrophages. (*A*) BMDMs were pretreated with 2-DG at the indicated concentrations for 2 hours, followed by *H pylori* infection (4 h; MOI, 50). The relative expression levels of the indicated genes were analyzed by quantitative RT-PCR. (*B*) BMDMs were pretreated with 2-DG (1 mmol/L) for 2 hours, followed by *H pylori* infection (24 h; MOI, 50). The levels of IL-1β, IL-6, and TNF-α were measured by ELISA. (*C*) BMDMs were pretreated with or without 2-DG (1 mmol/L) for 2 hours, followed by *H pylori* infection for 24 h. Protein levels of iNOS were immunoblotted as indicated. (*D*) BMDMs were pretreated with or without 2-DG (1 mmol/L) for 2 hours, followed by *H pylori* infection for 4 hours. Cells then were treated with actinomycin D (10 μg/mL) for the indicated time points and the relative expression levels of *Nos2* were measured by quantitative RT-PCR. (*E*) *Brd4*-deficient BMDMs were pretreated with or without 2 mmol/L sodium pyruvate for 1 hour, followed by *H pylori* infection for 4 hours. Cells then were treated with actinomycin D (10 μg/mL) for the indicated time points and the relative expression levels of *Nos2* were measured using quantitative RT-PCR. (*F*) WT and *Brd4*-deficient BMDMs were pretreated with or without 2 mmol/L sodium pyruvate for 1 hour, followed by *H pylori* infection for 24 hours. Protein levels of iNOS were immunoblotted as indicated. All results are presented as means ± SD of 3 independent experiments. $^{*}P < .05$, $^{**}P < .01$.

