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Differential effects of selective HDAC inhibitors on macrophage inflammatory responses to the Toll-like receptor 4 agonist LPS

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ABSTRACT

Broad-spectrum inhibitors of HDACs are therapeutic in many inflammatory disease models but exacerbated disease in a mouse model of atherosclerosis. HDAC inhibitors have anti- and proinflammatory effects on macrophages in vitro. We report here that several broad-spectrum HDAC inhibitors, including TSA and SAHA, suppressed the LPS-induced mRNA expression of the proinflammatory mediators *Edn-1*, *Ccl-7/MCP-3*, and *Il-12p40* but amplified the expression of the proatherogenic factors *Cox-2* and *Pai-1/serpine1* in primary mouse BMM. Similar effects were also apparent in LPS-stimulated TEPM and HMDM. The pro- and anti-inflammatory effects of TSA were separable over a concentration range, implying that individual HDACs have differential effects on macrophage inflammatory responses. The HDAC1-selective inhibitor, MS-275, retained proinflammatory effects (amplification of LPS-induced expression of *Cox-2* and *Pai-1* in BMM) but suppressed only some inflammatory responses. In contrast, 17a (a reportedly HDAC6-selective inhibitor) retained anti-inflammatory but not proinflammatory properties. Despite this, HDAC6$^{-/-}$ macrophages showed normal LPS-induced expression of HDAC-dependent inflammatory genes, arguing that the anti-inflammatory effects of 17a are not a result of inhibition of HDAC6 alone. Thus, 17a provides a tool to identify individual HDACs with proinflammatory properties. *J. Leukoc. Biol.* 87: 1103–1114; 2010.

Introduction

HDACs are enzymes that remove acetyl groups from specific lysine residues on histone proteins to regulate chromatin architecture and gene expression. These enzymes also post-translationally modify a number of nonhistone targets to control diverse cell functions [1]. HDACs are grouped into four distinct classes. Class I HDACs (HDAC1, -2, -3, and -8) generally localize to the nucleus [2], and class II HDACs (HDAC4, -5, -6, -7, -9, and -10) can shuttle between the nucleus and cytoplasm [2]. HDAC11, which has similarity to class I and II enzymes, is the sole member of the class IV group [1]. The class III HDACs or SIRT1–7 require the cofactor nicotinamide adenine dinucleotide for activity and act via a distinct mechanism to class I, II, and IV HDACs [3].

Small molecule inhibitors of HDACs generally cause growth arrest, cellular differentiation, and apoptosis and have been pursued as anticancer drugs [1]. In 2006, the U.S. Food and Drug Administration approved the HDAC inhibitor SAHA/vorinostat for the treatment of cutaneous T cell lymphoma [4]. Most HDAC inhibitors used in experimental animal models and in clinical trials as anticancer agents target the class I and class II enzymes. These include natural products such as butyrate and *R*-TSA [5], as well as synthetic compounds such as SAHA [4, 6]. Some inhibitors have reported selectivity for individual family members. MS-275 had the greatest potency

Abbreviations: BMM=bone marrow-derived macrophage(s), ChIP=chromatin immunoprecipitation assay, compound 17a=*(S)*-thioisobutyric acid *S*-(6-*tert*-butoxycarbonylamino-6-cyclopentylcarbamoylhexyl)ester, Cox-2=cyclooxygenase-2, Edn1=endothelin-1, EtOH=ethanol, HDAC=histone deacetylase, HIF-1α=hypoxia-inducible factor-1α, HMDM=human monocyte-derived macrophage(s), Hprt=hypoxanthine guanine phosphoribosyl transferase, MKP-1=MAPK phosphatase 1, NaB=sodium butyrate, PAI-1/serpine1=plasminogen activator inhibitor type 1, qPCR=quantitative PCR, SAHA=suberoylanilide hydroxamic acid, SIRT=sirtuin, TEPM=thioglycollate-elicited peritoneal macrophage(s), TSA=trichostatin A

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on the class I enzyme, HDAC1 [7], whilst tubacin [8], compound 17a [9], and compound 7 described by Kozikowski et al. [10] reportedly had some selectivity for the class II family member, HDAC6. Class III HDACs/SIRTs are insensitive to class I/II inhibitors as a result of mechanistic and structural differences between the enzymes [3].

In addition to their anticancer activity, pan-HDAC inhibitors have anti-inflammatory properties [5, 11, 12]. SAHA was therapeutic in mouse models of septic shock [13] and rheumatoid arthritis [14], TSA reduced disease severity in mouse models of multiple sclerosis [15], arthritis [16], and asthma [17], and butyrate reduced inflammation in a mouse model of colitis [18]. Several HDAC inhibitors were also neuroprotective in a rat model of ischemia-induced brain injury [19]. Despite these therapeutic effects, TSA actually exacerbated disease in a mouse model of atherosclerosis [20], and loss of HDAC activity has been implicated in disease exacerbation and insensitivity to treatment with glucocorticoids in chronic obstructive pulmonary disease [21, 22]. Such studies imply that although broad-spectrum HDAC inhibitors have potent anti-inflammatory properties, they are likely to have contraindications that could limit their use in such applications. Elucidation of the role that individual HDAC enzymes play in inflammatory pathways will aid in the design of specific inhibitors for anti-inflammatory applications.

