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Histone Deacetylase 7 Promotes Toll-like Receptor 4-dependent Proinflammatory Gene Expression in Macrophages*[S]

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Background: Histone deacetylase (HDAC) inhibitors reduce LPS-induced inflammatory mediator production from macrophages, but the relevant HDAC targets are unknown.
Results: A specific isoform of Hdac7 amplifies expression of LPS-inducible genes via a HIF-1α-dependent mechanism in macrophages.
Conclusion: The class IIa HDAC Hdac7 promotes inflammatory responses in macrophages.
Significance: Hdac7 may be a viable target for developing new anti-inflammatory drugs.

Broad-spectrum inhibitors of histone deacetylases (HDACs) constrain Toll-like receptor (TLR)-inducible production of key proinflammatory mediators. Here we investigated HDAC-dependent inflammatory responses in mouse macrophages. Of the classical Hdacs, Hdac7 was expressed at elevated levels in inflammatory macrophages (thioglycollate-elicited peritoneal macrophages) as compared with bone marrow-derived macrophages and the RAW264 cell line. Overexpression of a specific, alternatively spliced isoform of Hdac7 lacking the N-terminal 22 amino acids (Hdac7-u), but not the Refseq Hdac7 (Hdac7-s), promoted LPS-inducible expression of Hdac-dependent genes (*Edn1*, *Il-12p40*, and *Il-6*) in RAW264 cells. A novel class IIa-selective HDAC inhibitor reduced recombinant human HDAC7 enzyme activity as well as TLR-induced production of inflammatory mediators in thioglycollate-elicited peritoneal macrophages. Both LPS and Hdac7-u up-regulated the activity of the *Edn1* promoter in an HDAC-dependent fashion in RAW264 cells. A hypoxia-inducible factor (HIF) 1 binding site in this promoter was required for HDAC-dependent TLR-inducible promoter activity and for Hdac7- and HIF-1α-mediated transactivation. Coimmunoprecipitation assays showed that both Hdac7-u and Hdac7-s interacted with HIF-1α, whereas only Hdac7-s interacted with the transcriptional repressor CtBP1. Thus, Hdac7-u positively regulates HIF-1α-dependent TLR signaling in macrophages, whereas an interaction with CtBP1 likely prevents Hdac7-s from exerting this effect. Hdac7 may represent a potential inflammatory disease target.

Cells of the innate immune system utilize pattern recognition receptors such as TLRs[4] to detect molecular patterns derived from invading microorganisms (1). TLRs can also recognize endogenous danger signals, such as those produced through dysregulated biochemical pathways in pathological settings (*e.g.* oxidized low-density lipoprotein and β-amyloid) (2) or those released from cancerous or dying cells (*e.g.* versican and high-mobility group protein B1) (3, 4). Consequently, inappropriate TLR-mediated recognition of "self" has been linked to several inflammation-related pathologies, including atherosclerosis, lupus, rheumatoid arthritis (5), and tumor metastasis (3). Strategies that target TLR signaling pathways are, therefore, being pursued as potential anti-inflammatory therapies (6, 7).

TLR-mediated signaling is driven by phosphorylation and ubiquitination of target proteins (8, 9), which results in the induction of an array of host-protective, proinflammatory, and antimicrobial genes. Innate immune signaling pathways, including TLR signaling, can also be regulated by the reversible acetylation of lysine residues on target proteins (10, 11). This posttranslational modification is sometimes viewed as a histone-specific modification that regulates gene expression through effects on chromatin architecture. However, a wide array of proteins can be acetylated at lysines (12). Lysine acetylation is controlled by the opposing actions of two families of enzymes, histone acetyltransferases and HDACs. Small-molecule inhibitors of HDACs that have been developed as antican-

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[4] The abbreviations used are: TLR, Toll-like receptor; HDAC, human histone deacetylase; BMM, bone marrow-derived macrophage; TEPM, thioglycollate-elicited peritoneal macrophage; TSA, trichostatin A; DMSO, dimethyl sulfoxide; ANOVA, analysis of variance.

cer agents (13) also reportedly have therapeutic effects in a range of inflammatory disease models (14). These anti-inflammatory effects likely result from the regulation of multiple immune cell types, including T regulatory cells (15), Th17 cells, (16), macrophages (17–20), and dendritic cells (21). In macrophages, HDAC inhibitors reduce TLR-inducible production of a subset of proinflammatory cytokines, including TNFα, IL-12, IL-6, chemokines such as monocyte chemoattractant proteins 1 and 3, and other inflammatory mediators, including endothelin 1 (ET-1) (17, 18, 20, 22, 23). The mechanisms by which they do so remain poorly understood but may involve the impairment of transcription factor recruitment to target promoters (22) and inhibition of mitogen-activated protein kinase p38 signaling (10).

The anti-inflammatory effects of HDAC inhibitors imply that certain HDACs have proinflammatory functions (24). The HDAC family consists of 18 enzymes that have been divided into four classes on the basis of homology of the deacetylase domain to yeast proteins. The class I HDACs (HDAC 1–3 and 8) share an N-terminal deacetylase domain and generally localize to the nucleus where they deacetylate lysine residues on histone proteins, thus controlling chromatin architecture and gene expression. The class II HDACs have been divided into subclasses IIa (HDAC 4, 5, 7, and 9) and IIb (HDAC 6 and 10). HDAC 6 and 10 share duplication of the deacetylase domain and are localized in the cytoplasm (25), whereas many of the class IIa HDACs can shuttle between the nucleus and cytoplasm to regulate signaling and gene expression (26). A primary mechanism of action involves transcriptional derepression, in which the nuclear export of class IIa HDACs removes repressive activity, thus permitting inducible gene expression. In this study, we sought to determine whether class IIa HDACs regulate TLR signaling and, in so doing, identified a specific isoform of Hdac7 as a positive regulator of TLR responses in macrophages.

EXPERIMENTAL PROCEDURES

Cell Culture—Bone marrow-derived macrophages (BMMs) were obtained by differentiating bone marrow from 6- to 8-week-old C57Bl/6 mice in the presence of recombinant human colony-stimulating factor 1 (1×10^4 units/ml, a gift from Chiron) for 6 days. On day 6, BMMs were harvested and plated in complete medium containing colony stimulating factor 1 for treatment on day 7. Thioglycollate-elicited peritoneal macrophages (TEPMs) were generated by injection of 1 ml 10% thioglycollate broth into the peritoneal cavity of 6- to 8-week-old C57Bl/6 mice, followed by peritoneal lavage with PBS 5 days later. All animal studies were reviewed and approved by the appropriate University of Queensland animal ethics committee. The RAW264.7 cell line was obtained from the ATCC. Pools of stably transfected RAW264 cells (RAW-pEF6, RAW-Hdac7-u, and RAW-Hdac7-s) were created by electroporation of the indicated expression construct, followed by selection with 2 μg/ml blasticidin. BMMs and TEPMs were cultured in RPMI 1640 medium supplemented with 10% FCS, 20 units/ml penicillin, 20 units/ml streptomycin, and 2 mM L-glutamine. RAW264.7 cells were cultured as BMMs and TEPMs, except that the medium was supplemented with 5% FCS. HEK293 cells were cultured in DMEM (Invitrogen) supplemented with 10% FCS, 20 units/ml penicillin, 20 units/ml streptomycin, and 2 mM L-glutamine. All cells were cultured at 37 °C and 5% CO_2.

Reagents—Chromatographically purified LPS from *Salmonella enterica* subtype minnesota (catalog no. L2137, Sigma) was diluted in medium and used at 100 ng/ml. Trichostatin A (TSA) (Sigma) was dissolved in 100% EtOH, and compound 6 was dissolved in 100% dimethyl sulfoxide (DMSO) and then diluted in medium to be used at the indicated concentrations. Antibodies used for immunoblotting were anti-V5 (1:2500, Serotec), anti-V5-HRP (1:2500, Serotec), anti-FLAG-HRP (1:1000, Cell Signaling Technology), anti-Hdac7 (1:400, Santa Cruz Biotechnology), anti-Hdac4 (1:1000, Cell Signaling Technology), anti-Hdac1 (1:1000, Cell Signaling Technology), anti-acetylated H3 (1:2000, Cell Signaling Technology), anti-acetylated tubulin (1:2000, Sigma), anti-GAPDH (1:7000, Trevigen), anti-rabbit-HRP (1:3000, Cell Signaling Technology), anti-mouse-HRP (1:3000, Cell Signaling Technology), and anti-chicken-HRP (1:2500, Millipore).

NF-κB Reporter Assay—RAW264.7 cells stably transfected with the NF-κB-responsive E-selectin promoter driving GFP expression were used to monitor NF-κB-dependent gene expression (27). Cells were seeded in 24-well plates overnight and then treated, on the following day, with various stimuli for 6 h. The medium was removed and cells were washed in PBS and harvested from the plate in PBS containing 1 mM EDTA and 0.1% sodium azide. GFP expression was analyzed by flow cytometry using a BD FACSCantoII.