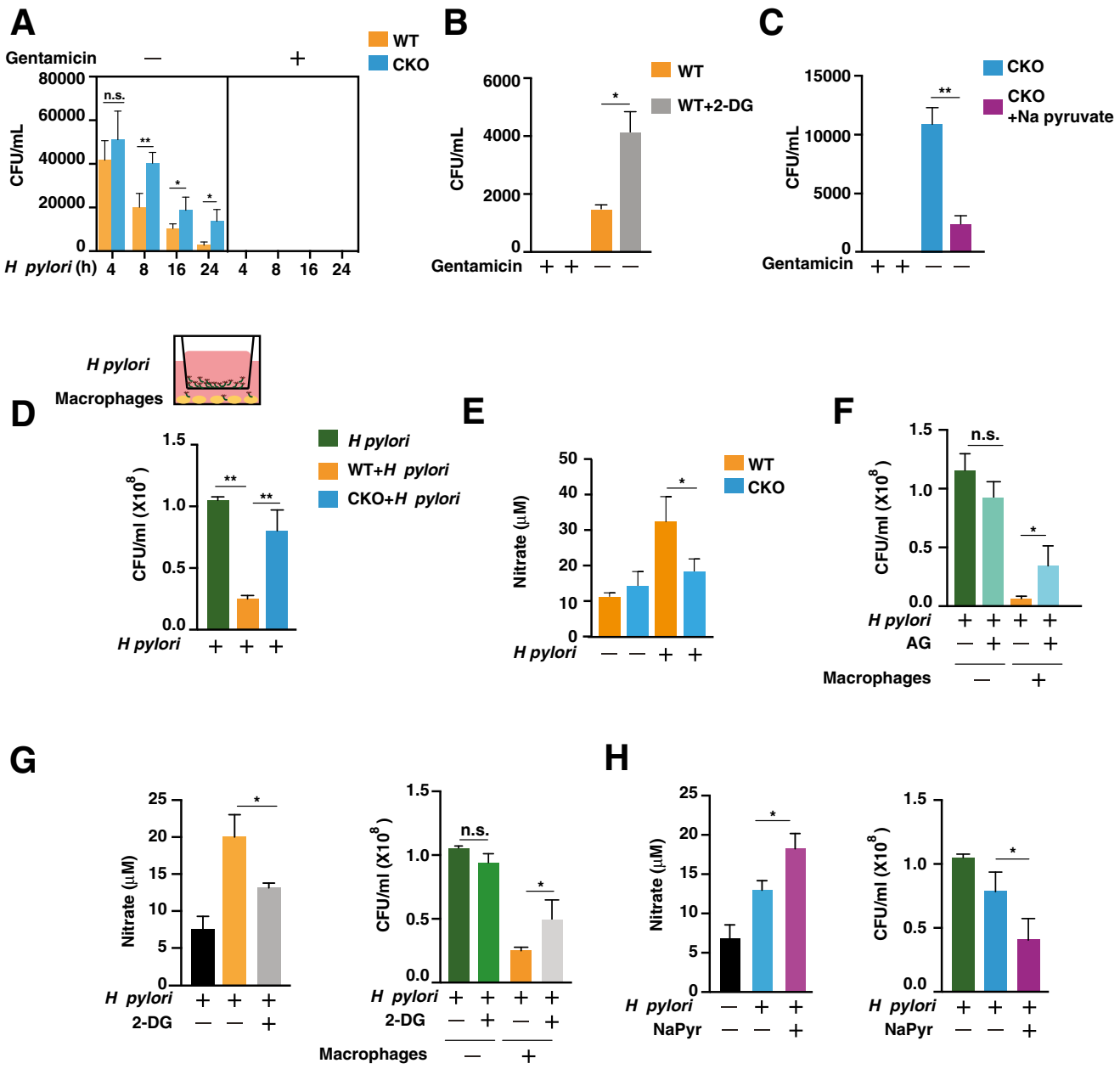


Figure 5. Glycolysis-dependent iNOS expression is essential for the killing of *H pylori*. (*A*) WT and *Brd4*-deficient BMDMs were infected with *H pylori* SS1 for the indicated time points (MOI, 100). Cells were washed and incubated in medium with or without gentamycin (100 μg/mL) for 1 hour. *H pylori* were enumerated after gentamicin-treated cells (*right*) and untreated cells (*left*) were lysed with 0.1% saponin and plated on Columbia agar plates with serial dilutions. Means ± SD are shown, data are representative of 3 independent experiments. (*B*) BMDMs were pretreated with 1 mmol/L 2-DG for 1 hour, followed by *H pylori* infection for 23 hours. *H pylori* were enumerated as in panel *A*. Means ± SD are shown, data are representative of 3 independent experiments. (*C*) *Brd4*-deficient BMDMs were pretreated with 2 mmol/L Na pyruvate for 30 minutes, followed by *H pylori* infection for 23 hours. *H pylori* were enumerated as in panel *A*. Means ± SD are shown, data are representative of 3 independent experiments. (*D* and *E*) *H pylori* SS1 was co-cultured with WT and *Brd4*-deficient BMDMs above 0.4-micrometer Transwell filter supports in DMEM containing 1.4 mmol/L arginine and dialyzed FBS for 24 hours (MOI, 100). (*D*) *H pylori* were enumerated by serial dilutions and (*E*) nitrate levels were measured in culture supernatants. *H pylori* SS1 cultured above Transwell supports without macrophages served as control. Means ± SD are shown, data are representative of 3 independent experiments. (*F*) WT BMDMs were pretreated with 10 mmol/L aminoguanidine (AG) for 1 hour, followed by co-culture with *H pylori* SS1 on Transwell filter supports for 24 hours (MOI, 100). *H pylori* were enumerated as in panel *D*. Means ± SD are shown, data are representative of 3 independent experiments. (*G*) WT BMDMs were pretreated with 1 mmol/L 2-DG for 2 hours, followed by the co-culture of *H pylori* SS1 for 24 hours (MOI, 100). Numbers of *H pylori* (*right*) and levels of nitrate (*left*) were measured as in panels *D* and *E*, respectively. Means ± SD are shown, data are representative of 3 independent experiments. (*H*) *Brd4*-deficient BMDMs were pretreated with 2 mmol/L sodium pyruvate for 1 hour, followed by co-culture with *H pylori* SS1 for 24 hours (MOI, 100). Levels of nitrate (*left*) and numbers of *H pylori* (*right*) were measured as in panels *D* and *E*, respectively. Means ± SD are shown, data are representative of 3 independent experiments. **P* < .05, ***P* < .01. CFU, colony-forming unit; NaPyr, sodium pyruvate.

to fail to internalize *H pylori*,[34] suggesting that the number of *H pylori* obtained in the killing assay (Figure 5*A*) represents the survived extracellular *H pylori* rather than the internalized bacteria not killed by macrophages. Indeed, there were no colonies on the plates when WT or *Brd4*-deficient BMDMs were treated with gentamicin for 1 hour after 23 hours of infection (Figure 5*A*).

We also assessed if glycolysis was involved in the macrophage-mediated killing of *H pylori*. When macrophages were treated with 2-DG, the ability of macrophages to kill extracellular *H pylori* was decreased, and the number of extracellular *H pylori* were increased significantly when cells were pretreated with 2-DG (Figure 5*B*). Furthermore, the addition of sodium pyruvate to *Brd4*-deficient BMDMs, which showed decreased glycolysis (Figure 2), recovered the ability of the macrophages to eradicate *H pylori* (Figure 5*C*). Together, these data show that BRD4-regulated glycolysis is critical for the bactericidal activity of macrophages toward *H pylori*.

To determine whether macrophage-mediated *H pylori* killing is through NO, we used an in vitro system in which Transwell filter supports separated *H pylori* and macrophages.[35] NO released from activated macrophages would diffuse through the filter and kill *H pylori* (Figure 5*D*, top diagram). When *H pylori* grown above the Transwell filter support were co-cultured for 24 hours with WT or *Brd4*-deficient BMDMs, preactivated with *H pylori* at a low multiplicity of infection (MOI) (MOI = 10), we observed that the *H pylori* numbers in a co-culture with activated WT BMDMs were reduced significantly compared with a co-culture of *H pylori* without macrophages (Figure 5*D*). However, the numbers of *H pylori* remained relatively unchanged when co-cultured with *Brd4*-deficient BMDMs (Figure 5*D*). In addition, the levels of nitrate, one of the oxidized products of NO, was increased in activated WT BMDMs, but decreased in *Brd4*-deficient BMDMs, indicating that NO is involved in the killing of *H pylori* (Figure 5*E*). Furthermore, an increase in *H pylori* numbers was observed in activated WT BMDMs treated with aminoguanidine (AG), an iNOS inhibitor involved in limiting arginine transport or utilization.[36] However, AG did not affect *H pylori* growth (Figure 5*F*), suggesting that the decreased ability of macrophages to kill *H pylori* resulted from reduced iNOS-mediated NO production in AG-treated cells. These data support the hypothesis that BRD4 is critical for macrophage-mediated *H pylori* killing by modulating iNOS-mediated NO production.

H pylori–activated glycolysis is critical for maintaining the stability of *Nos2* mRNA and iNOS production (Figure 4*A* and *G*), raising the possibility that glycolysis is involved in the production of NO and the killing of *H pylori.* Supporting this, we found that macrophages treated with 2-DG displayed reduced nitrate levels (Figure 5*G*, left panel) and a decreased ability to kill *H pylori* (Figure 5*G*, right panel). Moreover, the addition of sodium pyruvate to *H pylori*–infected *Brd4*-deficient BMDMs enhanced nitrate levels and the bacterial killing ability of *Brd4*-deficient macrophages (Figure 5*H*). These data indicate that BRD4-regulated glycolysis was involved in NO production and NO-mediated killing of *H pylori* in macrophages (Figure 5*F*).