We showed previously that HDAC inhibitors superinduced or repressed distinct subsets of inflammation-inducible genes in macrophages [23], whilst in other studies, classic, proinflammatory genes (e.g., IL-1β) were unaffected [13, 24, 25]. The suppressive effects of HDAC inhibitors on inflammatory mediator production from human and mouse macrophages and dendritic cells in response to TLR ligands have also been demonstrated in other studies [13, 24–26]. Differential effects of HDAC inhibitors on subsets of inflammation-associated target genes may relate to distinct functions of individual HDAC family members. For example, the class II HDAC, HDAC6, was required for virus-induced *IFN-β* expression in human fibroblasts, and the class I enzymes, HDAC1 and HDAC8, suppressed this response [27]. The suppressive effects of class I HDACs (HDAC1, -2, -3, and -8) on inflammatory gene expression have also been observed in other studies. We [23] and others [28] showed that class I HDACs abolished LPS-induced *Cox-2* expression or promoter activity in RAW264.7 murine macrophages, and HDAC2 was required for glucocorticoid-mediated suppression of inflammatory mediator production from human alveolar macrophages [22]. The implication is that targeting of class I HDACs may be undesirable in some inflammatory contexts. In this study, we set out to determine whether the pro- and anti-inflammatory effects of HDAC inhibitors on macrophage gene expression can be separated.

MATERIALS AND METHODS

Reagents

The thiazolyl blue tetrazolium bromide salt (MTT), TSA, NaB, mitomycin C, nocodazole, and *Salmonella minnesota* Re595 LPS were obtained from Sigma-Aldrich (St. Louis, MO, USA). MS-275 was obtained from Calbiochem (Darmstadt, Germany). MTT assay reagent was dissolved in PBS at 5 mg/mL and diluted to a final concentration of 1 mg/mL in complete media for use in cell culture. TSA was dissolved in EtOH, NaB was dissolved in milliQ H_2O, and LPS was dissolved at 5 mg/mL with sonication in 0.1% triethylamine and diluted further in complete media for cell-culture treatment. SAHA and compound 17a (reported by Suzuki et al. [9]) and compound 7 [10] were stored in powder form at 4°C after synthesis and were dissolved in DMSO for use in experiments. Mitomycin C powder was dissolved in milliQ H_2O immediately before use. Nocodazole was dissolved in DMSO at 3.3 mg/ml and diluted further in complete media before use.

Cell culture

The murine macrophage-like cell line RAW264.7 was obtained from American Type Culture Collection (Manassas, VA, USA) and was grown in bacteriological plastic dishes. TEPM were obtained by injecting C57BL/6J male 6- to 8-week mice i.p. with 1 ml 10% thioglycollate broth. Macrophages were harvested by peritoneal lavage with PBS after 5 days. Primary BMM were prepared by differentiating BM cells from C57BL/6J male 6- to 8-week mice or HDAC6$^{-/-}$ and wild-type littermate control mice with CSF-1. HMDM were prepared from CD14$^+$ monocytes from Red Cross blood donors [29]. In brief, mouse BM cells and CD14$^+$ monocytes were differentiated into BMM and HMDM, respectively, by culture with 1×10^4 U/mL human rCSF-1 (a gift from Chiron, Emeryville, CA, USA) for 6 days. On Day 6, BMM and HMDM were harvested and plated in complete media containing CSF-1 for treatment on Day 7. BMM, TEPM, and RAW264.7 cells were cultured in RPMI-1640 media supplemented with 10% FCS, 20 U/mL penicillin, 20 U/mL streptomycin, and 2 mM L-glutamine, and HMDM were cultured in IMDM (Invitrogen, Carlsbad, CA, USA) with 10% FCS, glutamine, and antibiotics, as described above. All cells were cultured in an incubator at 37°C with 5% CO_2.

MTT assay

Cell viability was measured using the MTT assay. Briefly, BMM or RAW264.7 cells were plated in 96-well plates at a density of 1×10^5 cells/well or 7×10^4 cells/well, respectively, and left overnight at 37°C to adhere. On the following day, cells were treated with compounds and incubated for 24 or 48 h at 37°C, after which, cell viability was determined by MTT assay. Absorbance at 570 nm was measured on a PowerWave XS micro-well plate reader (BioTek Instruments, Winooski, VT, USA), and raw data were analyzed using Prism v4 (GraphPad Software Inc., San Diego, CA, USA). Data were expressed as a percentage of the vehicle-treated control.

RNA extraction and real-time RT-qPCR

Cells were seeded onto six-well plates in complete media containing CSF-1 and allowed to adhere overnight at 37°C. On the following day, BMM or HMDM were treated as indicated with HDAC inhibitors in the presence or absence of 10 ng/mL LPS for the indicated time periods. RNA was prepared, and mRNA abundance was quantitated relative to the housekeeping gene *Hprt* using the SYBR Green system. Where data from multiple independent experiments were pooled and graphed + SEM, data for each experiment were normalized (by setting the LPS treatment as 1), except for the analysis of HDAC6$^{-/-}$ BMM. In this case, data are presented as gene expression relative to *Hprt* for wild-type versus HDAC6$^{-/-}$ BMM (mean+SEM for BMM from four wild-type and four HDAC6$^{-/-}$ mice). The statistical significance of deviation of means from samples stimulated with LPS versus the LPS + HDAC inhibitor was measured using data averaged from multiple independent experiments using a one-tailed one-sample *t*-test (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

Luciferase assays

RAW264.7 cells stably transfected with a 1.4-kb *Cox-2* promoter driving the luciferase reporter gene or the parent pGL2B control plasmid [23] were

treated for 8 h with varying concentrations of TSA in the presence or absence of LPS (10 ng/mL). Luciferase activity in cell lysates was assayed using the Luclite luminescence reporter gene assay system (PerkinElmer Life Sciences, Waltham, MA, USA), according to the manufacturer's instructions.

ELISAs

Levels of inflammatory mediators in macrophage culture supernatants were determined using sandwich ELISAs (IL-12p40, Becton Dickinson, San Jose, CA, USA; PAI-1, Sapphire Biosciences, Australia).