Mammalian Expression and Reporter Constructs—Mammalian expression plasmids were created by PCR cloning of the gene of interest from a mixed cDNA pool (generated from a mixture of RNAs from different tissues and cell types). PCR products were inserted into the pEF6-V5/6His vector (Invitrogen) using the topoisomerase I reaction for mHdac7-u, mHdac7-s, mHdac7-u-N-term (encoding amino acids 23–504 of Refseq Hdac7), mHdac7-u-C-term (encoding amino acids 498–938), mHdac9, hHIF-1α, mCtBP1, and mFam96A (irrelevant control protein). Hdac4 was inserted into the pcDNA3.1 V5/6His vector (Invitrogen). pEF6-FLAG, a modified pEF6-based vector, was used for expression of FLAG-tagged proteins. Hence, mHdac7-u (Kpn1 and Not1) and mHdac7-s (Spe1 and Xba1) were excised from pEF6-V5/6His and subcloned into pEF6-FLAG. mCtBP1.V5 was PCR-amplified using a reverse primer to add a FLAG tag followed by a stop codon, and then was cloned with topoisomerase I into pEF6-V5/6His. All mammalian expression plasmids that were generated were verified by sequencing. Plasmid DNA was purified using Endofree Maxiprep kits (Qiagen), and Hdac protein expression was confirmed by transient transfection and immunoblotting in HEK293 cells. The 270-bp *Edn1* promoter fragment was cloned from mouse genomic DNA using a forward primer that contained a 5′ SacI restriction site (AAGAGCTCGGTCTTATCTCTGGCTGCACGTTG (forward) and CTGGTCTGTGGCAGGAGAAGCAAAACG-TAAC (reverse)). The *Edn1-ΔHIF* promoter construct was created by site-directed mutagenesis using AAGAGCTCGGTCTT-ATCTCTGGCTGCTACTTGCCTGTGGGTGA (forward) and the same reverse primer as for *Edn1* (wild-type). Each fragment was sequentially digested with SacI and BglII and then ligated

into the pGL2 basic vector (pGL2B, Promega). Both constructs were verified by sequencing. pGL2 control (pGL2C, Promega) containing the SV40 promoter was used as a positive control. All plasmids were purified using Endofree Maxiprep kits (Qiagen).

Promoter Reporter Studies—RAW264 cells were electroporated (Bio-Rad Gene Pulser Xcell, 260 volts, 1000 microfarads) in 300 μl of volume with 10 μg of promoter-reporter plasmid and 5 μg of Hdac or 2 μg of HIF-1α expression plasmid unless indicated otherwise. Immediately following transfection, cells were washed in PBS, plated in 6-well plates, and incubated for 20 h before treatment with LPS and/or HDAC inhibitor for 8 h. Luciferase activity was measured using the Roche luciferase reporter gene assay according to the instructions of the manufacturer, using a MicroBeta trilux luminometer (PerkinElmer Life Sciences). Relative luciferase units were calculated by normalizing luciferase activity to total protein (Pierce BCA protein assay) in each sample.

RNA Preparation and Quantitative PCR Analysis of Gene Expression—Cells (2×10^6) were seeded in 60-mm tissue culture dishes (Nunc) and treated on the following day with LPS and/or HDAC inhibitors for the indicated times. Cells were then washed in ice-cold PBS. Cell lysates were harvested in RLT (guanidine thiocyanate) buffer (Qiagen), and total RNA was purified using RNeasy kits with on-column DNase digestion (Qiagen). cDNA was prepared using Superscript III (Invitrogen) and random hexamers, and quantitative RT-PCR was performed using SYBR Green (Applied Biosystems). Relative mRNA levels were determined using the ΔCt method, with *Hprt* used as the reference gene. All real-time PCR primer sequences are available on request.

Whole Cell Extracts and Immunoblotting—Whole cell lysates were prepared in either 2% SDS in 66 mM Tris-HCl or radioimmune precipitation assay buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Nonidet P-40) containing freshly added protease inhibitor mixture (Roche). BCA assays (Pierce) were used to quantify total protein concentration within lysates. Immunoblotting was performed on equal amounts of protein from lysates using precast NuPAGE gels (Invitrogen) and methanol-activated Immobilon-P PVDF membranes (Millipore). The membranes were probed with the indicated antibodies, and specific proteins were visualized using ECL (GE Healthcare).

Coimmunoprecipitation Assays—HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen) with expression constructs for Hdac7-u, Hdac7-s, Hdac7-Cterm, HIF-1α, CtBP1, or Fam96a. All constructs contained V5 or FLAG epitope tags as indicated in the figure legends. 24 h post-transfection, whole cell lysates were prepared in radioimmune precipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, protease inhibitors), homogenized through a 27-gauge needle, and centrifuged to remove insoluble fragments. Lysates were precleared with protein G magnetic beads (Invitrogen) and then incubated with 1 μg of anti-v5 (Serotec) or 1 μg of anti-FLAG (Sigma) at 4 °C overnight. Lysate + antibody was then incubated with washed protein G magnetic beads for 2 h at 4 °C. Beads were washed three times in radioimmune precipitation assay buffer, transferred to clean tubes, and bead-bound protein was eluted by resuspension in 1× LDS (Invitrogen) sample buffer containing 1× reducing agent (Invitrogen) and heating at 70 °C for 10 min. Proteins of interest were detected by immunoblotting using anti-FLAG-HRP (1:1000, Cell Signaling Technology) or chicken anti-V5 (1:2500, Genetex) with anti-chicken-HRP (1:2500, Millipore) or anti-v5-HRP (1:2500, Serotec).

ELISAs—The levels of inflammatory mediators in cell culture supernatants were measured using sandwich ELISAs according to the instructions of the manufacturer (IL-12p40, IL-6, and TNFα, BD Biosciences; ET-1, Cayman Chemical).

Inhibitor Synthesis—The class IIa HDAC inhibitor, compound 6, was described previously (28). Compound 6 was synthesized by dissolving diphenylacetic acid (800 mg, 3.73 mmol) in 10 ml of dichloromethane before adding thionyl chloride (280 μl, 3.87 mmol) under N_2. The reaction mixture was stirred for 1 h at room temperature before treating with hydroxylamine hydrochloride (1.22 g, 17.6 mmol) in 10 ml 10% Na_2CO_3. Compound 6 was precipitated from the solution and dried *in vacuo*. The yield was 810 mg (95%). Electrospray mass spectrometry, *m/z* 228.10 $[MH]^+$; high-resolution mass spectrometry calculated for $C_{14}H_{13}NO_2Na$ $[MNa]^+$, 250.0838; found, 250.0838; ^{1}H NMR (d6-DMSO), δ 10.7 (s, 1H), 8.98 (s, 1H), 7.32–7.20 (m, 10H), 4.72 (s, 1H). Prior to use, compound 6 was dissolved and stored in DMSO.

Cloning, Expression, and Purification of the Truncated Human HDAC7 Protein—Residues 518–991 of human HDAC7 were amplified by PCR from a pooled human cDNA template, and the product was inserted into the Champion pET small ubiquitin-like modifier vector (Invitrogen) using a TA cloning strategy. The resulting SUMO-hHDAC7 fusion protein was expressed in *Escherichia coli* BL21 (DE3) cells (Invitrogen) and grown in terrific broth medium in the presence of 50 μg/ml kanamycin. Cells were grown at 37 °C to an A_{600} of 0.5 before induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside, after which they were grown for a further 20 h at 37 °C. Cells were suspended in lysis buffer (20 mM sodium phosphate buffer (pH 7.4), 500 mM NaCl, 10 mM imidazole containing 1× protease inhibitor mixture, Roche) and were lysed by sonication. The lysate was purified using TALON resin (Clontech) and the bound protein was eluted in lysis buffer containing 150 mM imidazole. The eluted protein was dialyzed against 25 mM Tris-HCl (pH 8.0), 138 mM NaCl, and 0.05% Tween 20 overnight at 4 °C. The dialyzed protein was concentrated, and 10% glycerol was added before use in enzyme assays.

HDAC Enzyme Assays—Recombinant HDAC1 and HDAC6 enzymes were purchased from BPS Biosciences and Calbiochem. Protein concentrations were in the range of 0.1–0.7 mg/ml. Recombinant HDAC7 was generated as described above. Fluorescence readings were carried out on a CytofluorR Series 4000 fluorescence multiwell plate reader (Perspective Biosystems). Stock solutions of the HDAC inhibitor (10 mM) and substrates (10 mM) were freshly prepared in DMSO. The buffer for all experiments was 25 mM Tris/Cl (pH 8.0), 137 mM NaCl, 2.7 M KCl, and 1 mM $MgCl_2$. To avoid loss of enzyme activity through repeated freeze/thaw cycles, aliquots of HDAC1 and HDAC6 were prepared and stored at −80 °C, and recombinant HDAC7 enzyme was freshly prepared. The