BRD4 Regulates NO-Mediated Killing of H pylori *and* H pylori*–Induced Inflammation in Mice*

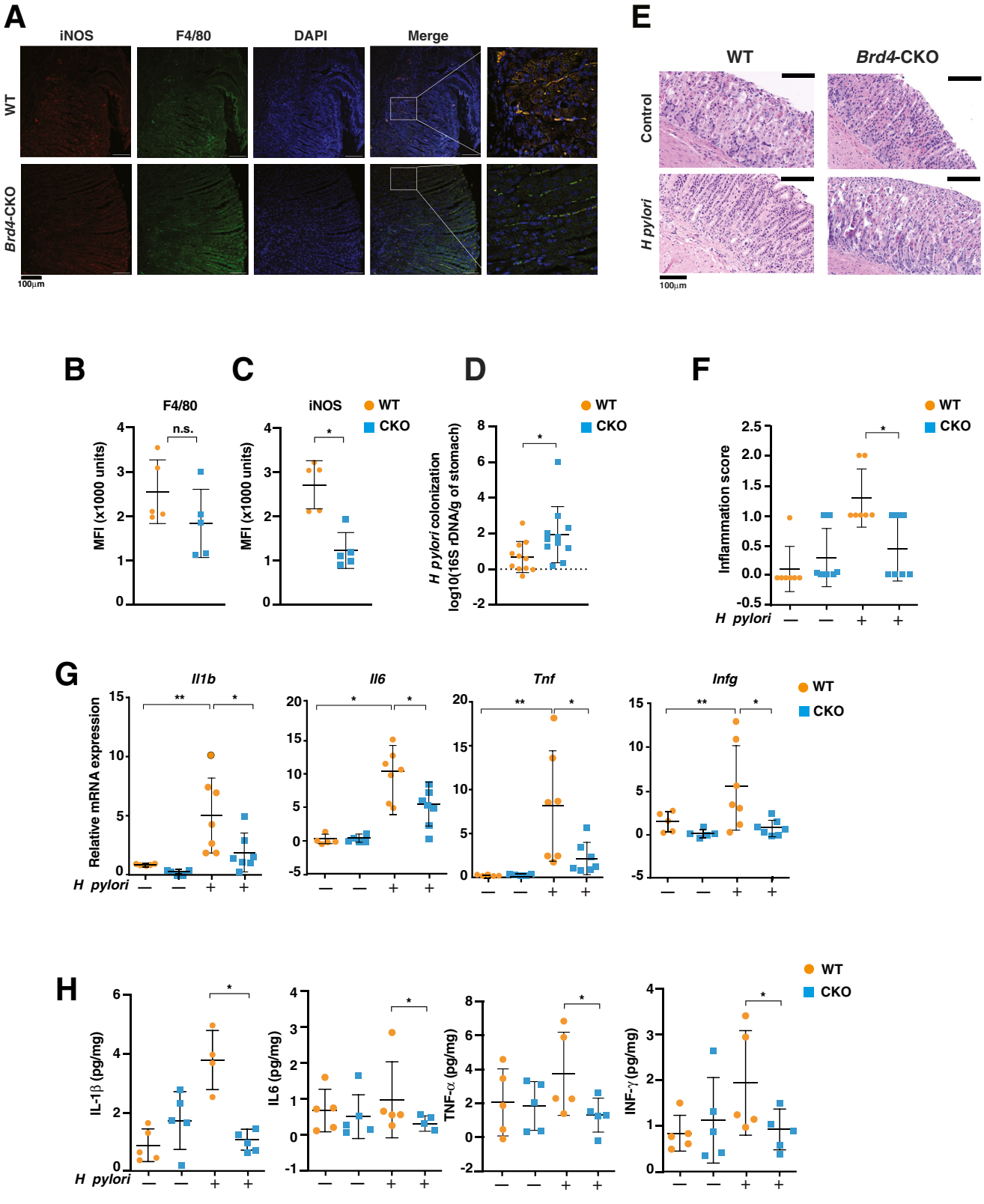
Having identified the critical role of BRD4 in glycolysis, NO production, and *H pylori* killing in BMDMs (Figure 5), we next sought to examine these functions of BRD4 in vivo using WT and myeloid lineage-specific *Brd4*-CKO mice. Gastric tissues from WT and *Brd4*-CKO mice after 4 weeks of *H pylori* infection were assessed for the expression of iNOS by immunofluorescent staining. Gastric tissues of infected WT mice displayed higher levels of iNOS than *Brd4*-CKO mice (Figure 6*A*). Macrophages have been shown to be a major source of iNOS in *H pylori*–infected mice. Increased infiltration of macrophages with increased iNOS expression are found in the gastric mucosa of *H pylori*–infected mice and in human beings with *H pylori*–associated atrophic gastritis.[37,38] We examined macrophage infiltration in gastric tissues with anti-F4/80 antibodies and found that *H pylori* Sydney strain 1 (SS1)–infected WT mice had a stronger F4/80 signal than infected *Brd4*-CKO mice but we found no significant difference in macrophage recruitment (Figure 6*B*), indicating that *Brd4*-CKO mice might have reduced macrophage infiltration. Importantly, the majority of iNOS and F4/80 staining was overlapped, supporting the notion that macrophages are the primary source of iNOS in *H pylori*–infected mice (Figure 6*A*). Quantification of iNOS staining in $F4/80^+$ macrophages showed higher levels of iNOS expression in gastric macrophages of infected WT mice than that of *Brd4*-CKO mice (Figure 6*C*). When we measured the numbers of *H pylori* colonized in gastric tissues of WT and *Brd4*-CKO mice by the 16S ribosomal DNA of *H pylori*, we observed that there were more *H pylori* colonized in *Brd4*-CKO mice than in WT mice (Figure 6*D*). The enhanced colonization of *H pylori* in *Brd4*-CKO mice likely was owing to the decreased NO-mediated *H pylori* killing by gastric macrophages.