Immunoblotting

Protein levels in BMM were assessed by Western analysis as described previously [23, 24]. Briefly, BMM were treated with HDAC inhibitors, and total cell extracts were prepared using boiling lysis buffer (66 mM Tris-HCl, pH 7.4, 2% SDS) with homogenization. Total protein concentration was determined using the bicinchoninic protein assay kit (Pierce, Rockford, IL, USA), and samples were resolved on pre-cast NuPAGE Bis-Tris 10–12% denaturing polyacrylamide gels (Invitrogen), which were transferred to methanol-activated Immobilon-P polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) and probed with specific antibodies for acetylated α-tubulin (1:2000 dilution, Sigma-Aldrich), acetylated histone H3 (1:5000, Cell Signaling Technology, Beverly, MA, USA) and H4 (1:2000, Upstate, Lake Placid, NY, USA), total histone H3 (1:1000, Cell Signaling Technology) and total H4 (1:750, Upstate), and STAT1 (1:1000, Cell Signaling Technology). Bands were visualized using a HRP-conjugated anti-rabbit IgG or anti-mouse IgG antibody (1:3000 dilution, Cell Signaling Technology) and chemiluminescence (Amersham Pcl, Buckinghamshire, UK).

ChIP assay

ChIP was performed as described previously [30, 31] using a specific anti-PU.1 antibody (sc-352x, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit IgG as an isotype control (Cell Signaling Technology). PU.1 enrichment at the PU.1-binding site within the *Ccl7* promoter was determined by qPCR analysis using specific primers to amplify that region (forward, 5′-CTTGGGATGTTGCTCGAAGG-3′; reverse, 5′-GCAGAGATGGCAGAGTCAAGG-3′). The specificity of PU.1 enrichment at the PU.1-binding site was confirmed using primers that amplified 1 kb upstream and downstream of this site within the *Ccl7* promoter (1 kb upstream forward, 5′-CTAGGCCATTTTTTCACCTTGTAAA-3′, reverse, 5′-GCAGACAAAGGTGGAAATAGGAA-3′; 1 kb downstream forward, 5′-AAGATCCCCAAGAGGAATCTCAA-3′, reverse, 5′-TTCCCAGGGACACCGACTACT-3′).

RESULTS

Differential effects of broad-spectrum inhibitors of HDACs on inflammatory gene expression in macrophages

Some HDAC-dependent inflammatory genes have been mapped by using microarrays to assess the effect of a single broad-spectrum HDAC inhibitor on macrophage responses to the TLR4 agonist LPS [23, 25]. Such studies have identified genes in which LPS-induced expression is suppressed (e.g., *Ccl-7*, *Edn-1*) or amplified (e.g., *Cox-2*) by HDAC inhibitors. We began by assessing the impact of several broad-spectrum inhibitors of HDAC enzymes on the expression of some of these genes to determine the generality of these effects. TSA, NaB, and SAHA all blocked LPS-induced *Ccl-7* mRNA expression effectively (**Fig. 1A**), but greatly enhanced LPS-induced *Cox-2* mRNA expression (Fig. 1B) at 10 h post-LPS stimulation. The regulated expression of inflammatory mediators in macrophages is dynamic, and some genes (e.g., *Edn-1*) are in-