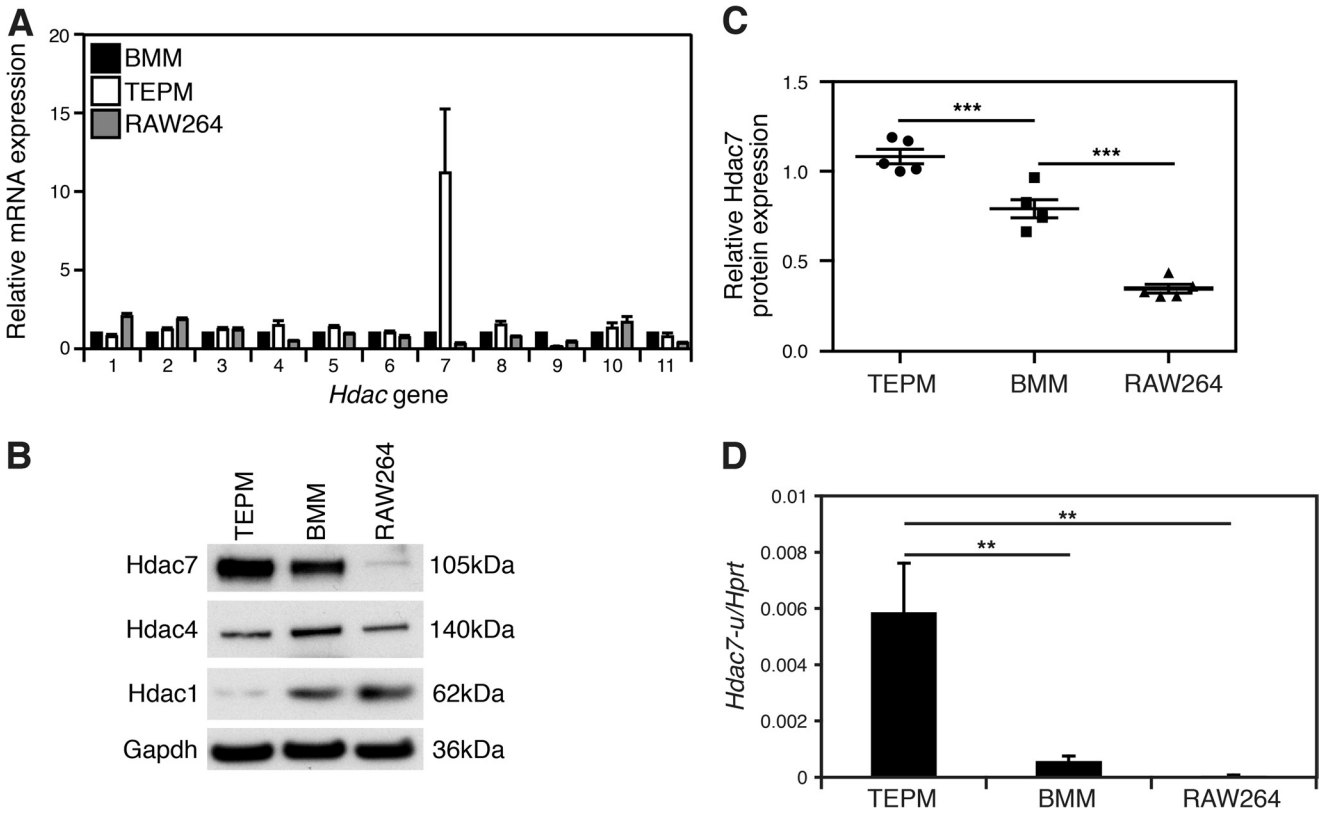

FIGURE 1. **Hdac7 expression is elevated in inflammatory macrophages.** *A*, quantitative PCR primers detecting the classical *Hdacs* were used to quantify mRNA levels relative to *Hprt* in BMMs (*black bars*), TEPMs (*white bars*), and RAW264 cells (*gray bars*). Data (mean ± S.E. of five independent cell preparations) are shown relative to BMMs for each gene. *B*, protein lysates prepared in 2% SDS from TEPMs, BMMs, and RAW264 cells were separated by SDS-PAGE and probed for Hdac7, Hdac4, Hdac1, and Gapdh. *C*, quantification of Hdac7 protein levels relative to Gapdh in TEPMs, BMMs, and RAW264 cells ($n = 5$, $p < 0.001$). *D*, primers that detect the extra exon in *Hdac7-u* were used to quantitate expression of *Hdac7-u* relative to *Hprt* in TEPMs, BMMs, and RAW264 cells. Data show the mean ± S.E. for five independent cell preparations. ANOVA with Tukey's test was used to compare all samples. **, $p < 0.01$).

enzyme was diluted with buffer to a final concentration of 0.005 ng/μl, and enzyme assays were carried out in 50-μl reaction volumes. Developer solution was used as described for HDAC1 by the supplier, and was added after 30-min incubation at 37 °C. The final substrate concentration was 50 μM. Bovine serum albumin was used at 100 μg/ml.

RESULTS

Identification of Hdac7 as a Candidate Promoter of TLR4 Responses in Macrophages—In view of recent evidence identifying macrophages as important cellular targets of HDAC inhibitors in inflammation models *in vivo* (29), we examined *Hdac* mRNA expression in primary mouse macrophages. Previously, we used comparisons of inflammatory macrophages (TEPMs) *versus* BMMs to identify genes that regulate macrophage inflammatory responses (30). Therefore, we analyzed the mRNA expression of all classical Hdacs (*Hdac1-11*) in TEPMs, BMMs, and RAW264 cells. *Hdac1–11* were all expressed at the mRNA level in mouse macrophages, but *Hdac7* was the only family member that was elevated substantially in TEPMs as compared with the other two cell populations (Fig. 1*A*). Hdac7 protein expression was also elevated in TEPMs compared with BMMs and RAW264 cells (Fig. 1, *B* and *C*), whereas another class IIa Hdac, Hdac4, was expressed at similar levels across the three macrophage populations (Fig. 1*B*). The class I Hdac Hdac1 was expressed at elevated levels in proliferating macrophages (BMMs and RAW264 cells) as compared with post-proliferative TEPMs (Fig. 1*B*).

Because of the reduced *Hdac7* mRNA expression in RAW264 cells in comparison with primary macrophages, we examined the effect of stable Hdac7 overexpression on TLR responses in this cell line. A previous study identified an alternative *Hdac7* mRNA transcript encoding an isoform lacking the N-terminal 22 amino acids of Hdac7 (Hdac7-u) (31). This transcript was also expressed at elevated levels in TEPMs in comparison with BMMs and RAW264 cells (Fig. 1*D*). Therefore, we also examined this variant in addition to full-length Hdac7 (Hdac7 spliced (Hdac7-s)). Both isoforms were overexpressed at similar levels in stably transfected pools of RAW264 cells (Fig. 2*A*), but, surprisingly, only Hdac7-u amplified LPS-induced mRNA expression of HDAC-dependent genes, including *Edn1* (~9-fold, Fig. 2*B*), *Il-12p40* (~6-fold, Fig. 2*C*) and *Il-6* (~20-fold, Fig. 2*D*). In contrast, LPS-inducible *Il-1β* mRNA expression, which was not reduced by HDAC inhibitors (22), was not affected by Hdac7-u overexpression (Fig. 2*E*). Studies with selective HDAC inhibitors imply that there are multiple mechanisms by which HDACs promote TLR responses (18). Consistent with this, LPS-inducible mRNA expression of *iNOS* and *Ccl7*, which were both induced by LPS in an HDAC-dependent manner in macrophages (10, 17), was not affected by Hdac7-u overexpression (Fig. 2, *F* and *G*). In comparison with

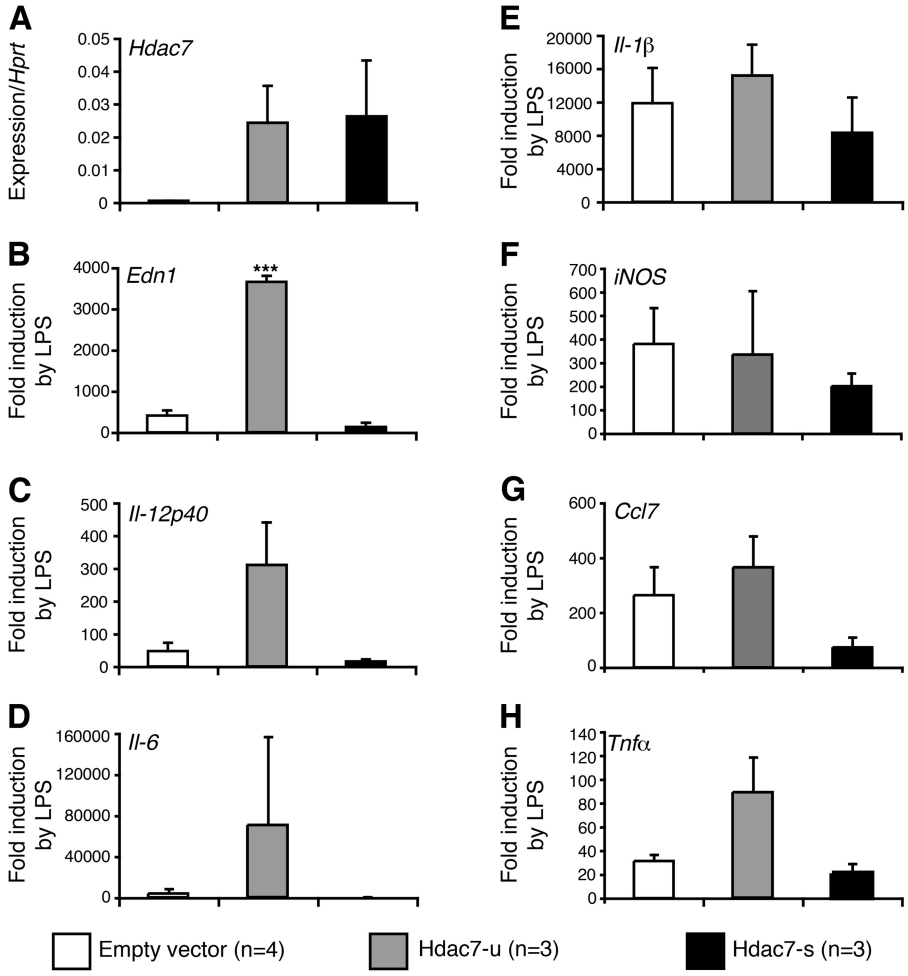

FIGURE 2. **Overexpression of Hdac7-u, but not Hdac7-s, in RAW264 cells amplifies the TLR4-inducible expression of a subset of inflammatory genes.** Independent pools of RAW264 cells stably transfected with either empty vector ($n = 4$), Hdac7-u ($n = 3$), or Hdac7-s ($n = 3$) were treated with LPS (100 ng/ml) for 4 h. Total *Hdac7* mRNA levels were determined in the different pools (*A*), as was LPS-regulated gene expression for *Edn1* (*B*), *IL-12p40* (*C*), *IL-6* (*D*), *IL-1β* (*E*), *iNOS* (*F*), *Ccl7* (*G*), and *Tnfα* (*H*). Data show the mean ± S.E. of fold induction in response to LPS across the independent pools of stable cell lines. ANOVA with Tukey's test was used. ***, $p < 0.001$.