We also evaluated gastric inflammation in *H pylori*–infected WT and *Brd4*-CKO mice. As expected, *H pylori*–infected WT mice developed pathologic changes with increased infiltration of immune cells and increased statistical inflammation scores compared with uninfected mice (Figure 6*E* and *F*). In contrast, infected *Brd4*-CKO mice showed attenuated inflammation with less infiltration of immune cells and decreased inflammation scores (Figure 6*E* and *F*). Furthermore, consistent with the gastric inflammation status, infected WT mice showed enhanced mRNA and protein levels of inflammatory cytokines, including IL-1B, IL-6, TNF-α, and interferon-γ. In contrast, *Brd4*-CKO mice had reduced expression levels of these inflammatory cytokines in gastric tissues (Figure 6*G* and *H*). Together, these data show that BRD4 is important for *H pylori*–induced gastric inflammation, which is partially attributable to BRD4-regulated iNOS expression and NO-mediated bacterial killing in gastric macrophages of *H pylori*–infected mice.

Discussion

Macrophages are critical mediators of the innate immune response, and their metabolic reprogramming plays an important role in regulating the functions of these immune

cells, including bacterial killing and cytokine production.[25] Glycolysis has been shown to be critical for the activation of macrophages upon bacterial infection, however, the detailed regulatory mechanisms are not fully understood. In this study, we identified BRD4 as a key regulator of glycolysis and M1 macrophage polarization in response to *H*



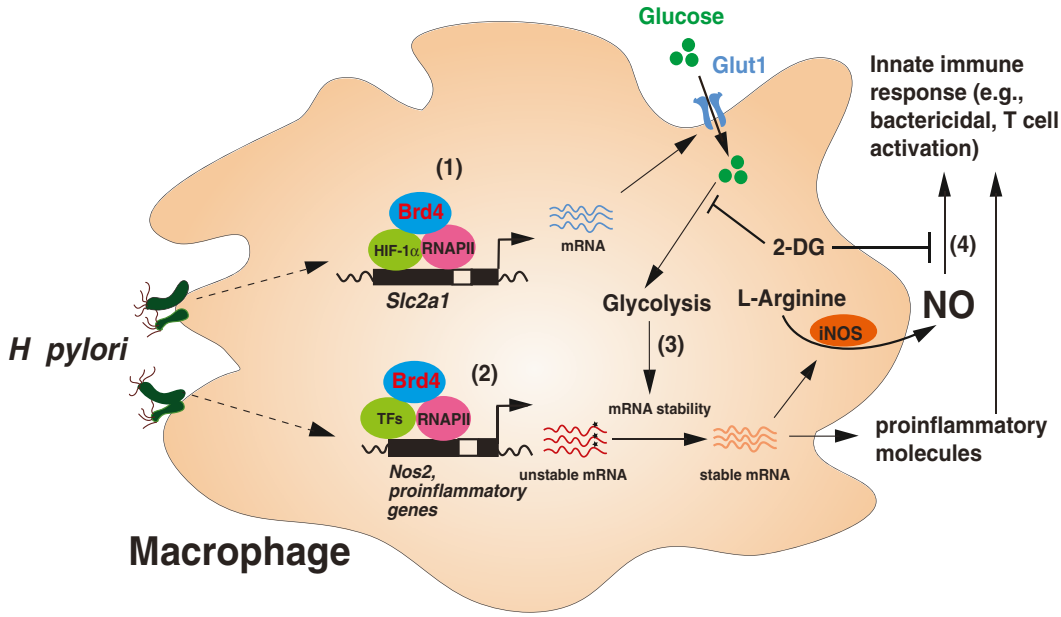


Figure 7. Schematic of BRD4-dependent iNOS regulation during *H pylori* infection in macrophages. Upon *H pylori* infection, BRD4 regulates HIF-1α dependent expression of *Slc2a1* and glycolysis in macrophages (1). BRD4 also regulates the transcription of *Nos2* mRNA (2). The activation of glycolysis stabilizes *Nos2* mRNA for the optimal expression of iNOS and the production of NO (3). NO, along with other inflammatory factors, contributes to the innate immune response during *H pylori* infection (4). TFs, transcription factors.

pylori infection. BRD4 facilitates HIF-1α–dependent expression of glycolytic genes, including *Glut1* and *Hk2*. In addition, BRD4-regulated glycolysis maintains the stability of *H pylori*–induced *Nos2* mRNA for the efficient production of iNOS and NO, a metabolite involved in the elimination of *H pylori* (Figure 7).

Polarization of gastric macrophages to M1 macrophages has been found in *H pylori*–infected mice and *H pylori*–induced atrophic gastritis in human beings.[35,38] Our current study shows that BRD4 is critical for *H pylori*–induced polarization and activation of M1 macrophages because many M1 proinflammatory genes, including *Nos2, Il1b, Il6*, and *Tnfa*, showed diminished up-regulation in *Brd4*-deficient macrophages (Figure 1).