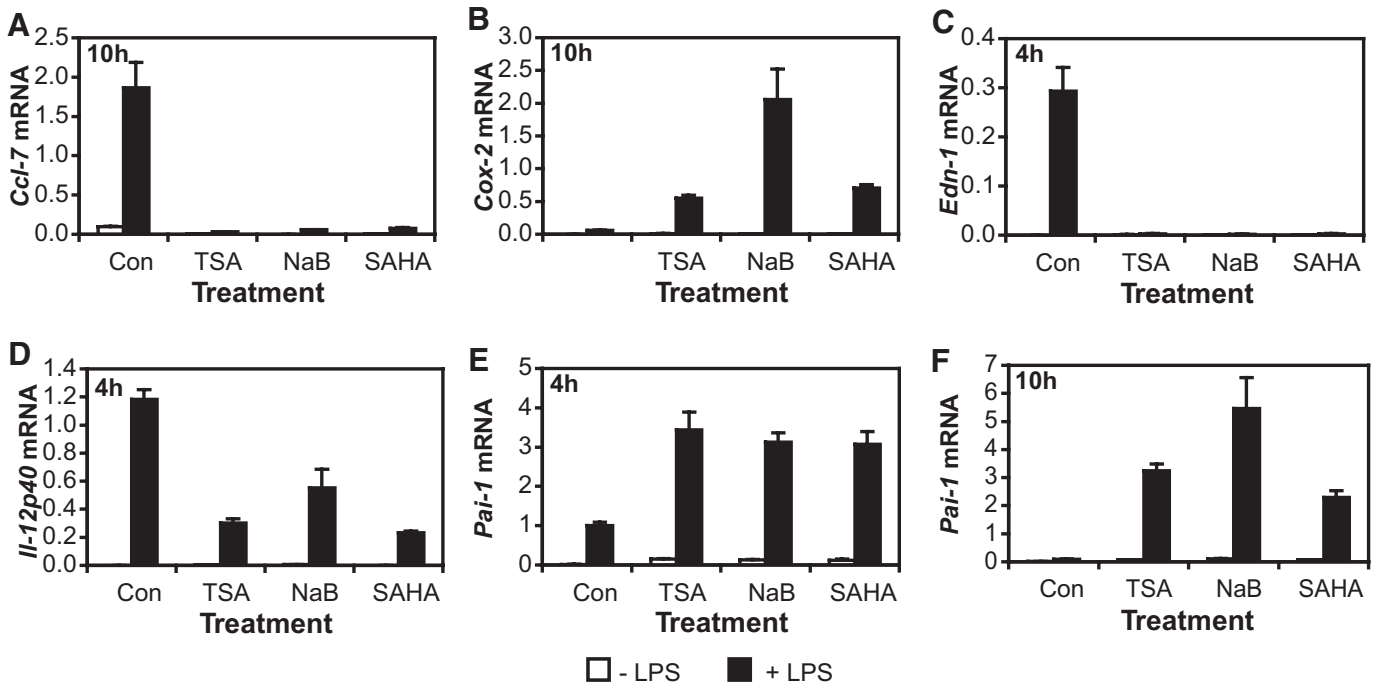


Figure 1. Divergent effects of broad-spectrum HDAC inhibitors on inflammatory gene expression in BMM. BMM were treated with medium [control (Con)] or with TSA (500 nM), NaB (10 mM), or SAHA (10 μM) in the presence (solid bars) or absence (open bars) of LPS (10 ng/mL) for 10 h (A, B, and F) or 4 h (C–E). Levels of mRNA for *Ccl-7* (A), *Cox-2* (B), *Edn-1* (C), *Il-12p40* (D), and *Pai-1* (E and F) were quantitated by RT-qPCR. Data points represent the mean of triplicates ± SD, and all data are representative of at least three independent experiments.

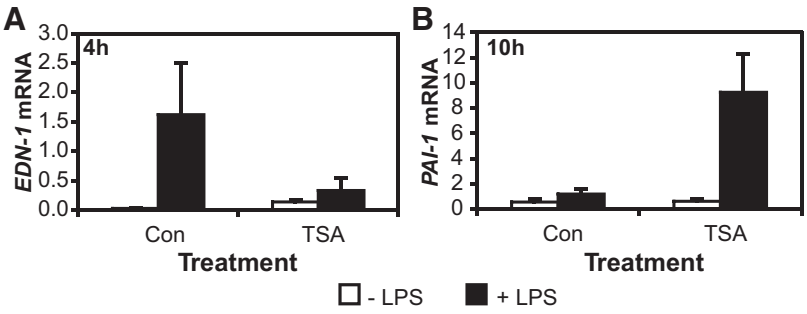


Figure 2. TSA suppresses *EDN-1* and amplifies *PAI-1* gene induction in LPS-stimulated HMDM. HMDM were treated with medium (Con), LPS (10 ng/mL), TSA (500 nM), or TSA + LPS, and mRNA was harvested and analyzed by RT-qPCR. Levels of mRNA for *EDN-1* at 4 h (A) and *PAI-1* at 10 h (B) were determined by RT-qPCR. Data points are the mean of triplicates + SD and are representative of four experiments from different donors.

duced rapidly and transiently. To examine the impact of broad-spectrum inhibitors on *Edn-1* expression, we examined an earlier time-point corresponding to the peak induction of *Edn-1* by LPS (data not shown). At 4 h post-stimulation, TSA, NaB, and SAHA all blocked LPS-inducible *Edn-1* expression effectively (Fig. 1C). The inducible expression of *Il-12p40* was also inhibited (Fig. 1D), as we reported previously for TSA [24].

From a therapeutic viewpoint, the amplification of *Cox-2* expression in macrophages by HDAC inhibitors is of concern, as the expression of this gene is elevated in macrophages in symptomatic atherosclerotic plaques [32], where it drives plaque rupture via PGE_2-mediated production of matrix metalloproteinases [33]. Elevated plasma levels of PAI-1 protein are also associated with an increased risk of thrombus formation and cardiovascular disease [34], so we assessed the impact of HDAC inhibitors on expression of this gene in macrophages. As with *Cox-2*, LPS-induced expression of *Pai-1* mRNA was amplified greatly by TSA, SAHA, and NaB at 4 and 10 h post-stimulation (Fig. 1, E and F). The differential effects of HDAC inhibitors on LPS responses were also apparent in HMDM, where TSA suppressed the LPS-inducible mRNA expression of *EDN-1* (**Fig. 2A**) but amplified LPS-induced *PAI-1* expression (Fig. 2B).

Opposing effects of HDAC inhibitors on IL-12p40 and PAI-1 protein secretion in macrophages

We next confirmed that the divergent effects of TSA on LPS-induced *Il-12p40* and *Pai-1* were also apparent at the protein level; TSA reduced IL-12p40 (**Fig. 3A**) but amplified PAI-1 protein secretion (Fig. 3B) in LPS-stimulated BMM. We also assessed whether the divergent effects of TSA on inflammatory responses were evident in another primary mouse macrophage population. As with BMM, TSA repressed LPS-inducible IL-12p40 (Fig. 3C) but amplified LPS-inducible PAI-1 (Fig. 3D) protein secretion from TEPM.

