Glycolysis-dependent histone deacetylase 4 degradation regulates inflammatory cytokine production

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ABSTRACT **Activation of the inflammatory response is accompanied by a metabolic shift to aerobic glycolysis. Here we identify histone deacetylase 4 (HDAC4) as a new component of the immunometabolic program. We show that HDAC4 is required for efficient inflammatory cytokine production activated by lipopolysaccharide (LPS). Surprisingly, prolonged LPS treatment leads to HDAC4 degradation. LPS-induced HDAC4 degradation requires active glycolysis controlled by GSK3β and inducible nitric oxide synthase (iNOS). Inhibition of GSK3β or iNOS suppresses nitric oxide (NO) production, glycolysis, and HDAC4 degradation. We present evidence that sustained glycolysis induced by LPS treatment activates caspase-3, which cleaves HDAC4 and triggers its degradation. Of importance, a caspase-3–resistant mutant HDAC4 escapes LPS-induced degradation and prolongs inflammatory cytokine production. Our findings identify the GSK3β-iNOS-NO axis as a critical signaling cascade that couples inflammation to metabolic reprogramming and a glycolysis-driven negative feedback mechanism that limits inflammatory response by triggering HDAC4 degradation.**

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INTRODUCTION

Macrophages bind invading agents and initiate the innate immune response by producing inflammatory cytokines. Although critical for host defense against infectious agents, the duration and strength of inflammatory response must also be tightly regulated, as prolonged activation can cause tissue damage and inflammatory disease (Marrack *et al.*, 2010). The inflammatory response is therefore tightly regulated at multiple levels. Activation of macrophages depends on the Toll-like receptors (TLRs), which recognize pathogens or pathogen-derived products and initiate the IκB kinase and mitogen-activated protein kinase cascades. These signal pathways activate transcription factors, including NF-κB and AP-1, leading to transcriptional induction of inflammatory cytokines (Fan and Cook, 2004; Palsson-McDermott and O'Neill, 2004). The production of inflammatory cytokines is further regulated at the translational level by tumor progression locus 2 (TPL2) and mammalian target of rapamycin (mTOR) kinase (Lopez-Pelaez *et al.*, 2012). In addition to these classical signaling pathways, a highly coordinated change in the metabolic program has also been recognized during macrophage activation. Macrophages challenged by bacterial lipopolysaccharide (LPS) activate glycolysis (Rodriguez-Prados *et al.*, 2010). Similarly, dendritic cells (DCs) switch from oxidative phosphorylation to predominantly aerobic glycolysis after exposure to LPS. This shift to glycolysis is required for DC maturation and production of key inflammatory cytokines (Krawczyk *et al.*, 2010). The metabolic program also plays an instructive role in polarizing macrophage into different subclasses with distinct functions (Vats *et al.*, 2006). Although the existence of an immunometabolic network is now well recognized, the signaling pathway that connects glycolysis and the inflammatory response in macrophages remains to be characterized.

Histone deacetylases (HDACs) are protein deacetylases with rapidly growing number of substrates and functions (Choudhary *et al.*, 2009; Norris *et al.*, 2009; Aka *et al.*, 2011). Inhibitors of HDACs have

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Abbreviations used: GSK3, glycogen synthase kinase 3; HDAC, histone deacetylase; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; mTOR, mammalian target of rapamycin; NAME, *N*-nitro-L-arginine methyl ester; NO, nitric oxide.

been investigated for their clinical utility in various diseases (Carafa *et al.*, 2013; Garbes *et al.*, 2013; Qiu *et al.*, 2013). In the immune system, HDAC inhibitors can suppress inflammatory cytokine production and demonstrate an antiinflammatory activity in isolated macrophages and in mice (Leoni *et al.*, 2002; Grabiec *et al.*, 2010). The mechanism by which HDAC inhibitors elicit this activity is not well understood, as the function of HDAC members in macrophages remains largely uncharacterized. It was recently reported that HDAC4, a member of the class IIA HDACs, regulates AP-1–dependent inflammatory cytokine transcription in skeletal muscle subject to denervation (Choi *et al.*, 2012). Inactivation of HDAC4 suppressed the production of inflammatory cytokines and partially spared denervated muscles from atrophy (Moresi *et al.*, 2010; Choi *et al.*, 2012). These findings reveal a regulatory activity of HDAC4 in the inflammatory cytokine program associated with skeletal muscle remodeling (Choi *et al.*, 2012). Whether HDAC4 plays a similar role in immune cells is not known.

In this study, we present evidence that HDAC4 is a new component of the immunometabolic network that controls inflammatory cytokine production. In BV2 microglia, a model for CNS macrophages, loss of HDAC4 impairs LPS-induced proinflammatory cytokine synthesis. Despite its requirement for inflammatory cytokine production, we found that HDAC4 is degraded upon prolonged LPS treatment. LPS-induced HDAC4 degradation requires active glycolysis. Sustained glycolysis activates caspase-3, which cleaves and promotes HDAC4 degradation. In LPS-challenged macrophages, we identified glycogen synthase kinase 3β (GSK3β)– and inducible nitric oxide synthase (iNOS)–dependent production of nitric oxide (NO) as the critical effector that activates glycolysis and caspase-3–dependent HDAC4 degradation. Of importance, forced expression of a caspase-3–resistant HDAC4 mutant prevents LPS-mediated degradation and extends the duration of inflammatory cytokine production. Our findings identify HDAC4 as a new regulatory component targeted by the immunoglycolytic program to affect the strength and duration of inflammatory cytokine production.