BRD4 likely cooperates with transcription factor NF-κB for the expression of these genes because BRD4 is a key coactivator of NF-κB and regulates the expression of several NF-κB–dependent proinflammatory genes in LPS-stimulated macrophages.[12,39]

A central characteristic of proinflammatory macrophages is the increasing reliance on glycolysis to meet energy and biosynthetic demands, while reinforcing the inflammatory response by modulating the epigenetic landscape.[40] *H pylori*–infected macrophages showed Warburg-like metabolism with increased uptake of glucose, glycolytic flux, and production of lactate (Figure 2). Consistent with the role of glycolysis and glucose uptake in the proinflammatory cytokine expression,[40,41] we observed that 2-DG reduced the expression of certain *H pylori*–induced proinflammatory genes (Figure 4).

An outcome of BRD4-regulated glycolysis is to maintain the stability of *Nos2* mRNA for the efficient production of iNOS (Figure 4*D*). It appears that BRD4 may directly regulate M1 inflammatory gene expression through its

Figure 6. (*See previous page*). BRD4 regulates inflammation in mice upon *H pylori* infection. (*A*) WT and *Brd4*-CKO mice were infected orogastrically with *H pylori* SS1 (10^8 colony-forming units) 3 times every 48 hours. Four weeks after infection, gastric tissues were assessed for the expression of iNOS and F4/80. Representative immunofluorescence images of gastric tissues from infected WT or *Brd4*-CKO mice immunostained for iNOS and F4/80. 4′,6-Diamidino-2-phenylindole (DAPI) represents nuclear counterstaining. (*B*) Quantification of F4/80+ macrophages from panel *A*. Results are shown as means ± SD (n = 5). (*C*) Quantification of iNOS staining in F4/80+ macrophages from panel *A*. Results are shown as means ± SD (n = 5). (*D*) The numbers of *H pylori* from the gastric mucosa of WT and *Brd4*-CKO mice infected for 4 weeks were measured by the relative levels of *H pylori* 16S ribosomal DNA (rDNA) using RT-PCR. Results are shown as means ± SD (n = 11). (*E*) Representative images of H&E staining of gastric tissues of WT and *Brd4*-CKO mice 4 weeks after *H pylori* infection. (*F*) Quantitative inflammation scores assessed from mice in panel *E*, and the results are shown as a median (n = 7 mice/group). (*G*) Relative mRNA levels of indicated cytokines were measured in gastric tissues of WT and *Brd4*-CKO mice with or without *H pylori* infection by quantitative RT-PCR. Results are shown as means ± SD (n = 7). (*H*) Levels of indicated cytokines in gastric tissues of uninfected or infected WT and *Brd4*-CKO mice were measured by enzyme-linked immunosorbent assay (n = 5). Results are shown as means ± SD. $^*P < .05$, $^{**}P < .01$. MFI, Mean flourescence intensity.

interaction with NF-κB or indirectly via its regulation of HIF-1α–mediated glycolysis.

HIF-1α, the master transcriptional regulator of glycolysis, is activated to sustain adenosine triphosphate production during hypoxia.[42] Our KEGG analysis showed a BRD4-dependent differential regulation of genes involved in the HIF-1α signaling pathway in response to *H pylori* (Figure 1). Recent studies also have suggested a role of HIF-1α in the activation and maintenance of M1 polarization upon *H pylori* infection.[43] Our data showed that BRD4 is critical for the polarization of M1 macrophages and HIF-1α–mediated glycolysis without affecting *Hif1a* expression in *H pylori*–infected macrophages (Figure 2*B*). BRD4-dependent activation of *Hif1a* expression has been reported in a melanoma cell line under hypoxic conditions, indicating that *Hif1a* and its target gene expression could be regulated by BRD4 in a cell type– and stimulation-specific manner.[44,45] BRD4 cooperates with HIF-1α to regulate glycolysis via the transcription of *Glut1*, *Hk2*, and likely other glycolytic genes (Figures 3 and 4). The reduced glycolysis in *Brd4*-deficient macrophages may be the result of the reduced HIF-1α–regulated expression of the glucose transporter Glut1 for glucose uptake and decreased expression of HK2. In addition to HIF-1α, BRD4 has been shown to facilitate various transcription factors, including NF-κB, peroxisome proliferator activated receptor γ, and IRF-8, to regulate the expression of distinct genes in macrophages.[12,18,19] The ability of BRD4 to differentially regulate the expression of genes in a context-dependent manner under physiological and pathologic conditions remains an important topic for further exploration.

Up-regulation of glycolytic metabolism in polarized M1 macrophages is critical for the fast generation of adenosine triphosphate needed to sustain phagocytosis and microbial killing.[46] NO is the major mediator responsible for the microbial killing of macrophages.[32] The production of NO and NO-mediated killing of *H pylori* was decreased in *Brd4*-deficient macrophages (Figures 4 and 5). The reduced NO production in *Brd4*-deficient macrophages likely reflected the BRD4-dependent transcriptional regulation of *Nos2* and iNOS (Figures 1*E* and 4*F*). BRD4 is able to regulate the expression of *Nos2* at 2 different levels. At the transcriptional level, BRD4 binds to the promoter of *Nos2* and facilitates NF-κB–dependent transcription of *Nos2*.[47] At the post-transcriptional level, the *Nos2* mRNA stability is regulated by BRD4 because *Nos2* mRNA had a reduced half-life in *Brd4*-deficient macrophages (Figure 4*E*). Importantly, the stability is regulated by BRD4-dependent glycolysis because rescuing glycolysis by sodium pyruvate reversed the diminished *Nos2* mRNA half-life and expression of iNOS in *Brd4*-deficient macrophages (Figure 4*E* and *F*). Therefore, our study highlights a second mechanism through the post-transcriptional regulation of *Nos2* expression by BRD4.