Pro- and anti-inflammatory effects of TSA on macrophage inflammatory responses occur at distinct concentrations

The divergent effects of HDAC inhibitors on inflammatory responses in macrophages could reflect distinct functions for individual HDAC family members. To determine whether the

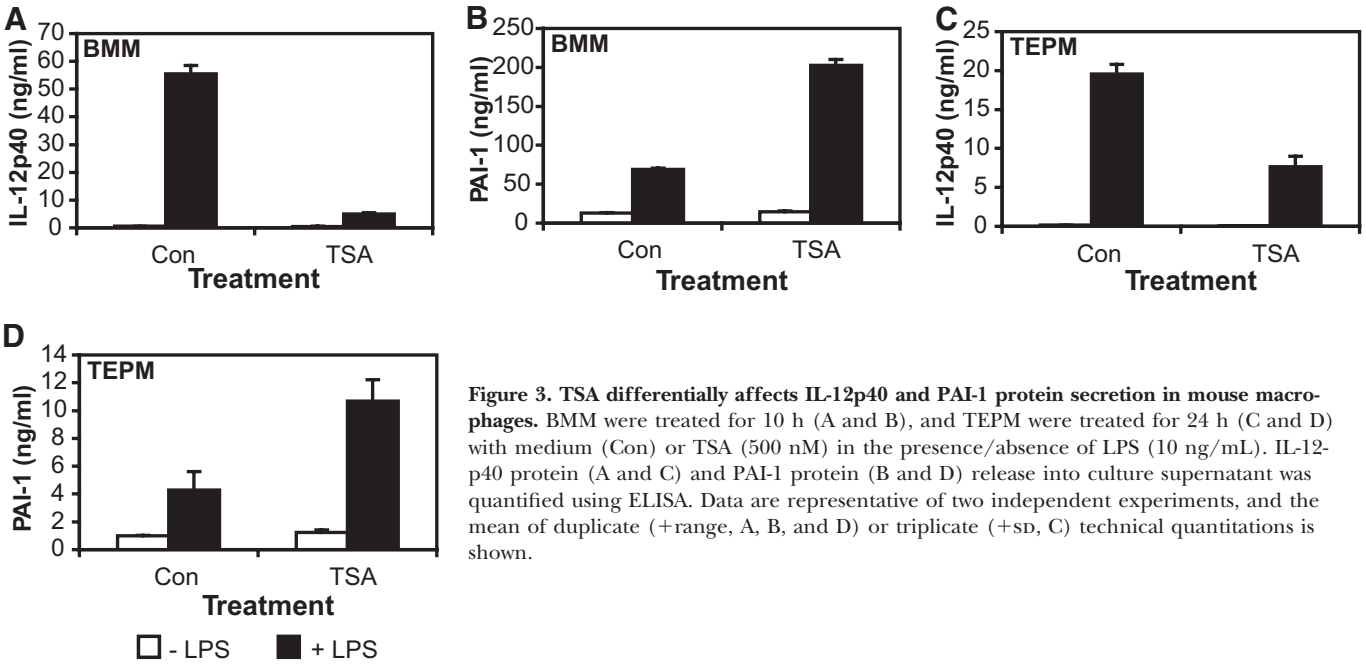


Figure 3. TSA differentially affects IL-12p40 and PAI-1 protein secretion in mouse macrophages. BMM were treated for 10 h (A and B), and TEPM were treated for 24 h (C and D) with medium (Con) or TSA (500 nM) in the presence/absence of LPS (10 ng/mL). IL-12-p40 protein (A and C) and PAI-1 protein (B and D) release into culture supernatant was quantified using ELISA. Data are representative of two independent experiments, and the mean of duplicate (+range, A, B, and D) or triplicate (+SD, C) technical quantitations is shown.

pro- and anti-inflammatory effects of broad-spectrum HDAC inhibitors could be distinguished, we assessed the effect of different TSA concentrations on inflammatory gene expression in BMM. TSA had minimal toxicity to BMM at concentrations below 30 nM, as assessed by MTT assay (**Fig. 4A**). Nonetheless, cytotoxic (100 and 500 nM) and noncytotoxic (10 and 30 nM) concentrations of TSA were active, as indicated by hyperacetylation of histone H3/H4 and α-tubulin in BMM (Fig. 4B). At 10 nM (a noncytotoxic concentration), TSA strongly repressed LPS-inducible mRNA expression of *Edn-1, Il-12p40*, and *Ccl-7* in BMM (Fig. 4, C–E). In contrast, 10 nM TSA did not amplify the LPS-induced expression of *Cox-2* (Fig. 4F), and *Pai-1* mRNA levels were increased only moderately (Fig. 4G). However, the 500-nM TSA concentration greatly increased LPS-induced *Cox-2* and *Pai-1* mRNA expression in BMM (Fig. 4, F and G). A similar phenomenon was apparent at the promoter level in mouse macrophage RAW264.7 cells stably transfected with a *Cox-2* promoter-luciferase reporter construct, where only concentrations of TSA that were cytotoxic to RAW264.7 cells (Fig. 4H) enhanced LPS-inducible *Cox-2* promoter activity (Fig. 4I).

We next determined whether the effects of HDAC inhibition on LPS-regulated gene expression, which we observed when examining single time-points (Figs. 1, 2, and 4), were