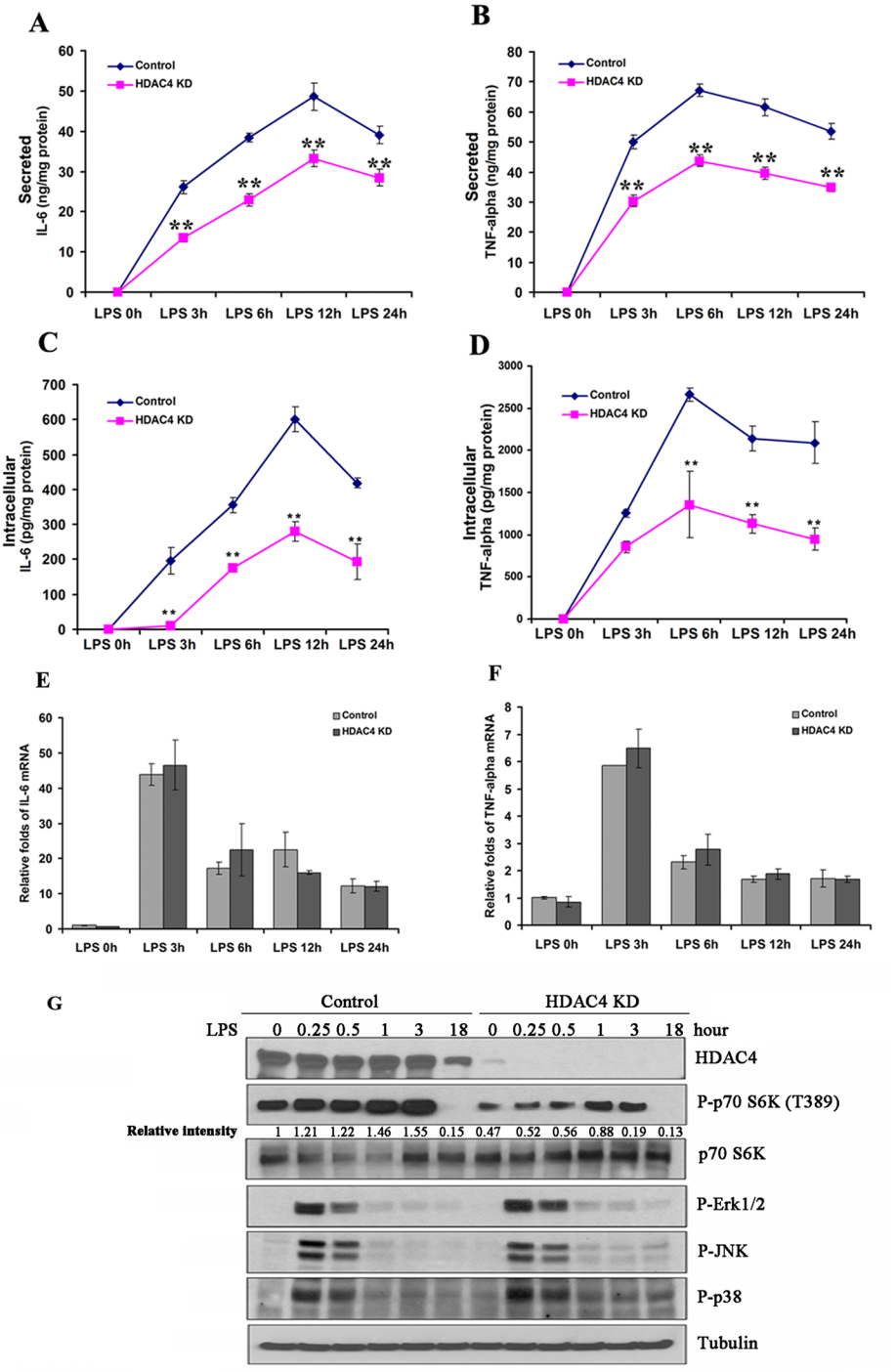

FIGURE 1: HDAC4 is required for inflammatory cytokines production and p70 S6K in activated BV2 cells. (A, B) Control and HDAC4 KD BV2 cells were treated with LPS (1 μg/ml) for indicated times. Medium was collected and analyzed for secreted IL-6 and TNF-α by ELISA. (C, D) Cell lysates were subject to ELISA to determine intracellular IL-6 and TNF-α production. Note that LPS-induced secreted and intracellular IL-6 and TNF-α were both much reduced in HDAC4-KD BV2 cells compared with control cells (**$p < 0.01$ vs. control). (E, F) BV2 cells treated by LPS were harvested at indicated time points. Total RNAs were extracted, and real-time PCR was performed for IL-6 and TNF-α. (G) BV2 cells were treated with LPS at indicated time points, and cell lysates were subject to immunoblotting with antibodies for HDAC4, phosphorylated Erk1/2, JNK, p38, and p70 S6K (T389) as indicated.