The ability of NO to freely diffuse across the cell membrane gives it a unique advantage as an antimicrobial agent. Several studies have shown that *H pylori* are more sensitive to NO and reactive nitrite species than other enteric bacteria, possibly owing to the lack of NO-sensing machinery and detoxification proteins.[48] *Brd4*-deficient macrophages were defective in the killing of *H pylori* (Figure 5*A* and *D*), indicating a mechanism of BRD4-dependent *H pylori* killing by macrophages. The ability of macrophages to kill *H pylori* can be attributed to NO because inhibition of NO production by aminoguanidine attenuated the bacterial killing ability of macrophages (Figure 5*F*). In a mouse model of *H pylori* infection, we also found increased colonization of *H pylori* in the stomach of *Brd4*-CKO mice compared with WT mice (Figure 6*C*). The increased bacterial colonization in the *Brd4*-CKO mice after 4 weeks of infection reflected a defect in NO-mediated killing of *H pylori* by macrophages owing to reduced iNOS expression in gastric macrophages of *Brd4*-CKO mice (Figure 6*A*–*C*). The increased colonization of *H pylori* in *Brd4*-CKO mice was coupled with reduced inflammation, decreased inflammation scores, and lower proinflammatory cytokine production (Figure 6*D*–*H*). Similar observations were reported in other animal studies, likely owing to the impaired immune response for *H pylori* clearance.[49,50]

iNOS-mediated production of NO also has been shown to contribute to host pathology because *H pylori*–induced atrophic gastritis is associated with increased iNOS expression.[38] Furthermore, *Nos2*-deficient mice showed reduced incidence of gastric adenocarcinoma in a mouse model of gastric cancer by challenging *H* pylori–infected mice with N-methyl-N-nitrosourea.[51] Therefore, iNOS-mediated NO production might have dual roles: first, it contributes to the host's innate immune response by killing *H pylori* during the earlier stage of acute infection as shown in the present study with 4-week infection in mice. Conversely, the continuous production of NO during the later stage of chronic infection might contribute to the development of gastric cancer by inducing DNA damage and by affecting the activity of p53 and poly (adenosine diphosphate-ribose) polymerase.[52] Whether BRD4-regulated NO production contributes to *H pylori* infection–triggered gastric cancer remains unclear and merits further investigation in *Brd4*-CKO mice.

In conclusion, this study shows that BRD4 cooperates with HIF-1α to regulate a glycolysis-mediated innate immune response upon *H pylo*ri infection. BRD4 is a critical regulator of inflammation by not only regulating macrophage cytokine expression but also governing NO production. We previously showed that BRD4 inhibitor JQ1 suppressed gastric inflammation in *H pylori*–infected mice.[15] The anti-inflammatory effect of JQ1 on *H pylori*–infected mice likely result from its effects on both epithelial cells and macrophages. Considering the critical role of macrophage BRD4 in gastric inflammation against *H pylori* infection and the role of iNOS-mediated NO production in the pathogenicity of *H pylori* infection, targeting BRD4 in gastric macrophages may be a rationale for new therapeutic approaches for *H pylori* infection–associated gastric diseases and gastric cancer.

Materials and Methods

Reagents and Antibodies

Sodium pyruvate (P5280) and aminoguanidine (396494) were purchased from Sigma Aldrich. Vancomycin

(97062) was purchased from VWR. Echinomycin (11049) was purchased from Cayman Chemical. 2-DG (4515) was from Tocris Biosciences. Anti-BRD4 antibody (A301-985A) was from Bethyl Laboratories. Antibody against RNAPII (sc-899) was purchased from Santa Cruz Biotechnologies. Antibody against HIF-1α (NB100-449) was purchased from Novus Bio. Anti-GLUT1 antibody (ab115730) was purchased from Abcam. Anti–β-tubulin (T5168) antibody was purchased from Sigma.

Mice

C57BL/6 WT (*Brd4*$^{Flox/Flox}$) and *Brd4*-CKO (*Brd4*$^{Flox/Flox}$-*LyzM*$^{Cre\text{-}cre}$) mice have been described previously.[12] Mice were kept under specific pathogen-free conditions at the animal facilities of University of Illinois at Urbana-Champaign (UIUC). All animal experiments were approved by the UIUC Institutional Animal Care and Use Committee.

Preparation of BMDMs

BMDMs were generated as previously described.[12] Briefly, bone marrow cells were isolated from the femur and tibia of 8- to 12-week-old mice under aseptic conditions. Extracted bone marrow cells were differentiated for 7 days in Petri dishes in Dulbecco's modified Eagle medium (DMEM)/F12 with 10% fetal bovine serum (FBS), L-glutamine (2 mmol/L), penicillin/streptomycin (1:100), HEPES buffer (10 mmol/L), and 20% conditioned medium of L929 cells. After differentiation, macrophages were cultured in media lacking L929 conditioned medium and penicillin/streptomycin for experiments. Immortalized BMDMs have been described previously.[18]

H pylori *Culture and Infection*

The *H pylori* SS1 strain was cultured as previous described.[15] For in vivo infection, 8- to 12-week-old female WT and *Brd4*-CKO mice were infected with 0.2 mL *H pylori* SS1 (10^8 colony-forming units) in brucella broth through oral gavage 4 times every 48 hours (on days 1, 3, 5, and 7) while the control group received only broth. Mice were killed 4 weeks after final infection for analysis. For in vitro experiments, BMDMs were infected with *H pylori* SS1 for the indicated time at a MOI of 50 or 100.