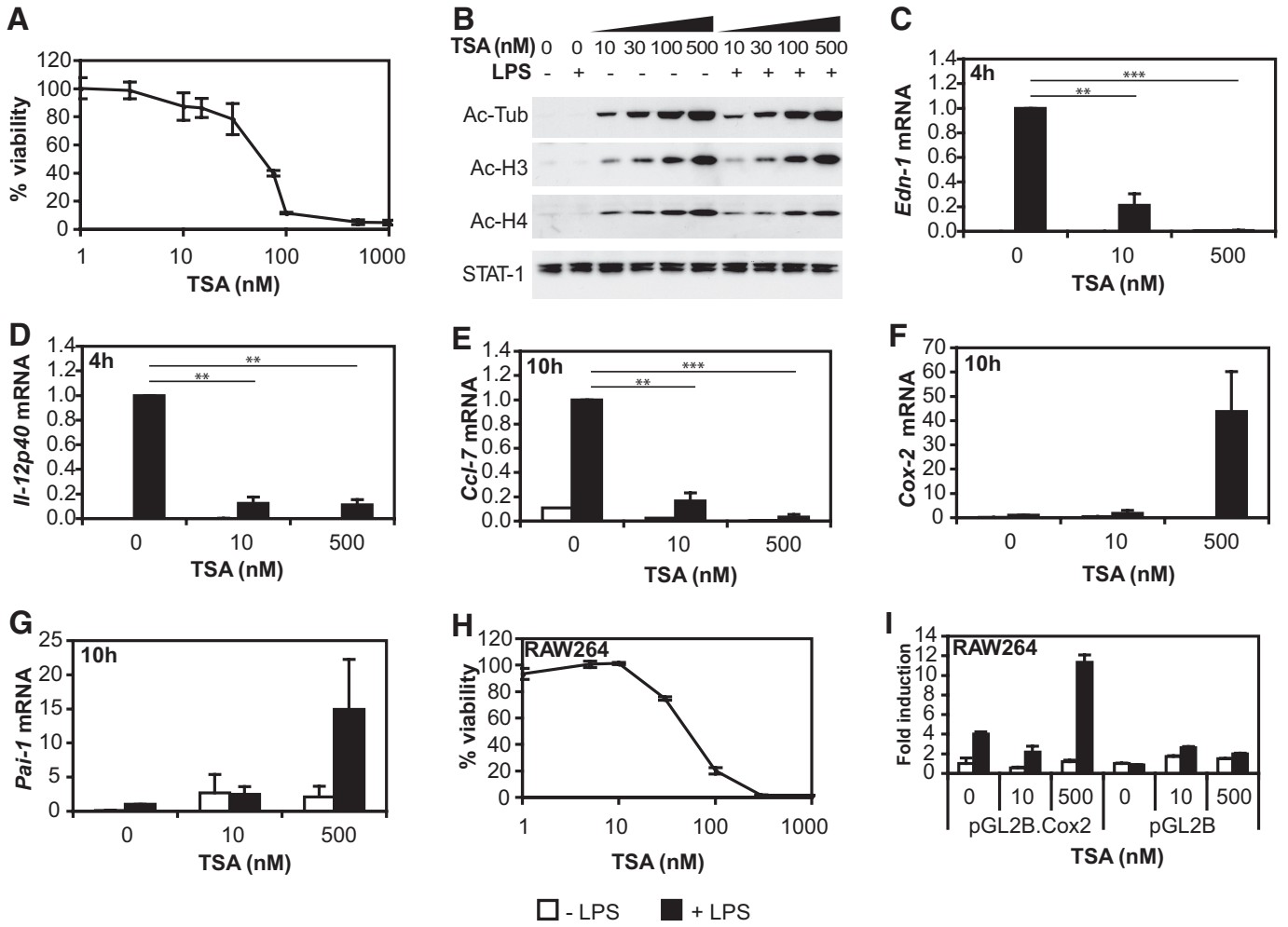


Figure 4. Pro- and anti-inflammatory effects of TSA occur at distinct concentrations in BMM. (A) BMM were treated with varying concentrations of TSA for 24 h, and cell viability was assessed by MTT assay. Data points are the mean of triplicates ± SD. (B) Cells were treated with various concentrations of TSA for 10 h, with (+) or without (–) LPS (10 ng/mL), and hyperacetylation of α-tubulin (Ac-Tub) and histone H3/H4 (Ac-H3/Ac-H4, respectively) was assessed by immunoblotting. STAT-1 protein was used as a loading control. (C–G) BMM were treated with medium or 10 or 500 nM concentrations of TSA in the presence (solid bars) or absence (open bars) of LPS (10 ng/mL) for 4 h (C and D) or 10 h (E–G). Levels of mRNA for *Edn-1* (C), *Il-12p40* (D), *Ccl-7* (E), *Cox-2* (F), and *Pai-1* (G) were determined by real-time PCR. Data points represent the average + SEM of three independent experiments (**, $P<0.01$; ***, $P<0.001$). (H) RAW264.7 cells were treated with varying concentrations of TSA for 24 h, and cell viability was assessed by MTT assay. Data points are means of triplicates ± SD and are representative of three independent experiments. (I) RAW264.7 cells were stably transfected with pGL2B.*Cox2* or pGL2B and were treated with 10 or 500 nM TSA in the presence (solid bars) or absence (open bars) of LPS (10 ng/mL) for 8 h. Luciferase activity was quantified, and results are displayed as fold induction relative to control values. Data are average of triplicates ± SD and representative of two independent experiments.

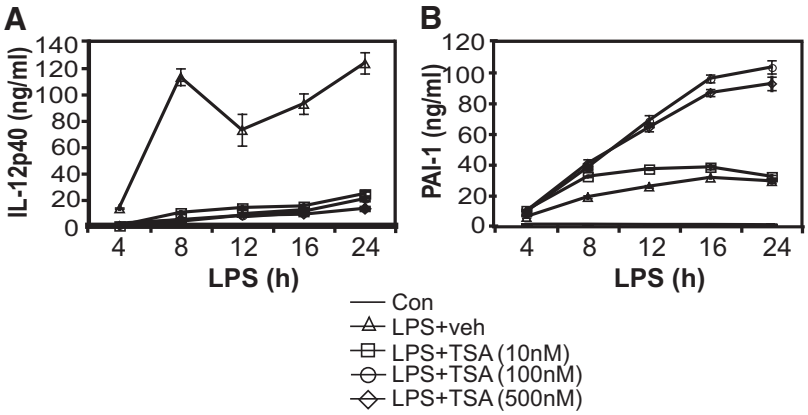


Figure 5. Time course of effects of TSA on LPS-inducible IL-12p40 and PAI-1 protein secretion in BMM. BMM were treated with LPS (10 ng/ml) plus vehicle (veh; EtOH, control for TSA), LPS plus varying concentrations of TSA (10 nM, 100 nM, or 500 nM) or were left untreated (Con). Culture supernatants were harvested over a 24 h time course, and ELISA was used to determine levels of IL-12p40 (A) and PAI-1 (B). Data are displayed as mean (n=3) + SD and are representative of two independent experiments.