the effects of Hdac7-u on *Edn1*, *Il-12p40*, and *Il-6*, LPS-inducible *Tnfα* mRNA expression was increased more modestly (~3-fold, Fig. 2*H*). The amplifying effect of Hdac7-u on expression of a subset of TLR4-inducible genes was apparent over an LPS time course (Fig. 3, *A–D*) and was also observed at the protein level, as assessed by levels of IL-12p40 and IL-6 in culture supernatants (*E* and *F*). As was apparent with mRNA expression, TNFα protein secretion was affected more modestly (Fig. 3*G*).

Targeting Hdac7 Reduces Inflammatory Mediator Production from Inflammatory Macrophages—We next determined whether pharmacological inhibition of Hdac7 function impaired HDAC-dependent TLR4 responses. Compound 6, a previously reported class IIa HDAC inhibitor (28), inhibited the activity of recombinant human HDAC7 (Fig. 4*A*) and displayed selectivity for this enzyme over HDAC1 (class I) and HDAC6 (class IIb) (IC_{50} for HDAC7, 354 nM; IC_{50} for HDAC6, 5000 nM; IC_{50} for HDAC1, >10,000 nM). Consistent with this selectivity for Hdac7, treatment of TEPM with compound 6 did not promote hyperacetylation of tubulin (Hdac6 substrate) or histone H3 (class I Hdac substrate), whereas the broad-spectrum HDAC inhibitor TSA caused hyperacetylation of both proteins (Fig. 4*B*). However, compound 6 did reduce levels of ET-1, IL-12p40, IL-6, and TNFα in culture supernatants from LPS-activated TEPMs (Fig. 4, *C–F*) without affecting cell viability at the concentrations used (data not shown). Thus, overexpression of Hdac7 amplifies a subset of TLR4 responses, whereas pharmacological inhibition reduces these responses.

The Edn1 Promoter Activity Is LPS-inducible in an HDAC-dependent Manner—LPS-inducible *Edn1* expression is almost completely HDAC-dependent (17, 18). *Edn1* encodes a proprotein that is processed sequentially to generate the secreted peptide ET-1. ET-1 has both vasoconstrictive and proinflammatory functions and has been linked to numerous inflammatory diseases (32–34). Therefore, we used the *Edn1* proximal promoter in reporter assays to investigate mechanisms by which Hdac7 promotes TLR4 responses. As expected, the broad-spectrum HDAC inhibitor TSA blocked LPS-inducible *Edn1* promoter activity, indicating that LPS-mediated transcriptional activation is HDAC-dependent (Fig. 5*A*). This effect was not apparent with all LPS-inducible promoters because the NF-κB-dependent E-selectin promoter was not inhibited by TSA (supplemental Fig. S1). In fact, consistent with a previous study (10), this response was actually slightly enhanced. As with the effects of Hdac7 overexpression (Fig. 2), Hdac7-u, but not full-length

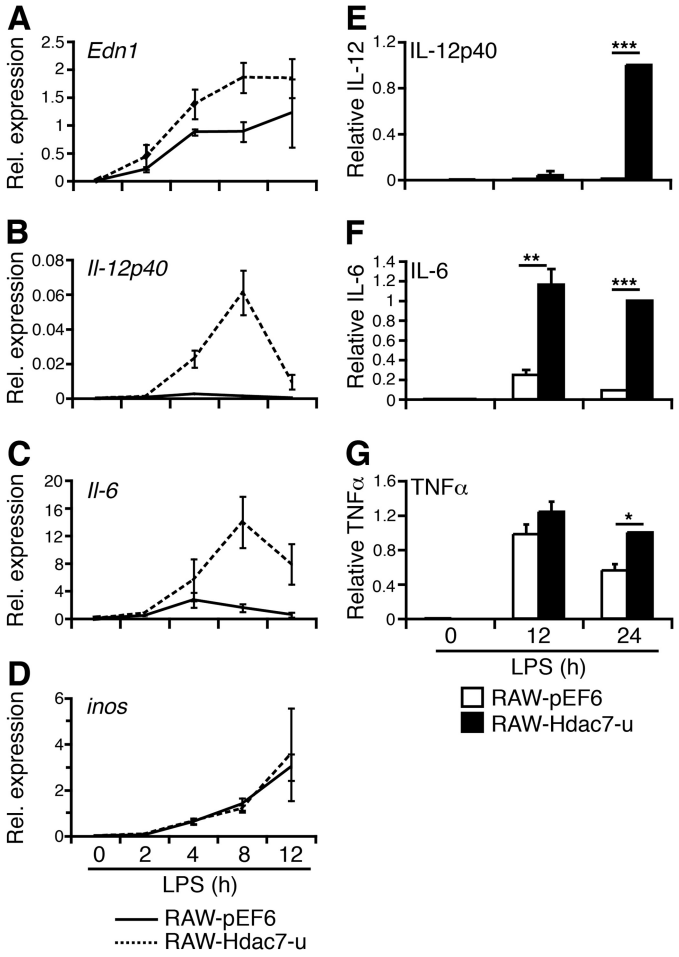

FIGURE 3. **Hdac7-dependent amplification of TLR4-inducible gene expression and cytokine release in macrophages.** Time course of LPS-inducible *Edn1* (*A*), *Il-12p40* (*B*), *Il-6* (*C*), and *iNOS* (*D*) mRNA expression in RAW264 cells overexpressing empty vector (RAW-pEF6, *solid line*) or Hdac7-u (RAW-Hdac7-u, *dotted line*). Data (mean ± S.D. of technical triplicates) are representative of two independent experiments. Equal numbers of RAW-pEF6 (*open bars*) and RAW-Hdac7-u (*filled bars*) cells were stimulated with LPS for 12 or 24 h, and culture supernatants were analyzed for IL-12p40 (*E*), IL-6 (*F*), and TNFα (*G*). Data (relative to RAW-Hdac7-u at 24 h LPS) are combined from three independent experiments (mean ± S.E.) (Student's *t* test and one-sample Student's *t* test for 12- and 24-h data, respectively. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Hdac7 (Hdac7-s), enhanced basal and LPS-inducible *Edn1* promoter activity (Fig. 5*B*). Hdac7-N-term, a truncation mutant of Hdac7-u lacking the C-terminal deacetylase domain, did not activate the *Edn1* promoter (Fig. 5*C*). TSA inhibited trans-activation of the *Edn1* promoter by Hdac7-u (Fig. 5*D*). Although the effect of compound 6 was less pronounced, it reduced the Hdac7-u + LPS response to a level similar to that of LPS alone (Fig. 5*E*). The ability of Hdac7-u to activate the *Edn1* promoter appeared to be specific to this family member because the class IIa Hdacs, Hdac4 and Hdac9, when expressed ectopically (Fig. 5*F*), did not enhance *Edn1* promoter activity (Fig. 5*G*). Hence, HDAC-dependent trans-activation of the *Edn1* promoter was specific to Hdac7-u and required deacetylase activity.

HDAC-dependent Edn1 Promoter Activity Is Dependent on HIF-1α—HIF-1α promotes TLR4-dependent inflammatory responses in macrophages (35, 36). Therefore, we hypothesized that an HIF-binding site in the *Edn1* promoter (37) might be involved in Hdac7-u-dependent amplification of this TLR4 response. Accordingly, mutation of the HIF-binding site (Fig. 6*A*) greatly reduced basal, LPS-inducible, and Hdac7-u-mediated up-regulation of the *Edn1* promoter (Fig. 6*B*). Overexpression of HIF-1α also activated the *Edn1* promoter, and this effect was again dependent on an intact HIF binding site (Fig. 6*C*). In cells cotransfected with HIF-1α, LPS further increased *Edn1* promoter activity only marginally (< 2-fold, Fig. 6, *C* and *D*), suggesting that ectopic HIF-1α expression delivered an LPS-like signal. In accordance with this, the HIF-1α response was sensitive to TSA, as was observed for LPS (Fig. 6*D*).