RNA Sequencing

BMDMs were infected with *H pylori* (MOI, 50) for 4 hours and total RNA was prepared using the Bio-Rad Aurum Total RNA Minikit (7326820). A quality check of RNA samples was performed using the Agilent 2100 BioAnalyzer. Complementary DNA library construction and sequencing were performed by BGI with the BGI-SEQ-500 platform. Clean reads were mapped to Mus musculus genome (mm9) using STAR (Spliced Transcripts Alignment to a Reference). Differentially expressed genes were identified with the DEGseq package. Genes with a fold change of $\geq$1.5 and false discovery rates of $\leq$0.1% were considered statistically significant. Gene Ontology enrichment and KEGG pathway enrichment analysis were used for further analysis using the R package, Clusterprofiler.

Quantitative Real-Time PCR and Immunoblotting

BMDMs were infected with *H pylori* SS1 at the indicated time points and total RNA was isolated with the Aurum Total RNA Mini Kit (7326820; Bio-Rad Laboratories) and reverse-transcribed with the iScript Complementary DNA Synthesis Kit (170-8891; Bio-Rad Laboratories) according to the manufacturer's instructions. Quantitative real-time PCR was performed on an ABI 7300 instrument using iTaq Universal SYBR Green Supermix (172-5124; Bio-Rad Laboratories) and appropriate primers. A list of primer sequences can be found in Supplementary Table 1. Immunoblotting was performed as previously described.[39] All Western blots are representative of at least 3 independent experiments.

Metabolic Profiling

The ECAR was measured using the Seahorse xFe96 Extracellular flux analyzer (Agilent Technologies). Briefly, WT and *Brd4*-deficient macrophages were plated at 5×10^4 cells per well on XFe96 plates 1 day before the experiment. Cells then were infected with or without *H pylori* SS1 (5 h; MOI, 50). Cells were washed and plated in XF DMEM with 10% dialyzed FBS and incubated in a non-CO_2 incubator for 1 hour. ECAR was measured using the glycolysis stress kit assay, with addition of 25 mmol/L glucose, 1 mmol/L oligomycin, and 50 mmol/L 2-DG according to the manufacturer's protocol. The drugs were loaded into calibrated XFe96 sensor cartridges (Agilent). All data was background corrected and normalized to cell numbers using Cytation 5 (BioTek). Experiments were performed at the Tissue Engineering and Phenotype Resource core facility at the Cancer Center at Illinois.

mRNA Stability

For measurement of mRNA half-life, WT and *Brd4*-deficient BMDMs were treated with *H pylori* SS1 at a MOI of 50 for 4 hours. Actinomycin D (10 μg/mL) was added to the cells to stop transcription and samples were collected at 15, 30, and 60 minutes after treatment. In each experiment, specific mRNA levels at zero time were arbitrarily assigned a value of 100 and all RNA levels were determined relative to zero time. Cells were pretreated with 2-DG (1 mmol/L) for 2 hours or sodium pyruvate (2 mmol/L) for 1 hour where indicated.

Enzyme-Linked Immunosorbent Assay

Levels of IL-1β (88-7013-88; Invitrogen), TNF-α (88-7324-88; eBiosciences), IL-6 (431304; BioLegend), and interferon γ (430801; BioLegend) in cell supernatants and mouse tissue samples were measured using enzyme-linked immunosorbent assay kits according to the manufacturer's instructions.

Luciferase Reporter Assay

HEK293T cells were transfected using the calcium phosphate transfection method. Luciferase activity was

measured 48 hours after transfection of plasmids using the dual luciferase assay system (E1910; Promega). *Slc2a1* promoter-luciferase reporter plasmid was made by cloning PCR products of *Slc2a1* promoter sequence (-47 to -871 base pairs from TSS (transcription start site)) from mouse genomic DNA into pGL3-basic plasmid (Promega). HRE-luciferase reporter plasmid was provided by Dr Zhang (University of Texas Southwestern).

ChIP–Quantitative PCR

ChIP assays were performed as described previously.[18] Briefly, immortalized BMDMs ($\sim 2 \times 10^7$ cells) were cross-linked with 1% formaldehyde for 10 minutes at room temperature. The cross-link was quenched by 125 mmol/L glycine and cells were sonicated with the Diagenode Bioruptor 300 (Diagenode) to reach the desired genomic fragment length (~300–700 bp). Immunoprecipitation was performed overnight at 4°C by mixing cell lysates, antibodies of interest, and Protein A Magnetic Dynabeads (10001D; Thermo Fisher Scientific). Protein–DNA complexes were reverse cross-linked at 65°C overnight with the addition of proteinase K (0.2 mg/mL). DNAs were purified further with the Qiagen quick spin column and quantitative PCR was performed as described earlier.

Measurement of Nitrate Levels

Levels of nitrate were measured in culture supernatants using the nitrate/nitrite colorimetric assay kit (780001; Cayman Chemicals) according to the manufacturer's instructions.

Glucose Uptake and Lactate Assays

Cells (4×10^4 per well) were plated 1 day before infection on 96-well plates in complete DMEM/F12 medium lacking antibiotics. Cells were infected with *H pylori* SS1 for 5 hours (MOI, 50). Cell culture supernatants were collected and assayed for lactate release by the Lactate-Glo assay kit (J5021; Promega) according to the manufacturer's instructions. Glucose uptake assay was performed using the Glucose uptake-Glo assay kit (J1341; Promega) according to the manufacturer's instructions. Briefly, cells were plated 1 day before infection in DMEM/F12 medium lacking antibiotics on 96-well plates. Cells were infected with *H pylori* SS1 (MOI, 50) for 5 hours. Cells were washed with phosphate-buffered saline (PBS) and incubated with 2-DG for 10 minutes followed by stop buffer and neutralization buffer. Samples were incubated with luciferin detection reagent for 1 hour at room temperature and luminescence was recorded.