RESULTS

HDAC4 is required for efficient p70 S6K signaling and inflammatory cytokine production

To determine whether HDAC4 is involved inflammatory cytokine production in macrophages, we knocked down HDAC4 in a microglia cell line (BV2) by a small interfering RNA (siRNA) and/or short hairpin RNA (shRNA; Figure 1G; see *Materials and Methods*). Microglia are resident macrophages in the CNS and play important roles in neuroinflammation (Perry and Gordon, 1988). As shown in

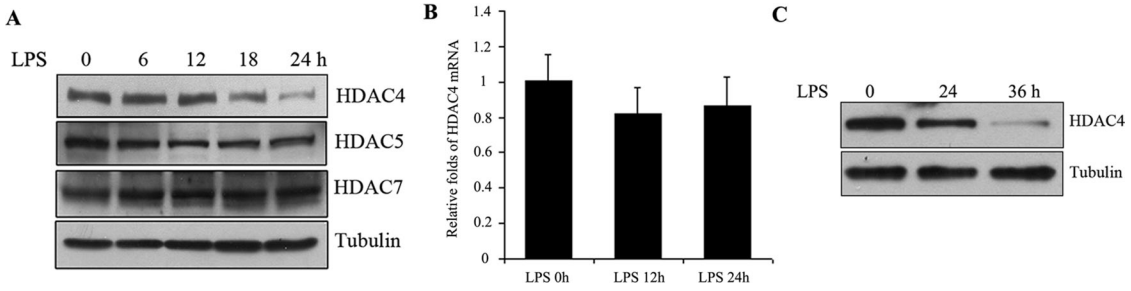

FIGURE 2: HDAC4 was degraded after prolonged LPS treatment. (A) BV2 cells were treated with LPS (1 μg/ml) for indicated times, followed by immunoblotting with antibodies for HDAC4, HDAC5, HDAC7, and α-tubulin as indicated. Note that HDAC4, but not HDAC5 or HDAC7, was markedly reduced after prolonged LPS treatment (18–24 h). (B) HDAC4 mRNA level was checked by real-time PCR after LPS treatment. LPS only modestly affected HDAC4 mRNA after LPS treatment. (C) Bone marrow–derived primary macrophages were treated with LPS (1 μg/ml) at indicated times, followed by immunoblotting for HDAC4. Note that HDAC4 protein level also was decreased after LPS treatment for 24 and 36 h.

Figure 1, A and B, HDAC4-knockdown (KD) BV2 cells secreted much less interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) than control cells (by ~30–50%) upon LPS treatment. Intracellular IL-6 and TNF-α protein levels were also significantly decreased in HDAC4 KD cells, indicating that synthesis of cytokines is reduced (Figure 1, C and D). The induction of IL-6 and TNF-α mRNA by LPS, however, was not affected by HDAC4 KD (Figure 1, E and F). These results indicate that HDAC4 regulates inflammatory cytokine production posttranscriptionally, possibly at the translation level.

In macrophages, efficient translation of inflammatory cytokines requires a MAP3 kinase, TPL2, which regulates translation by activating the mTOR kinase (Lopez-Pelaez *et al.*, 2012). We found that phosphorylation of an mTOR downstream effector, p70 S6K, was markedly reduced in HDAC4 KD macrophages compared with their control counterparts (Figure 1G). In contrast, phosphorylation of the mitogen-activated protein kinases (MAPKs; ERK, JNK, and p38) was not affected by HDAC4 KD (Figure 1G). Thus HDAC4 deficiency selectively affects mTOR-p70 S6K but not MAPK signaling. These results indicate that HDAC4 is required for normal inflammatory cytokine production and mTOR-p70S6K signaling in BV2 cells activated by LPS.

Prolonged LPS activation leads to HDAC4 degradation

Although HDAC4 is required for inflammatory cytokine production, a time-course analysis revealed that HDAC4 level in BV2 cells was markedly reduced after prolonged LPS treatment (18–24 h; Figure 2A). This effect is specific, as the closely related class IIA HDAC members HDAC5 and HDAC7 were not affected by the same treatment (Figure 2A). HDAC4 mRNA level was not obviously affected by LPS treatment (Figure 2B). A similar loss of HDAC4 was also apparent in primary bone marrow–derived macrophages challenged by LPS (Figure 2C). These results show that prolonged LPS treatment leads to HDAC4 degradation in macrophages.

LPS-induced HDAC4 degradation is activated by glycolysis-dependent caspase-3 activation

At the time when HDAC4 protein degradation became apparent, 18–24 h after LPS treatment, we noticed that the culture medium had turned visibly yellow, indicative of acidification. Because LPS-challenged macrophages activate glycolysis and produce lactate (Rodriguez-Prados *et al.*, 2010), whose accumulation would acidify the medium, we asked whether HDAC4 degradation was linked to glycolysis. We confirmed that glucose uptake and glycolysis were indeed elevated in BV2 cells upon LPS treatment, and knockdown of HDAC4 had no apparent effect on this activity (Figure 3, A–C). Of importance, inhibition of glycolysis by a nonmetabolizable glucose analogue, 2-deoxyglucose (2-DG), or a lactate dehydrogenase inhibitor, oxamic acid, effectively blunted LPS-induced HDAC4 degradation in BV2 cells (Figure 3, D and E, top). These results indicate that active glycolysis is required for LPS-induced HDAC4 degradation.