LPS-dependent Up-regulation of HIF-1α Requires HDAC Activity—We next addressed the involvement of HDACs in regulating LPS-inducible HIF-1α expression in macrophages. In RAW264 cells, ectopically expressed HIF-1α protein was undetectable in the basal state but was readily detectable after 2 h of LPS stimulation (Fig. 7*A*). LPS-induced HIF-1α protein levels were substantially reduced by TSA at 2 h post-stimulation, but interestingly, this inhibition was not observed at 4 h of LPS stimulation (Fig. 7*A*). Similar effects were observed at the mRNA level (specific detection of the ectopically expressed HIF-1α mRNA) in these cells (Fig. 7*B*). Thus, the early up-regulation of HIF-1α protein expression by LPS is dependent upon HDAC activity, but this effect is overcome at later time points. In contrast to TSA, compound 6 did not reduce LPS-induced HIF-1α protein expression (Fig. 7*C*), thus indicating that class IIa Hdac activity is not required for this response. This suggests that Hdac7-u likely regulates LPS-inducible *HIF-1α* protein function rather than expression.

Hdac7 Synergizes with HIF-1α in the LPS Response—It has been reported that HDAC7 promotes HIF-1α-dependent responses to hypoxia (38). Similarly, we found that substimulatory amounts of Hdac7-u that were insufficient to activate the *Edn1* promoter alone synergized with HIF-1α for this response in RAW264 cells (Fig. 8*A*). Given that the effect of Hdac7 on LPS responses was selective for Hdac7-u, we next determined whether there was a selective interaction between Hdac7-u and HIF-1α. In coimmunoprecipitation experiments, we found that both Hdac7-u and Hdac7-s interacted with HIF-1α (Fig. 8*B*), implying that a differential interaction between HIF-1α and Hdac7-u *versus* Hdac7-s was not responsible for the selective effect of Hdac7-u in promoting inflammatory responses. The N-terminal region of Hdac7-s has a documented consensus binding site (PMDLR) for the CtBP1 transcriptional repressor (39, 40). The absence of the first 22 amino acids from Hdac7-u results in the loss of the proline reside in this motif. Therefore, we reasoned that this might reduce or disrupt binding of CtBP1 to Hdac7-u. Fig. 8*C* shows that Hdac7-s, but not Hdac7-u, pulled down CtBP1. Similarly, the C-terminal region of Hdac7 containing the deacetylase domain as well as an irrelevant control protein (Fam96a) failed to interact with CtBP1. These data suggest that although both Hdac7-s and Hdac7-u interact with HIF-1α, the interaction of Hdac7-s with CtBP1 likely constrains its capacity to promote inflammatory responses. Thus, the selective capacity for Hdac7-u to promote inflammatory responses may require both its interaction with HIF-1α as well as its inability to be constrained by CtBP1-dependent transcriptional repression.

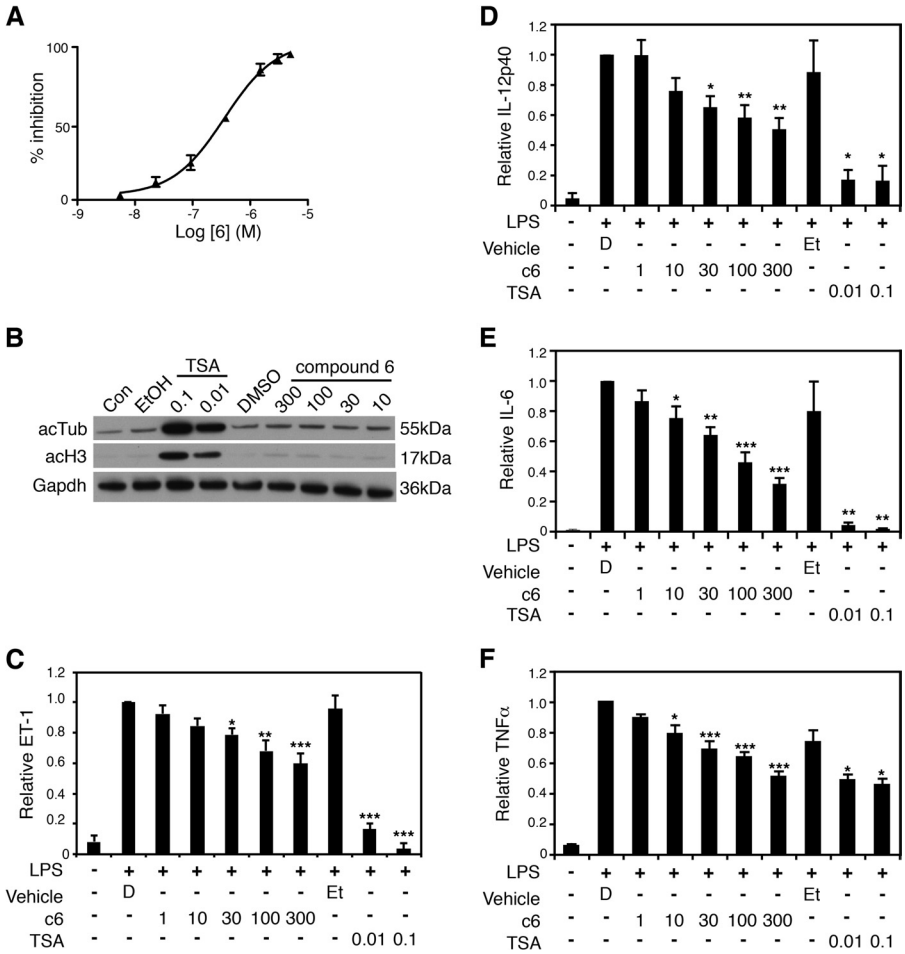

FIGURE 4. **A class IIa HDAC inhibitor inhibits TLR-inducible inflammatory mediator production from primary mouse macrophages.** *A*, inhibition of recombinant hHDAC7 enzyme activity with compound 6. *M*, molar. *B*, TEPMs were treated with HDAC inhibitor (shown in micromolar) or vehicle control (*Con*) for 4 h. Protein lysates in 2% SDS were analyzed by immunoblotting to detect acetylated tubulin (*acTub*), acetylated histone H3 (*acH3*), and Gapdh as a loading control. Data are representative of three independent experiments. *C–F*, TEPMs were treated with LPS (100 ng/ml), and the indicated concentration (shown in micromolar) of compound 6 (*c6*), TSA, or appropriate vehicle (DMSO (*D*) for c6 and EtOH (*Et*) for TSA) for 8 h. Levels of secreted ET-1 (*C*), IL-12p40 (*D*), IL-6 (*E*), and TNFα (*F*) in culture supernatants were determined by ELISA. Data (mean $\pm$ S.E.) are combined from four independent experiments and are displayed relative to the LPS + DMSO-treated sample. ANOVA with Dunnett's multiple comparison test was used to compare the c6- and TSA-treated samples to the relevant vehicle control. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

DISCUSSION

Many studies have demonstrated suppressive effects of HDAC inhibitors on TLR-inducible inflammatory responses (16, 17, 19–22, 41, 42). Here we identified elevated Hdac7 expression in inflammatory macrophages (Fig. 1) and defined a role for a specific isoform of this Hdac (Hdac7-u) in promoting the expression of a subset of TLR-inducible, proinflammatory genes in macrophages. The response was selective because this amplification was not observed for the class IIa HDACs Hdac4 and Hdac9 (Fig. 5*G*). Deletion of the C-terminal deacetylase domain (Fig. 5*C*), treatment with TSA (Fig. 5*D*), and treatment with compound 6 (Fig. 5*E*) all inhibited Hdac7-mediated activation of the *Edn1* promoter, implying that Hdac7 deacetylase activity is required for amplification of a subset of TLR4 responses. Nonetheless, HDAC7 can interact with and utilize the enzymatic activity of other HDACs, for example, the class I HDAC HDAC3 (43), so it is also possible that the deacetylase dependence partly involves the recruitment of other deacetylases. Indeed, it has been reported recently that 45% of LPS-inducible genes were down-regulated in Hdac3$^{-/-}$ mouse macrophages (44), among them *Il-6* and *Edn1*. Interestingly, Hdac3 has also been shown recently to constrain alternative macrophage activation (45). Thus, it is plausible that Hdac7 and Hdac3 cooperate to regulate macrophage inflammatory responses.

Our analysis of the *Edn1* gene indicates that Hdac7 acts, at least in part, by regulating HIF-1α. Both Hdac7- and HIF-1α-dependent trans-activation of the *Edn1* promoter required a functional HIF-1α binding site (Fig. 6, *B* and *C*). Furthermore, an interaction between Hdac7 and HIF-1α in cells was demonstrated (Fig. 8*B*), and these proteins synergistically amplified LPS-inducible *Edn1* promoter activity (Fig. 8*A*). Finally, Hdac7-u promoted the production of IL-6, IL-12p40, and, to a lesser extent, TNF-α (Figs. 2 and 3). HIF-1α was required for LPS-inducible production of these inflammatory mediators *in vivo*, and, indeed, HIF-1 binding sites exist within the *Il-6* and *Tnf*α gene regulatory regions (35). Although the precise mechanism(s) by which Hdac7 promotes HIF-mediated LPS responses still remain(s) to be determined, a previous study showed that HDAC7 promoted HIF-1α transcriptional activity