also apparent when examining functional responses in BMM (IL-12p40 and PAI-1 protein secretion) across a 24 h LPS time course. TSA inhibited LPS-induced IL-12p40 production in BMM at all concentrations examined (10, 100, and 500 nM) and at all points in the time course (**Fig. 5A**). In contrast, PAI-1 secretion from BMM was amplified across the same time course, and higher cytotoxic TSA concentrations (100 and 500 nM) were particularly effective (Fig. 5B). Similar to the findings with regulated gene expression (Fig. 4G), the noncytotoxic TSA concentration (10 nM) did increase PAI-1 production but had a more modest effect than cytotoxic TSA concentrations.

Only cytotoxic concentrations of TSA strongly amplified LPS-induced *Pai-1* and *Cox-2* mRNA levels (Fig. 4) and PAI-1 protein secretion in BMM (Fig. 5). This is likely a result of specific HDAC enzymes having an essential, dual function in cellular proliferation, as well as negative regulation of inflammatory gene expression. For example, HDAC1 is required for normal cellular proliferation [35] but also negatively regulates LPS-inducible *Cox-2* promoter activity [28], and separating these two activities may not be possible in proliferating cells. Nonetheless, it was technically possible that the amplifying effect of HDAC inhibitors on LPS-induced *Pai-1* and *Cox-2* mRNA expression was triggered as a generalized response to cell death, rather than HDAC inhibition per se. This seemed unlikely, as HDAC inhibitors amplified *PAI-1* gene expression in HMDM (Fig. 2) without having any obvious effect on cell viability (data not shown), and we showed previously that HDAC inhibitors amplified *Cox-2* gene expression by interfering with negative-feedback regulation by HDACs [23]. To exclude this possibility formerly, however, the effect of two other cytotoxic drugs (mitomycin C and nocodazole) on LPS-induced PAI-1 protein secretion from BMM was examined. Although treatment of BMM for 24 h with mitomycin C or nocodazole reduced cell viability profoundly (**Fig. 6A**), neither drug amplified LPS-induced PAI-1 protein secretion (Fig. 6B). These data indicate that not all cytotoxic drugs amplify a subset of macrophage inflammatory responses and imply that broad-spectrum HDAC inhibitors do not simply do so by killing cells.

Comparison of the effects of selective HDAC inhibitors on protein acetylation in macrophages

The class I HDACs, HDAC1 and HDAC8, repressed LPS-inducible *Cox-2* expression or promoter activity [23, 28]. As HDAC1 is LPS-inducible in macrophages [23], it is likely that this HDAC acts as a negative-feedback regulator of inducible *Cox-2* expression. The amplification of LPS-inducible *Cox-2* expression by broad-spectrum inhibitors could thus result from inhibition of HDAC1. We therefore predicted that MS-275, which has reported selectivity for HDAC1 [7], would have a similar effect to broad-spectrum HDAC inhibitors in amplifying *Cox-2* expression. For comparison, we also assessed the effects of two compounds, compound 17a [9] and compound 7 [10], which were reportedly selective for HDAC6. We hypothesized that HDAC6 might promote some TLR-initiated, inflammatory responses in macrophages, because HDAC6 has been reported to sta-

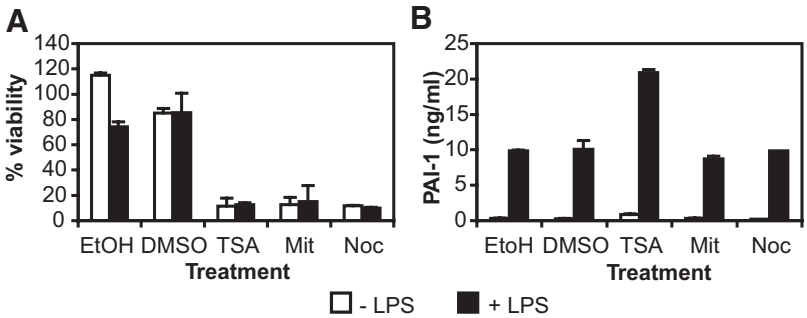


Figure 6. PAI-1 induction by TSA in BMM is not a generalized response to cytotoxicity. BMM were treated with vehicle (EtOH, control for TSA; DMSO, control for nocodazole), 500 nM TSA, 100 μg/ml mitomycin C (Mit), or 10 μM nocodazole (Noc) in the presence (solid bars) or absence (open bars) of 10 ng/ml LPS. (A) Cell viability at 24 h was examined by MTT assay, and (B) PAI-1 protein secretion at 10 h was assessed by ELISA. Data are average of duplicate quantitations + range and represent profiles from two independent experiments.

bilize the transcription factor HIF-1α and promote its transcriptional activity [36], and because HIF-1α is a key transcription factor in TLR signaling pathways and inflammatory responses in macrophages in vitro and in vivo [37, 38]. We therefore predicted that these HDAC6-selective inhibitors would diminish TLR-driven inflammatory responses in macrophages.