We next searched for the molecular mechanism that connected LPS-induced glycolysis and HDAC4 degradation. HDAC4 was previously identified as a substrate of caspase-3 (Paroni *et al.*, 2004). Cleavage by caspase-3 promotes HDAC4 degradation (Liu *et al.*, 2004). We therefore investigated whether caspase-3 was involved in LPS-induced HDAC4 degradation. As shown in Figure 3F, LPS treatment induced a cleaved and active form of caspase-3, whose appearance was temporally correlated with the loss of HDAC4 protein (16–24 h posttreatment). Of importance, the application of glycolysis inhibitors, 2-DG and oxamic acid, which prevented HDAC4 degradation, also blunted LPS-induced caspase-3 activation (Figure 3, D and E, middle). These data suggest that prolonged activation and glycolysis by LPS activates caspase-3, which then triggers HDAC4 degradation. Supporting this proposal, a pan-caspase inhibitor, Z-VAD-FMK, blocked LPS-induced caspase-3 activation and HDAC4 degradation (Figure 3G). Knockdown of caspase-3 by siRNA also markedly prevented LPS-induced HDAC4 degradation (Figure 3H). Of note, HDAC4 degradation is not a consequence of apoptosis and necrosis, as Z-VAD-FMK treatment increased rather than decreased apoptosis and necrosis associated with LPS treatment (Figure 3I), an observation consistent with a previous report (Kim *et al.*, 2001). These results show that LPS-induced HDAC4 degradation requires glycolysis-dependent caspase-3 activation.

GSK3β-dependent NO production activates LPS-induced glycolysis and HDAC4 degradation

Previously, GSK3β was implicated in HDAC4 degradation via an unknown mechanism (Cernotta *et al.*, 2011). Because GSK3β is also a critical regulator in proinflammatory cytokine production (Martin *et al.*, 2005), we asked whether GSK3β was involved in LPS-induced glycolysis and HDAC4 degradation. We found that GSK3β inhibitors LiCl (Figure 4A) and SB216763 (Supplemental Figure S1A) both effectively suppressed LPS-induced HDAC4 degradation (top), as well as caspase-3 activation (middle). Of importance, GSK3β inhibition also decreased glucose uptake and glycolysis in LPS-activated BV2 cells (Figure 4, B and C, and Supplemental Figure S1B). These results indicate that GSKβ signaling is critical for LPS-induced glycolysis, caspase-3 activation, and HDAC4 degradation.

In LPS-activated BV2 cells, GSK3β regulates the induction of iNOS, which produces the NO important for mounting an effective inflammatory response (Yuskaitis and Jope, 2009). Of interest, NO