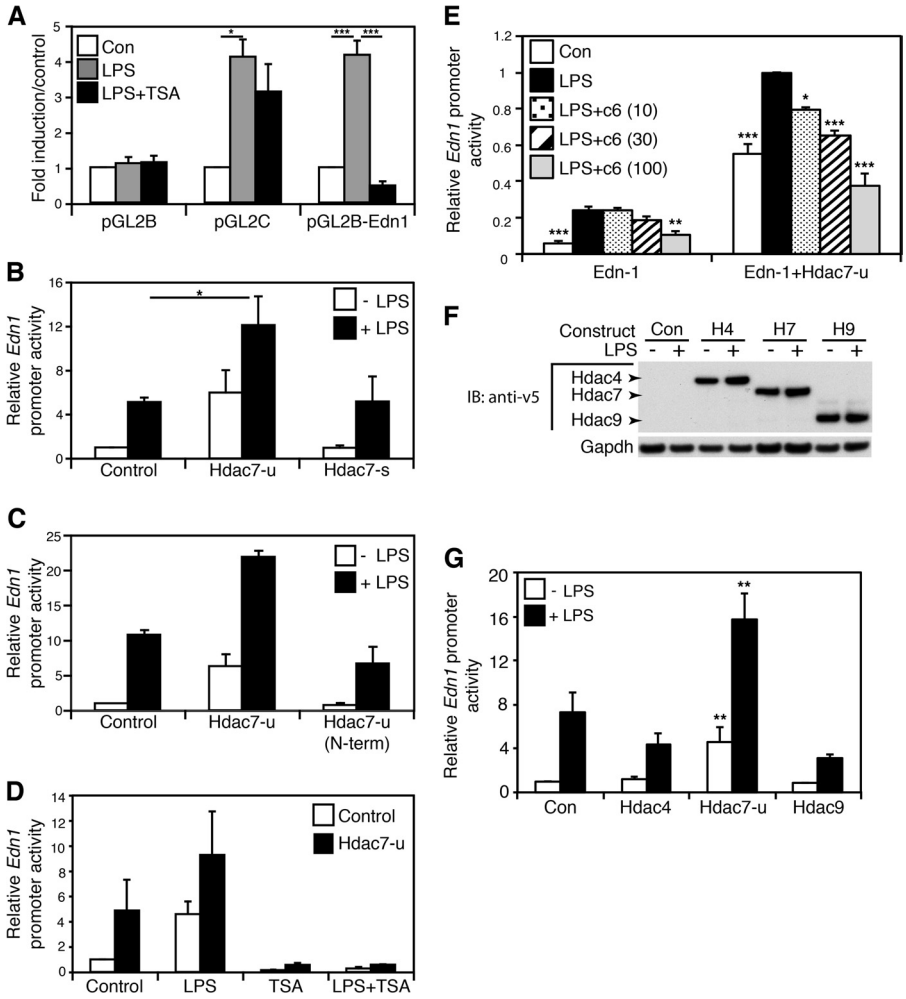

FIGURE 5. **Hdac7 activates the *Edn1* promoter in an Hdac-dependent fashion in mouse macrophages.** *A*, RAW264 cells were transiently transfected with an *Edn1* promoter construct driving luciferase, the empty vector pGL2B, or the LPS-responsive positive control pGL2C (*Con*). After 20 h, cells were treated with LPS (100 ng/ml) or LPS + TSA (500 nM) for 8 h. Luciferase activity is shown relative to the control. Data (mean ± S.E., ANOVA and Tukey-Kramer test) are combined from three independent experiments. *, $p < 0.05$; ***, $p < 0.001$. *B*, RAW264 cells were transfected with *Edn1* promoter alone or with *Edn1* plus Hdac7-u or Hdac7-s. After 20 h, cells were treated with LPS for 8 h, after which luciferase activity was analyzed. Data (mean ± S.E. for three independent experiments) are shown relative to the unstimulated control. *, $p < 0.05$, Student's *t* test. *C*, RAW264 cells were transfected with *Edn1* promoter alone (control), *Edn1* plus Hdac7-u, or *Edn1* plus the N-terminal region of Hdac7-u, Hdac7 (*N-term*, amino acids 23–504). Luciferase activity was measured after 8-h stimulation with LPS. Data (mean + range of duplicate transfections within the experiment) are displayed relative to the *Edn1* promoter alone and are representative of three independent experiments. *D*, RAW264 cells were transfected with *Edn1* plus empty vector (*open bars*) or *Edn1* plus Hdac7-u (*filled bars*) and treated with EtOH (vehicle control), LPS, TSA, or LPS + TSA for 8 h. Luciferase activity was measured and is shown relative to the vehicle control (mean ± S.E. for three independent experiments). *E*, experiments were performed as for *D*, except that a concentration range of compound 6 (in micromolar) was examined. Data (mean ± S.E. for three independent experiments) are shown relative to the LPS-treated *Edn1* promoter plus a Hdac7-u sample. ANOVA with Dunnett's multiple comparison was used to compare LPS alone to LPS + compound 6 for either the *Edn1* promoter or the *Edn1* promoter + Hdac7-u groups. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *F*, RAW264 cells were transiently transfected with the *Edn1* promoter construct plus class IIa Hdac expression constructs or an empty vector (control). After 20 h, transfected cells were treated for 8 h with LPS (*filled bars*) or left untreated (*open bars*), after which cell lysates were immunoblotted (*IB*) for the V5 tag of the ectopically expressed Hdacs. Data are representative of two independent experiments. *G*, experiments were performed as above, except that luciferase activity was monitored. Pooled data from five independent experiments (mean ± S.E.) are shown relative to the *Edn1* promoter alone (*Con*), and ANOVA with Dunnett's multiple comparison test was used to compare the Hdac expression constructs to the relevant control (control - LPS or control + LPS). **, $p < 0.01$.

during hypoxia (38), so a similar mechanism is likely to apply during LPS responses. The observed interaction between Hdac7 and HIF-1α in cells (Fig. 8*B*) is consistent with this.

A previous study reported differential expression of two distinct Hdac7 isoforms that differ by 22 amino acids at the N terminus during smooth muscle cell differentiation (31). Both isoforms were expressed by primary macrophages (Fig. 1*D* and data not shown), and, surprisingly, the amplifying effect on the TLR4 response was restricted to the shorter isoform, Hdac7-u (Figs. 2 and 5*B*). Although differential interactions between these two Hdac7 isoforms and MEF2C and/or serum response factor (31) could account for the effects observed in our study, our identification of a selective interaction between Hdac7-s and CtBP1 provides an alternative explanation for the selective capacity of Hdac7-u to promote HIF-1α-dependent transcriptional responses (Fig. 9). The relative levels of Hdac7-s, Hdac7-u, and CtBP1 may, thus, act to fine-tune inflammatory responses in different cellular contexts. For example, a reduced expression of CtBP1 might license Hdac7-s, and potentially other class IIa Hdacs, to activate inflammatory pathways. Although the CtBP1 binding motif is present in all class IIa HDACs, there are transcript variants of human