In Vitro Bacterial Killing Assay

WT or *Brd4*-deficient BMDMs were infected with *H pylori* SS1 (MOI, 100) for 23 hours. Cells were washed 3 times with 1× PBS and cultured in complete DMEM/F-12 medium with or without 100 μg/mL gentamycin for 1 hour to kill all external bacteria. Cells then were washed and incubated in 0.1% saponin at 37°C for 30 minutes. Serial dilutions of cell lysates were plated on Columbia agar plates for colony-forming unit determination after incubation at 37°C in 10% CO_2 for 2 days.

Transwell Bacterial Killing Assay

WT or *Brd4*-deficient BMDMs were plated at the bottom of the wells of a 12-well plate in complete DMEM/F-12 medium 1 day before the co-incubation experiment. A 0.4-μmol/L Transwell insert (3460; Corning) was placed in the wells and *H pylori* SS1 (MOI, 100) was added above the Transwell support in complete DMEM/F-12 medium for 24 hours. WT and *Brd4*-deficient BMDMs were activated at a MOI of 10 at the time of infection. After 24 hours, *H pylori* was collected and plated as serial dilutions on Columbia agar plates and incubated at 37°C in 10% CO_2 for 4 days.

H&E Staining

Paraffin gastric tissues were sectioned into 4-μm sections and stained by H&E at the Histopathology Laboratory (Department of Comparative Biosciences, College of Veterinary Medicine, UIUC). Images were taken using an EVOS XL Core Microscope (Life Technologies). Inflammation in gastric tissues was quantified by a single pathologist, blinded to each group. Inflammation was graded on an ordinal scale of 0–3 based on the Sydney System: grade 0, no inflammation; grade 1, mild inflammation (slight increase in mononuclear cells); grade 2, moderate inflammation (dense but focal mononuclear inflammatory cells); and grade 3, severe inflammation (dense and diffuse mononuclear inflammatory cells).

Immunofluorescence Staining

Deparaffinized gastric tissue section slides were blocked in 2% FBS in Phosphate Buffered Saline with Tween (PBST) buffer for 1 hour, then incubated with anti-iNOS or anti-F4/80 antibodies (cat. 14-4801-82; ThermoFisher) overnight at 4°C in a humidified chamber. Slides were washed in PBST and incubated with Alexa Fluor 488 donkey anti-mouse (A21206; Invitrogen) or Alexa Fluor 546 donkey anti-rabbit (A10040; Invitrogen) for 1 hour at room temperature in a humidified chamber. Staining solution (1 μg/mL 4′,6-diamidino-2-phenylindole) was added to the slides for 5 minutes. Coverslips were mounted and images were taken using a ZEISS LSM 510 confocal laser scanning microscope. The mean fluorescence intensity of iNOS expression was quantified in F4/80-positive macrophage cells using the Fiji image processing package of Image J2.[53]

H pylori *Colonization Assay*

WT and *Brd4*-CKO mice were killed 4 weeks after infection with *H pylori* SS1. Stomachs of mice were collected and approximately half of a whole stomach was cut into pieces 1 mm in size and homogenized in PBS. DNA was extracted using the QIAamp DNA mini kit according to the manufacturer's protocol. Bacterial colonization was quantified by measuring *H pylori*–specific 16S ribosomal DNA normalized with mouse B2-microglobulin in the same

specimen. The density of *H pylori* was shown as the number of bacterial genomes per nanogram of host genomic DNA.

Statistical Analysis

All data are presented as means ± SD unless otherwise stated. The Student *t* test and Mann–Whitney test were used to analyze the data. Statistical significance was determined using GraphPad Prism9 software (GraphPad). For all data, $P \leq .05$ was considered statistically significant.

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CRediT Authorship Contributions
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Conflicts of interest
The authors disclose no conflicts.

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Supplementary Table 1. Primer Sequences for Quantitative RT-PCR and Plasmid Cloning

Gene	Forward	Reverse
Slc2a1-promoter cloning primer	CTAGCTAGCGGTGTCCGGCTGGGATCG	CCCAAGCTTTTCATCTACCATTTGTTAGG
Nos2	GTTCTCAGCCCAACAATACAAGA	GTGGACGGGTCGATGTCAC
Il1b	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
Tnfa	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG
Il12b	GTCCTCAGAAGCTAACCATCTCC	CCAGAGCCTATGACTCCATGTC
Cxcl10	CCAAGTGCTGCCGTCATTTTC	GGCTCGCAGGGATGATTTCAA
Slc2a1	CAGTTCGGCTATAACACTGGTG	GCCCCCGACAGAGAAGATG
Hk2	GAGCCACCACTCACCCTACT	CCAGGCATTCGGCAATGTG
Ldha	TGTCTCCAGCAAAGACTACTGT	GACTGTACTTGACAATGTTGGGA
Eno1	TGCGTCCACTGGCATCTAC	CAGAGCAGGCGCAATAGTTTTA
Pfk1	GGAGGCGAGAACATCAAGCC	CGGCCTTCCCTCGTAGTGA
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
18s rDNA	GCAATTATTCCCCATGAACG	GGCCTCACTAAACCATCCAA
Slc2a1-ChIP primer	CCAGACTGTGGTCAGTAGCC	TTTTTATAGGACCGCCGCCA
Hk2-ChIP primer	CAGGCTAGTCTTTCTGCGCC	TGTTGAAGAGGCCTTCGGTT
H pylori 16S rDNA	TTTGTTAGAGAAGATAATGACGGTATCTAAC	CATAGGATTTCACACCTGACTGACTATC

rDNA, ribosomal DNA.