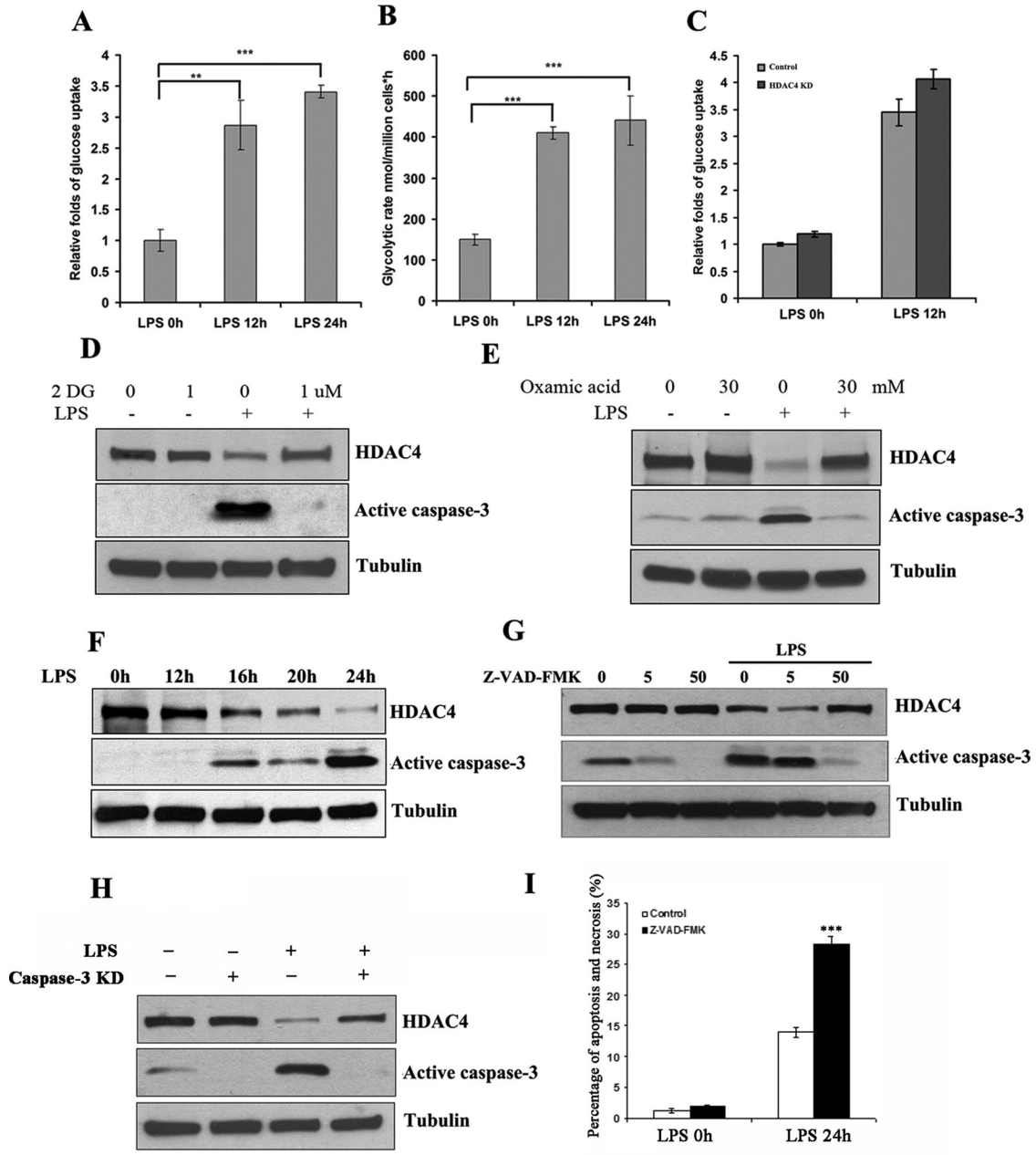

FIGURE 3: Glycolysis-coupled caspase-3 activation leads to LPS-induced HDAC4 degradation. (A) LPS promoted glucose uptake in BV2 cells at 12 and 24 h (**$p < 0.01$ and $p < 0.001$ vs. LPS 0h). (B) Glycolytic rate was determined in activated BV2 cells. LPS dramatically increased glycolytic rate at 12 and 24 h (***$p < 0.001$ vs. LPS 0h). (C) HDAC4 KD had no apparent effect on glucose uptake in BV2 cells upon LPS treatment at 12 h. (D, E) BV2 cells were pretreated with 2-dexoy-D-glucose (2DG; 1 μM) or oxamic acid (30 mM) for 4 h, followed by LPS treatment for 24 h. Both 2DG and oxamic acid pretreatment markedly blunted LPS-induced HDAC4 degradation. (F) HDAC4 and active caspase-3 protein levels in BV2 cells were determined by immunoblotting at indicated time points after LPS treatment. Note that the appearance and abundance of active caspase-3 coincided with the loss of HDAC4. (G) BV2 cells were pretreated with pan-caspase inhibitor Z-VAD-FMK for 4 h at indicated concentrations, followed by LPS treatment for 24 h. High dose (50 μM) of Z-VAD-FMK effectively abolished caspase-3 activation and prevented HDAC4 degradation. (H) BV2 cells were transfected with a siRNA for caspase-3, followed by 24 h LPS treatment. Caspase-3 KD effectively abolished LPS-induced HDAC4 degradation. (I) BV2 cells were untreated or pretreated with Z-VAD-FMK for 4 h, followed by LPS treatment. At the indicated time points, apoptosis was analyzed by fluorescence-activated cell sorting using Annexin V-PE and 7-AAD Apoptosis Detection Kit. Pretreatment of Z-VAD-FMK did not suppress LPS-induced apoptosis (***$p < 0.001$ vs. control).

was previously shown to activate glycolysis in astrocytes (Almeida *et al.*, 2004). We therefore investigated whether NO played a role in LPS-induced glycolysis and HDAC4 degradation. We confirmed that iNOS protein and NO were robustly induced in BV2 cells by LPS treatment, and these inductions were clearly reduced by GSK3β inhibitors (Figure 4, D and E, and Supplemental Figure S1, C and D). Of importance, treatment with *N*-nitro-L-arginine methyl ester (NAME), an irreversible inhibitor for iNOS and NO production, effectively suppressed LPS-induced NO, glucose uptake, and glycolysis (Figure 4, F–H). In addition, NAME treatment