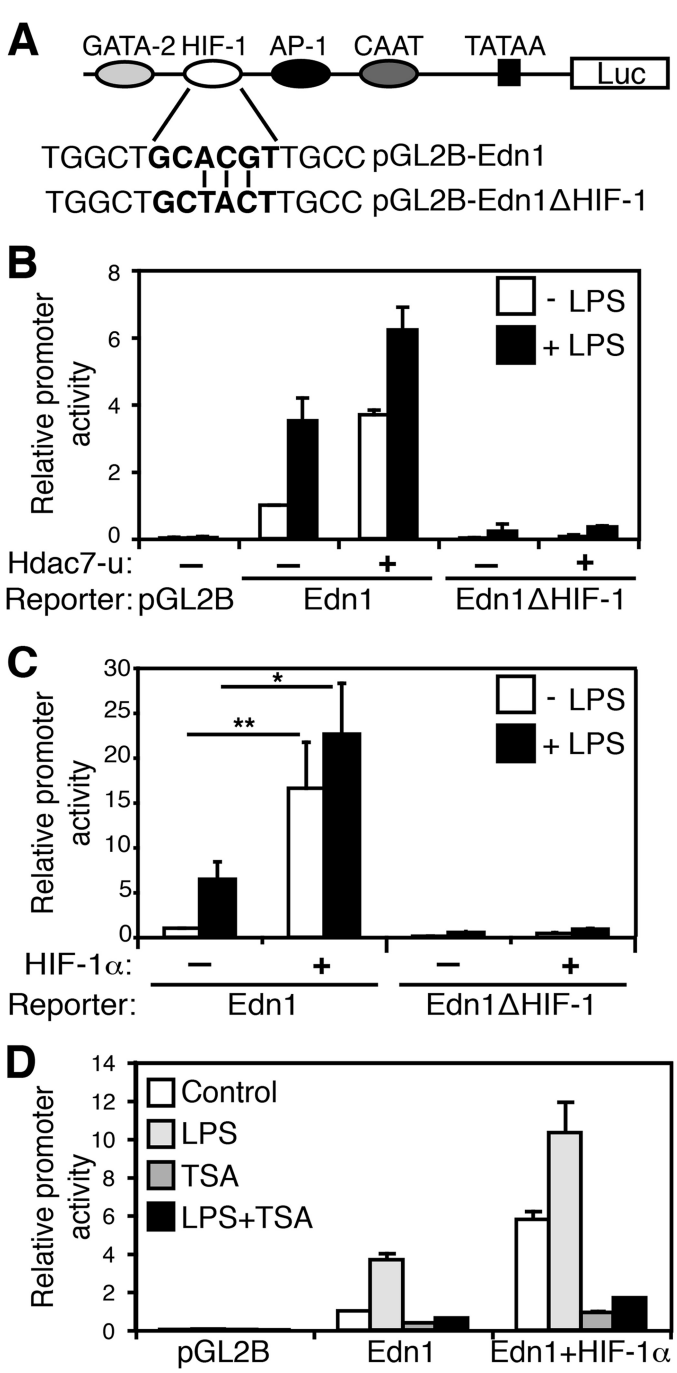

FIGURE 6. **Amplification of TLR4 responses by Hdac7 involves HIF-1α.** *A*, schematic diagram of the HIF-1 binding site in the *Edn1* promoter and the three nucleotide residues mutated to create the *Edn1-ΔHIF* promoter construct (37). *Luc*, luciferase. *B*, RAW264 cells were transiently transfected with the *Edn1* (wild-type) or *Edn1-ΔHIF* promoter constructs, with or without an Hdac7-u expression construct and treated with LPS for 8 h. Data (relative to the *Edn1* promoter alone) are the mean + range of duplicate transfections and are representative of two independent experiments. *C*, RAW264 cells were transfected with *Edn1* or *Edn1-ΔHIF* promoter constructs with or without an HIF-1α expression construct and were treated with LPS for 8 h. Promoter activity was assessed by luciferase assay. Data (mean ± S.E.) are combined from three independent experiments and are shown relative to the *Edn1* promoter untreated control. ANOVA with Dunnett's multiple comparison test was used. *, $p < 0.05$; **, $p < 0.01$). *D*, the *Edn1* promoter construct was transfected into RAW264 cells with either an HIF-1α expression construct or empty vector. pGL-2B was also included as a negative control. Cells were treated with EtOH (vehicle control), LPS (100 ng/ml), TSA (500 nM), or LPS + TSA. Data (average of duplicate transfections + range) are representative of two independent experiments and are displayed relative to the *Edn1* promoter alone.

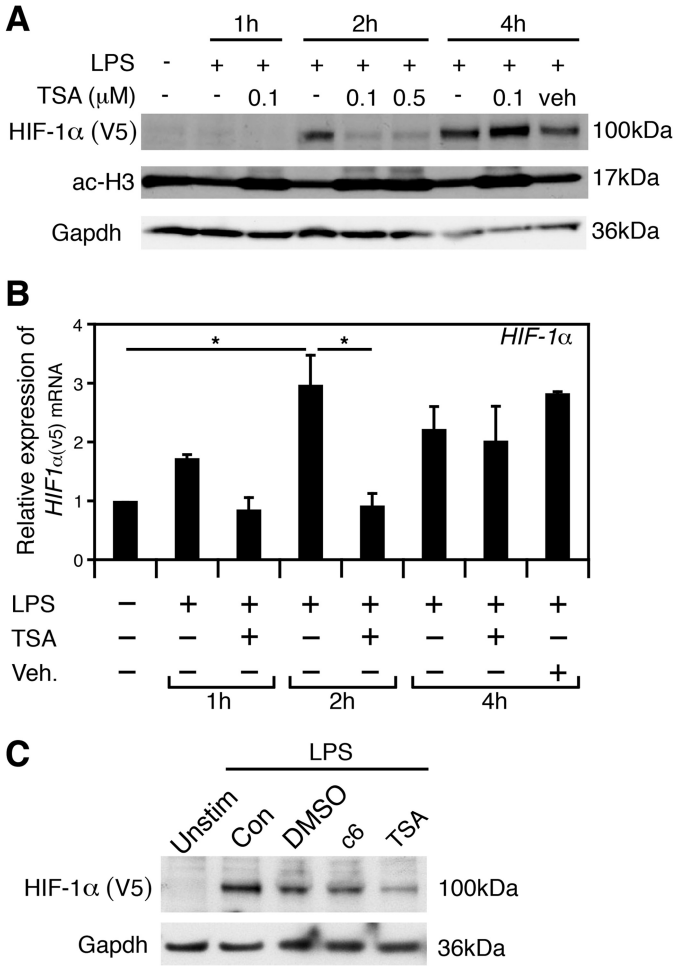

FIGURE 7. **LPS-inducible HIF-1α expression in macrophages requires HDAC activity.** *A*, RAW264 cells stably expressing hHIF-1α-V5 were treated with LPS or LPS + TSA for 1, 2, or 4 h. hHIF-1α was detected by Western blot analysis using an anti-v5 antibody, and the activity of TSA was confirmed by monitoring acetylated histone H3 (*ac-H3*). Gapdh levels are shown as a loading control. Data are representative of three independent experiments. *veh*, vehicle. *B*, RAW-HIF-1α-V5 cells were treated as in *A*, and mRNA levels of ectopically expressed *HIF-1α* were determined by quantitative PCR. Data (mean ± S.E.) are combined from three independent experiments and are displayed as expression relative to untreated control cells. ANOVA with Bonferroni's multiple comparison test was used. *, $p < 0.05$. *C*, RAW264 cells stably expressing hHIF-1α-V5 were treated with LPS (100 ng/ml), LPS + DMSO, LPS + compound 6 (*c6*, 100 μM), and LPS + TSA (0.1 μM) or were left untreated (*Unstim.*) for 2 h. HIF-1α-protein levels in whole cell lysates were assessed by immunoblotting. Data are representative of three independent experiments. *Con*, control.

HDAC7 (Ensembl code ENST00000427332) and human HDAC4 (UCSC code uc010fyy.3) in which this motif is disrupted through the loss of the proline residue (*i.e.* translation starts immediately after this), as occurs in mouse Hdac7-u. It remains to be determined whether these HDAC isoforms also promote inflammatory responses. Differential interactions between CtBP1 and Hdac7-s *versus* Hdac7-u may also contribute to selective roles for these Hdac7 isoforms in regulating other transcriptional activators in other biological systems, such as during smooth muscle cell differentiation.

Beyond Hdac7, our findings also provide further insight into TLR-regulated HIF-1α function. In diseased tissue, hypoxia and inflammatory stimuli are intimately associated. Current models propose that migration of innate immune cells into

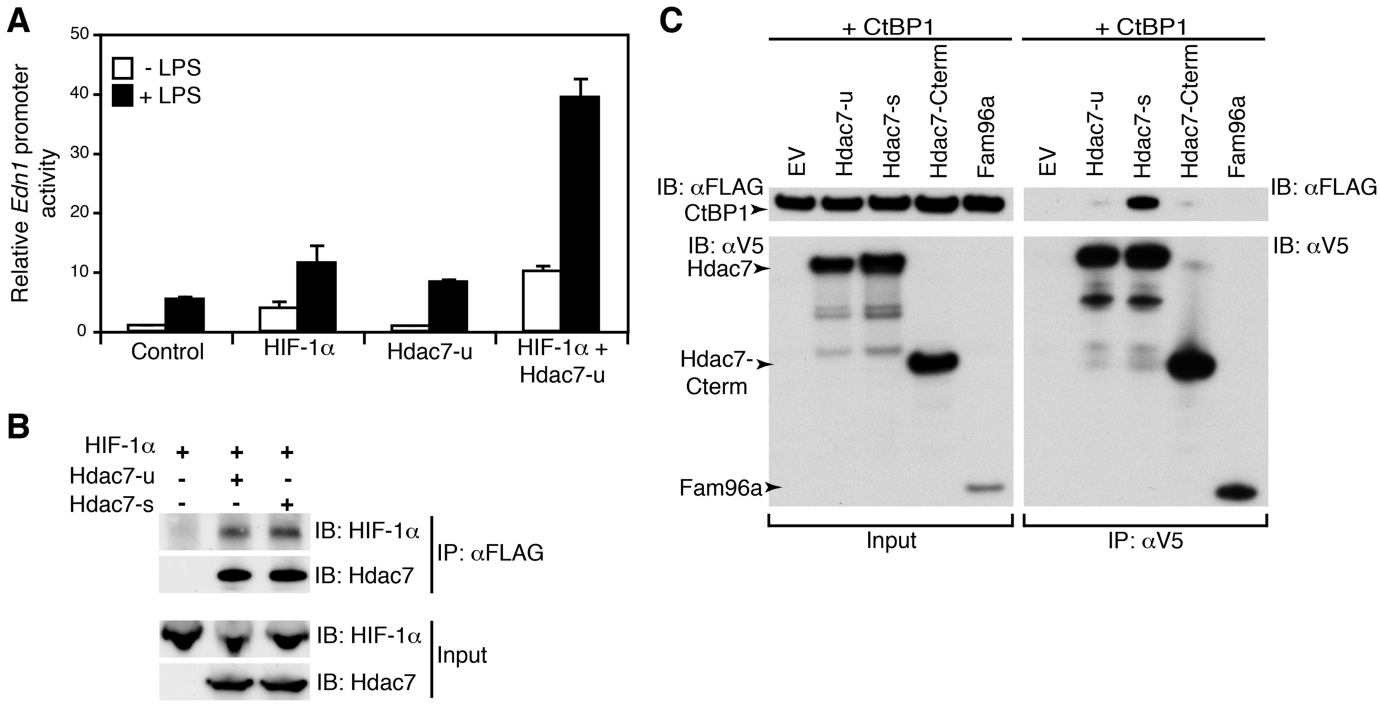

FIGURE 8. **Hdac7 and HIF-1α interact and synergize.** *A*, RAW264 cells were transfected with the *Edn1* promoter construct alone (*control*), the *Edn1* promoter construct plus 1 μg (suboptimal) of HIF-1α expression construct, the *Edn1* promoter construct plus 2 μg (suboptimal) of Hdac7-u expression construct, or the *Edn1* promoter construct plus HIF-1α and Hdac7-u. Cells were treated with LPS (*filled bars*) for 8 h or were left untreated (*open bars*) before analysis of luciferase activity. Data (mean + range of duplicate transfections) are representative of two independent experiments and are displayed relative to the *Edn1* promoter alone (control). *B*, both Hdac7-u and Hdac7-s interact with HIF-1α. Coimmunoprecipitation (*IP*) experiments were performed in HEK293 cells using Hdac-FLAG expression constructs as bait. Immunoprecipitated HIF-1α was detected by anti-V5 immunoblotting (*IB*). Data are representative of three independent experiments. *C*, HEK293 cells were cotransfected with CtBP1-FLAG and either V5 empty vector (*EV*) or V5-tagged Hdac7-u, Hdac7-s, Hdac7-C-term (*Cterm*), or Fam96a (irrelevant control protein). Immunoprecipitation was performed with an anti-V5 antibody, and immunoprecipitated CtBP1-FLAG was detected with an anti-FLAG antibody. Data are representative of two independent experiments.