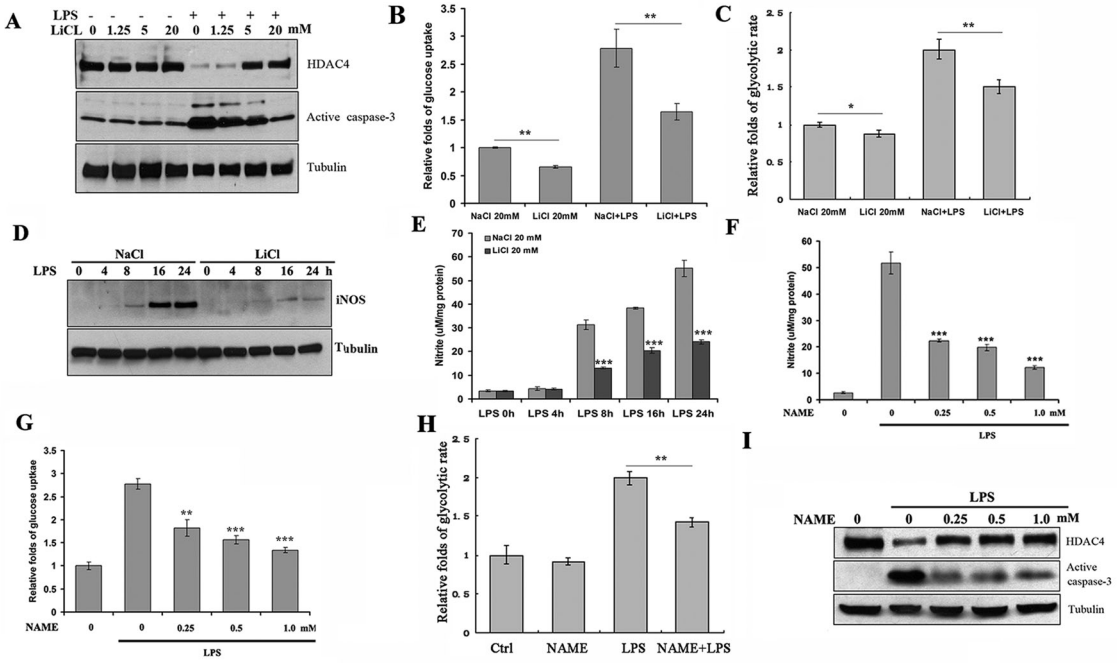

FIGURE 4: GSK3β activated LPS-induced glycolysis and HDAC4 degradation through NO. (A) BV2 cells were treated with a GSK3β inhibitor LiCl at indicated concentrations alone or with LPS (1 μg/ml) for 24 h. The levels of active caspase-3 and HDAC4 were determined by immunoblotting. LiCl markedly abolished LPS-induced caspase-3 activation and HDAC4 degradation. (B, C) BV2 cells were treated with LiCl (20 mM) and LPS alone or in combination as indicated. Glucose uptake assay was performed after 24 h of LPS treatment. LiCl markedly blunted LPS-induced glucose uptake and glycolysis, respectively (*$p < 0.05$ and **$p < 0.01$ vs. only LPS treatment). (D, E) BV2 cells were cotreated with LiCl (20 mM) and LPS for indicated times. iNOS protein expression and NO production were determined. LiCl strongly inhibited LPS-induced iNOS protein expression and NO production (***$p < 0.001$ vs. only LPS treatment). (F) BV2 cells were treated with indicated concentrations of an iNOS inhibitor, NAME, and LPS. Production of NO was strongly inhibited by NAME in a dose-dependent manner (***$p < 0.001$ vs. only LPS treatment). (G, H) BV2 cells were pretreated with indicated concentrations of an iNOS inhibitor, NAME, for 4 h, followed by LPS treatment for another 24 h. NAME pretreatment inhibited LPS-induced glucose uptake and glycolysis. (I) NAME pretreatment abolished LPS-induced caspase-3 activation and HDAC4 degradation (***$p < 0.001$ vs. only LPS treatment).

also decreased caspase-3 activation and HDAC4 degradation (Figure 4I). These results identify GSK3β and NO as critical regulators of LPS-induced glycolysis and further confirm the critical role of glycolysis and caspase-3 activation in HDAC4 degradation.

Caspase 3-resistant HDAC4 mutant extends the duration of inflammatory cytokine production

The degradation of HDAC4 occurred late during the LPS response, when the production of inflammatory cytokines has begun to decline (Figure 2A). As HDAC4 is required for a robust production of IL-6 and TNF-α (Figure 1, A and B), these observations suggest that HDAC4 degradation might contribute to the attenuation of inflammatory cytokine production. To test this hypothesis, we mutated the caspase-3 cleavage site in HDAC4 by converting aspartic acid (D289) to glutamic acid (E). The HDAC4-D289E mutant was previously shown to be resistant to caspase-3 cleavage (Liu *et al.*, 2004; Paroni *et al.*, 2004). We electroporated wild-type and D289E mutant HDAC4 into BV2 cells and subjected them to LPS treatment. As shown in Figure 5A, ectopic wild-type HDAC4, similar to endogenous HDAC4, was clearly degraded after prolonged LPS treatment. In contrast, the level of caspase-3–resistant HDAC4-D289 mutant remained steady, supporting the importance of caspase-3–mediated cleavage in promoting HDAC4 degradation. Of importance, BV2 cells expressing HDAC4-D289E mutant, but not wild-type HDAC4, produced markedly more IL-6 and TNF-α than control cells at the later time points of LPS treatment (Figure 5, B and C, 12–36 h). Consistent with HDAC4 regulating inflammatory cytokine posttranscriptionally (Figure 1), IL-6 and TNF-α mRNA levels were not affected by HDAC4-D289E mutant (Figure 5, D and E). Furthermore, phosphorylation of the mTOR target, p70 S6 kinase, was also elevated in HDAC4-D289E–expressing cells at 24 and 36 h compared with control BV2 (Figure 5A). These results indicate that HDAC4 degradation contributes to the attenuation of inflammatory cytokine production after prolonged LPS activation.

DISCUSSION

In macrophages activated by LPS, the robust induction of glycolysis is an intricate part of the inflammatory response. The signaling cascade that couples macrophage activation to glycolytic reprogramming remains poorly characterized. In this article, we identified GSK3β, iNOS, and NO as a key signaling axis that coordinates inflammation and metabolic reprogramming in macrophages. Our analysis indicates that the production of NO is required for both the classical inflammatory response and the glycolytic switch associated with macrophage activation. We further identified HDAC4 as a new component of this immunometabolic network, where it is required for full mTOR signaling and normal cytokine production. Of importance, prolonged LPS activation triggers glycolysis-dependent and caspase-3–mediated HDAC4 degradation. The glycolysis-driven HDAC4 degradation suggests a metabolism-based feedback mechanism that could attenuate inflammatory response upon extended LPS exposure.

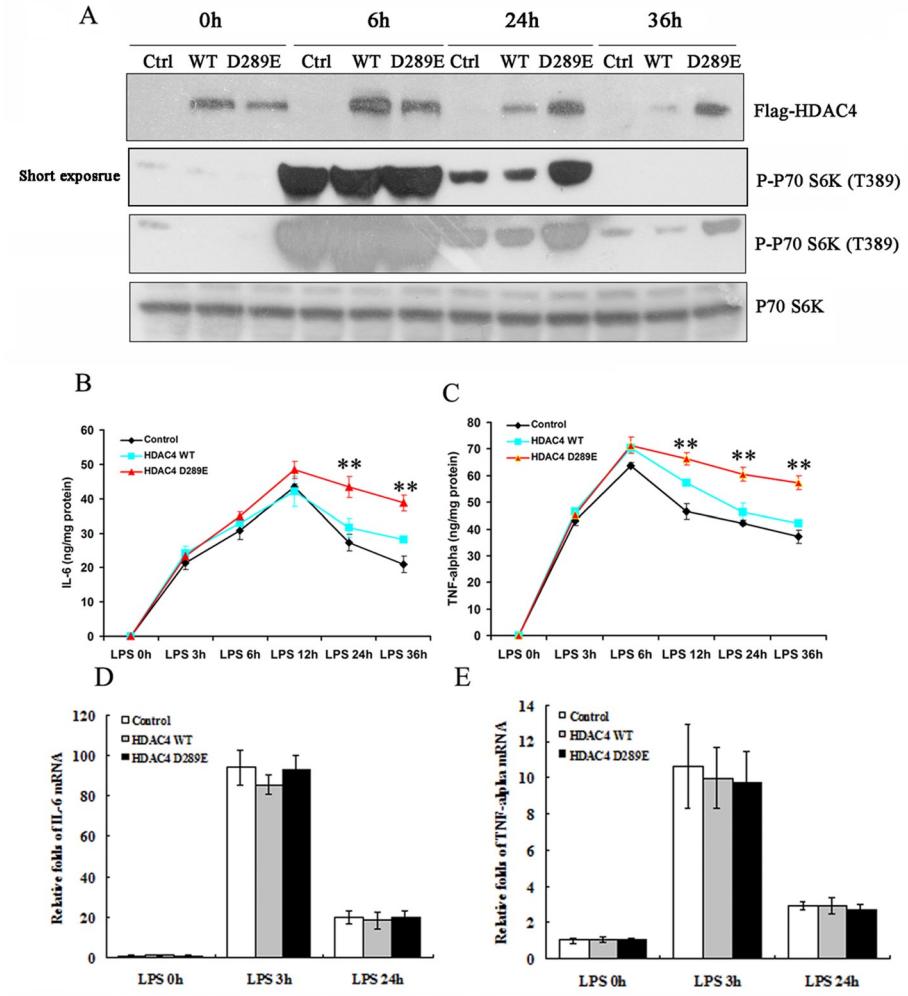

FIGURE 5: Caspase-resistant HDAC4 mutant extends the duration of inflammatory cytokine production. (A) FLAG-tagged wild-type (WT) and D289E mutant HDAC4 were transfected into BV2 cells by electroporation, followed by LPS treatment for 36 h. The ectopic HDAC4 was detected by an FLAG antibody. Note that caspase-3–resistant mutant HDAC4 (D289E) is resistant to LPS-induced degradation and caused elevated p70 S6K (T389) phosphorylation at 24 and 36 h after LPS treatment. (B, C) Media from WT and D289E mutant HDAC4–expressing BV2 cells were analyzed for secreted IL-6 (B) and TNF-α (C) by ELISA at indicated time points after LPS treatment. Note that both IL-6 and TNF-α levels were markedly higher in D289E mutant HDAC4–expressing BV2 cells than in control and WT HDAC4–expressing cells at later but not early time points (**$p < 0.01$ vs. HDAC4 WT). (D, E) Real-time PCR showed that mRNA expression of IL-6 and TNF-α was not affected by overexpressing caspase-3–resistant HDAC4 (D289E).

Our results identified HDAC4 as a positive regulator of inflammatory cytokine production in macrophages. HDAC4 was reported to promote the transcription of inflammatory cytokine genes in denervated muscle by deacetylating and activating MEKK2, which stimulates the MAPK–AP1 transcriptional axis (Choi *et al.*, 2012). In macrophages, our evidence suggests that HDAC4 regulates cytokine production posttranscriptionally, at least in part, by promoting mTOR-p70 S6K signaling, that which cytokine translation. Rapamycin, an mTOR inhibitor, did not affected glucose uptake, but it dramatically inhibited LPS-induced P70 S6K activation and proinflammatory cytokine productions (Supplemental Figure S2). Although it remains to be determined how HDAC4 regulates mTOR activity and whether a specific deacetylation event is involved, the characterization of HDAC4 in the inflammatory network has revealed a unique regulatory relationship between HDAC4 and kinase signaling, underscoring the cross-talk between acetylation- and phosphorylation-dependent signaling cascades.

We found that HDAC4 was degraded after prolonged LPS stimulation. This degradation requires active glycolysis, suggesting that it is a part of the immunometabolic program required for proper inflammatory response. Glycolysis-dependent HDAC4 degradation requires caspase-3, which becomes activated after prolonged LPS treatment. Of note, although our data do not support a major role of caspase-3–dependent apoptosis in HDAC4 degradation (Figure 3I), our results do not exclude a potential involvement of apoptosis in this process. Of importance, the expression of a caspase-3–resistant HDAC4 mutant escaped LPS-induced degradation and prolonged the production of TNF-α and IL-6 (Figure 5). Given the positive role of HDAC4 in the production of TNF-α and IL-6 (Figure 1), temporally regulated HDAC4 degradation might contribute to the natural attenuation of inflammatory cytokine production. Our results suggest a potential metabolism-based safeguard mechanism by which glycolysis not only drives the proinflammatory response, but also limits the duration of inflammatory cytokine production by triggering the eventual degradation of HDAC4.