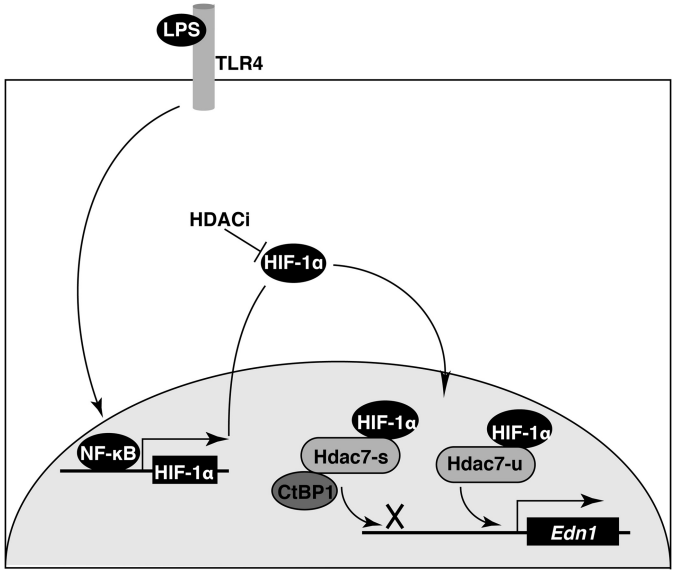

FIGURE 9. **Proposed model of Hdac7-u involvement in TLR4 responses.** LPS signaling up-regulates HIF-1α mRNA and protein expression in macrophages. The early response is dependent upon HDAC activity (but is independent of class IIa Hdacs), whereas the later response is HDAC-independent. Both Hdac7-u and Hdac7-s can interact with HIF-1α, but an interaction between CtBP1 and Hdac7-s prevents this isoform from promoting HIF-1α-dependent transcriptional responses. In contrast, Hdac7-u promotes HIF-1α-dependent expression of *Edn1* as well as coregulated TLR4 target genes.

hypoxic tissues stabilizes HIF-1α, thus priming cells for an encounter with TLR ligands and activation of HIF-1-dependent inflammatory responses (46). Multiple mechanisms have been implicated in TLR-activated HIF-1α responses in macrophages, including increased transcription of the *Hif-1α* gene (47, 48) as well as decreased degradation of HIF-1α protein (35). LPS-mediated production of succinate has also been shown very recently to stabilize HIF-1α protein (36). In our studies, LPS up-regulated mRNA and protein levels of ectopically expressed HIF-1α (Fig. 7, *A* and *B*), so effects beyond activation of the endogenous promoter must contribute to this response. Stabilization of Hif-1α mRNA and/or protein are obvious possibilities. Because TSA (Fig. 7*A*), but not compound 6 (Fig. 7*C*), blocked the early up-regulation of HIF-1α expression by LPS, non-class IIa Hdacs are likely to be involved in promoting this response. In contrast, at later time points, LPS-induced HIF-1α was not inhibited by TSA (Fig. 7, *A* and *B*), thus suggesting alternative mechanisms of control. It is possible that this delayed HDAC-independent response involves succinate-mediated stabilization of HIF-1α (36). Our data thus suggest that multiple Hdacs are involved in regulating HIF-1α during TLR4 responses, non-class IIa Hdacs being required for the initial LPS-induced expression of this protein, whereas Hdac7-u subsequently promotes HIF-1α-dependent transcription. Although a number of HDACs are known to regulate HIF-1α (38, 49, 50), to the best of our knowledge, this is the first report of HDAC-dependent regulation of HIF-1α in TLR pathways.

In addition to promoting HIF-1α-dependent responses, Hdac7 has a well characterized role acting as a transcriptional derepressor during T cell development. In this setting, Hdac7 inhibits the transcriptional activity of members of the MEF2

transcription factor family. T cell receptor signaling promotes the PKD1-dependent nuclear export of Hdac7 (51), thus enabling inducible gene expression. Hence, Hdac7 can regulate inducible gene expression through modulation of both the HIF-1α pathway and the MEF-2 pathway. Whether Hdac7-mediated regulation of MEF2 family members has a function in innate immune cells remains to be clarified. This would seem possible because others have shown that MEF2A and MEF2D are up-regulated during human macrophage differentiation and interact with HDAC7 (52).

Although there is some literature documenting evidence for the potential of HDAC inhibitors in the treatment of inflammatory diseases (14), the specific HDAC enzymes that promote inflammation are still poorly defined. At least some of the anti-inflammatory effects of HDAC inhibitors may reflect the fact that certain HDACs constrain immunoregulatory pathways. For example, Hdac9 is a negative regulator of Treg cell development (53), and Hdac11 inhibits IL-10 production from antigen-presenting cells (54). Hence, inhibition of each of these enzymes might be predicted to have anti-inflammatory effects *in vivo*. In contrast, our data are consistent with Hdac7-u directly promoting inflammatory responses in macrophages, although we cannot exclude the possibility that it also inhibits the expression of anti-inflammatory genes in these cells. However, several lines of evidence indicate that the anti-inflammatory effects of HDAC inhibitors on macrophages cannot be due to Hdac7 inhibition alone. Firstly, studies with HDAC-selective inhibitors implicate multiple HDAC-dependent mechanisms in regulating even a small number of TLR4-inducible genes (18). Secondly, some of the known HDAC-dependent TLR target genes (*e.g. iNOS* and *Ccl7*) were not affected by Hdac7-u overexpression (Figs. 2 and 3). Finally, others have reported recently that Hdac3 promotes TLR4-dependent inflammatory responses in macrophages (44). Hence, Hdac7-u is likely to promote the expression of a subset of HDAC-dependent, TLR4-inducible, proinflammatory genes in macrophages.

The *in vivo* functions of Hdac7 in TLR pathways remain to be determined. *Hdac7*$^{-/-}$ mice die during embryonic development through defects in vasculature development, so an *in vivo* functional analysis will require the generation of innate immune cell-specific knockouts and/or transgenic mice. Nonetheless, our *in vitro* data suggest that Hdac7 is a candidate target for diseases in which innate immune cells contribute to pathology. In this respect, HDAC7 has been proposed previously as a potential proinflammatory target in systemic sclerosis (55), a disease in which both macrophages (56) and ET-1 (57) are implicated. HDAC7 expression was also up-regulated in cartilage from osteoarthritic patients and correlated with an increase in matrix metalloproteinase 13 expression and cartilage degradation (58). However, although we observed that Hdac7 inhibition reduced the LPS-induced production of key inflammatory mediators (Fig. 4, *C–F*), we cannot discount the possibility that inhibition of other class IIa Hdacs contributes to these effects. A recent study also showed that Hdac7 down-regulation was required for trans-differentiation of B cells into macrophages and for optimal acquisition of TLR4 responses (59). This suggests that specific Hdac7 isoforms may have distinct functions in mature macrophages *versus* during myeloid development. Thus, further studies are required to determine the contribution of HDAC7 to inflammation-related pathologies and to map the precise mechanisms through which it promotes HIF-1α-dependent TLR4 responses.

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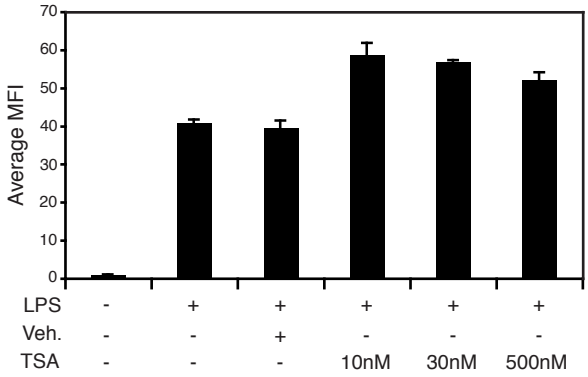
Figure S1



SUPPLEMENTARY S1A. RAW264 cells stably expressing the E-selectin promoter driving GFP were treated with LPS, LPS+EtOH (vehicle), TSA, or LPS+TSA for 6h. Mean fluorescence intensity (MFI) of GFP expression was analysed by flow cytometry. Data shows mean+range of 2 independent experiments.

Histone Deacetylase 7 Promotes Toll-like Receptor 4-dependent Proinflammatory Gene Expression in Macrophages

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