MATERIALS AND METHODS

Antibodies and reagents

The following antibodies were used: HDAC5, HDAC7, and MyD88 (Abcam, Cambridge, MA); active caspase-3, p-P70 S6K (T389), and P70 S6K antibodies (Cell Signaling, Danvers, MA); α-tubulin and FLAG antibodies (Sigma-Aldrich, St. Louis, MO). Polyclonal anti-HDAC4 antibody was described previously (Zhao *et al.*, 2001). GSK3 inhibitor SB216763, iNOS inhibitor NAME, Griess reagent, and LPS were purchased from Sigma-Aldrich.

Cell culture

The murine microglial BV2 cell line was cultured in DMEM (GIBCO) supplemented with 10% FBS, penicillin G 100 U/ml, and streptomycin 100 U/ml and incubated in humidified incubators at 37°C with 5% CO_2. BV2 cells were seeded into six-well plates (1×10^6 cells/well) and stimulated by LPS (1 μg/ml) on the next day. Cell culture supernatant and cell lysates were collected at indicated time points and stored at −70°C before analysis. Murine bone marrow–derived primary macrophages were prepared as described previously (Chin *et al.*, 2002).

Plasmids and siRNA

HDAC4 knockdown in BV2 cells was accomplished by two sequential transfections with a HDAC4 siRNA (5′-GAGCAGCAGAGGAUCCACCAGUUAA-3′) (Choi *et al.*, 2012) or a single transfection into a BV2 line that stably expresses a HDAC4 shRNA

(5′-GCCAAAGATGACTTCCCTCT-3′) transduced by lentivirus. Lipofectamine RNAiMAX was used for siRNA transfection. Both methods resulted in efficient HDAC4 knockdown and comparable phenotypes in cytokine production. The human caspase–resistant pcDNA3-HDAC4 (D289E) with a FLAG tag was constructed by mutating Asp-289 into glutamic acid. Transient transfections of BV2 were performed using nucleofection (Amaxa Kit V; Lonza).

Real-time reverse transcription-PCR

Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. A 2.0-μg amount of total RNA from each sample was reversely transcribed to cDNA using Random primers and M-MLV Reverse Transcriptase. A 2.0-μl amount of cDNA product was used for quantitative PCR by using SsoFast EvaGreen Supermix kit (172-5201; Bio-Rad). The following primers were used: IL-6, 5′-TCAATTCCAGAAACCGCTATGA-3′ and 5′-CACCAGCATCAGTCCCAAGA-3′; TNF-α, 5′-ATGGCCTCCCTCTCATCAGT-3′ and 5′-CTTGGTGGTTTGCTACGACG-3′; glyceraldehyde-3-phosphate dehydrogenase (internal control), 5′-ACAACTTTGGCATTGTGGAAG-3′ and 5′-GTTGAAGTCGCAGGAGACAAC-3′.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) for detecting TNF-α and IL-6 in cell culture supernatant and cell lysates was carried out in 96-well plates according to the manufacturer's instruction (R&D Systems) and analyzed using a plate reader at 450 nm. The concentration of each cytokine was determined according to the standard provided with the kits. Final values were normalized to total protein concentrations.

Western blotting analysis

After treatment, cells were washed twice with cold phosphate-buffered saline (PBS) and then lysed in ice-cold NETN buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylmethanesulfonylfluoride, 0.2 mM leupeptin, and protease inhibitor cocktail [Sigma-Aldrich]). Lysates were cleared by centrifugation (13,000 rpm for 20 min). Protein concentrations were determined using the bicinchoninic acid assay (Pierce). Equal amounts of cell extracts were then resolved by SDS–PAGE, transferred to nitrocellulose membranes, and probed with antibodies. Blots were detected using an ECL system (Amersham).

Nitric oxide assay

Nitric oxide in culture medium was determined using the Griess Reagent (Sigma-Aldrich) according to the manufacturer's instructions, and absorbance was measured at 540 nm. Final values were normalized to total protein concentrations.

Glucose uptake and glycolytic rate assay

BV2 cells were seeded into 12-well plates (2×10^5 cells/well). After treatment, cells were washed with warm PBS and supplemented with [^{3}H]2-deoxy-D-glucose to a final concentration of 100 μM and 0.5 μCi/well in 0.5 ml of Krebs-Ringer-HEPES buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.4, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, mM KH_2PO4 1.2, 120 mM NaCl, and 10 mM dextrose). After 10-min incubation at 37°C, cells were washed with cold PBS, followed by three rinses with 20 mM glucose, and then lysed in 200 μl of 0.05% SDS for 30 min at room temperature. Lysates were centrifuged at 12,000 rpm for 3 min. We transferred 175 μl of supernatant to scintillation vials to count for radioactivity and used the rest to measure protein concentration. Glycolytic rates were measured as previously described (Vander Heiden *et al.*, 2001). Briefly, cells were plated in 24-well plates and treated by LPS. Cells were washed twice and incubated with 10 Ci of 5-^{3}H-glucose at 37°C for 1 h. The reaction was stopped by adding 0.1 M HCl solution. ^3H_2O generated by the enolase activity was separated from 5-^{3}H-glucose by diffusion, and counts were measured with a Microbeta scintillation counter.

Statistical analysis

Student's *t* tests were applied for comparisons. All data are expressed as mean ± SEM. The significance threshold was at a level of 5% ($p < 0.05$).

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