To assess the selectivity of MS-275, compound 17a, and compound 7 in macrophages, we first examined the hyperacetylation of HDAC substrates, HDAC1 and HDAC6 deacetylate histone H3, [35] and α-tubulin [39], respectively. Although TSA caused hyperacetylation of histone H3 and α-tubulin in BMM (**Figs.** 4B and **7A**), MS-275 caused hyperacetylation of histone H3 but not α-tubulin (Fig. 7A). Conversely, compound 17a preferentially hyperacetylated α-tubulin, although higher concentrations also enhanced histone H3 acetylation (Fig. 7A). Even at the highest concentration of 17a examined (100 μM), histone H3 acetylation was modest by comparison with that observed with TSA and MS-275 (see Fig. 9C). We conclude that 17a does display some selectivity for HDAC6 over HDAC1 in BMM, although potency against other HDACs in these cells is currently unknown. Conversely, compound 7, which was reported to inhibit HDAC6 preferentially over HDAC3 and -10 [10], did not show any differential effects on histone H3 versus α-tubulin acetylation in BMM (Fig. 7A). Thus, we focused on a comparison of MS-275 and 17a. The differential effects of MS-275 and 17a were also apparent at the level of cell survival; like broad-spectrum HDAC inhibitors, MS-275 was cytotoxic to BMM (Fig. 7B). By contrast, 17a did not affect BMM viability at concentrations up to 300 μM (data not shown).

Differential effects of selective HDAC inhibitors on macrophage inflammatory responses

Consistent with the known functions of class I HDACs as negative regulators of *Cox-2* expression, MS-275 amplified LPS-induced C*ox-2* mRNA levels in BMM at 10 h (**Fig. 8A**). *Pai-1* mRNA expression was similarly affected (Fig. 8B). In contrast, 17a did not enhance the inducible expression of these genes, but it did effectively inhibit LPS-induced *Edn-1* (Fig. 8C) and *IL-12p40* (Fig. 8D) gene expression. MS-275 failed to affect IL-12p40 mRNA expression and was also much less potent at reducing levels of secreted IL-12p40; TSA and 17a caused a dramatic reduction in LPS-induced IL-12p40 production, and MS-275 had more modest effects over a concentration range (Fig. 8E).

Previously, we reported that *Ccl-7* gene expression in BMM was dependent on the transcription factor PU.1 and that TSA reduced PU.1 protein expression and *Ccl-7* mRNA expression [23]. In contrast to broad-spectrum inhibitors, neither MS-275 nor 17a affected the basal or LPS-inducible expression of *Ccl-7* (**Fig. 9A**). Consistent with this, TSA repressed basal and LPS-induced *Pu.1* mRNA expression in BMM, whilst MS-275 but not 17a reduced the expression of this gene modestly (Fig. 9B). Similar effects were apparent when examining PU.1 protein levels; TSA greatly reduced PU.1 protein expression in LPS-activated BMM, whilst MS-275 and 17a had more modest effects (Fig. 9C). Finally, ChIP assays demonstrated that TSA ablated PU.1 association with a previously characterized PU.1-

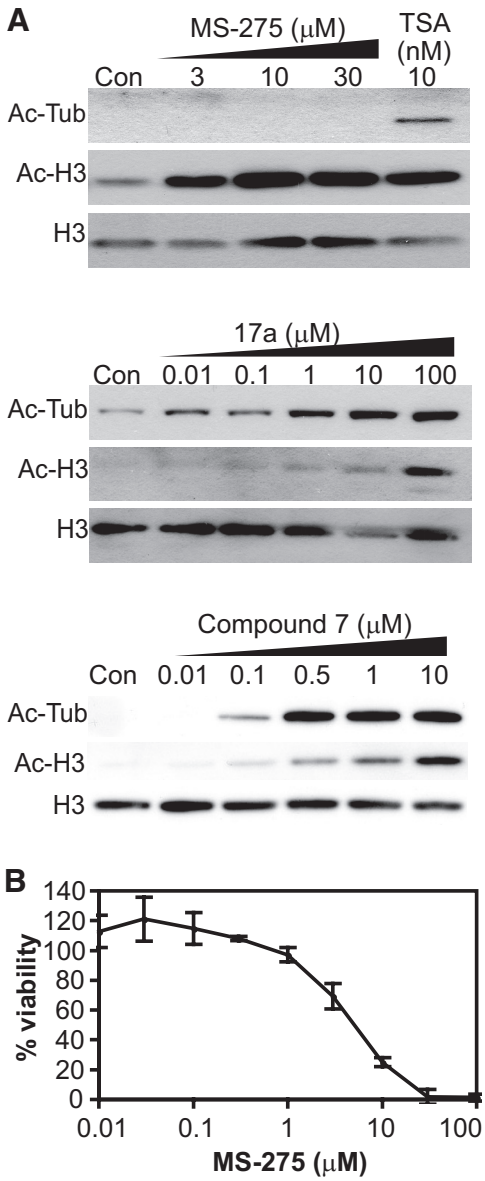


Figure 7. Differential effects of selective HDAC inhibitors on protein acetylation in BMM. (A) BMM were treated with the HDAC1 inhibitor MS-275, the broad-spectrum inhibitor TSA, or the HDAC6 inhibitors 17a and compound 7 at varying concentrations for 4 h. Whole cell lysates were prepared, and histone H3 and α-tubulin acetylation was assessed by immunoblotting. Total histone H3 is shown as a loading control. Data represent three independent experiments. (B) BMM were treated with varying concentrations of MS-275 for 24 h, and cell viability was assessed by MTT assay. Data points are the mean ± SD of triplicate experiments and represent three independent experiments.

binding site in the *Ccl7* promoter (Fig. 9D). In contrast, PU.1 still associated with this site in LPS-activated BMM treated with MS-275, albeit at somewhat reduced levels (Fig. 9D). The specificity of the association was confirmed by demonstrating no enrichment for PU.1 association when using primers detecting genomic DNA 1 kb upstream and 1 kb downstream of the

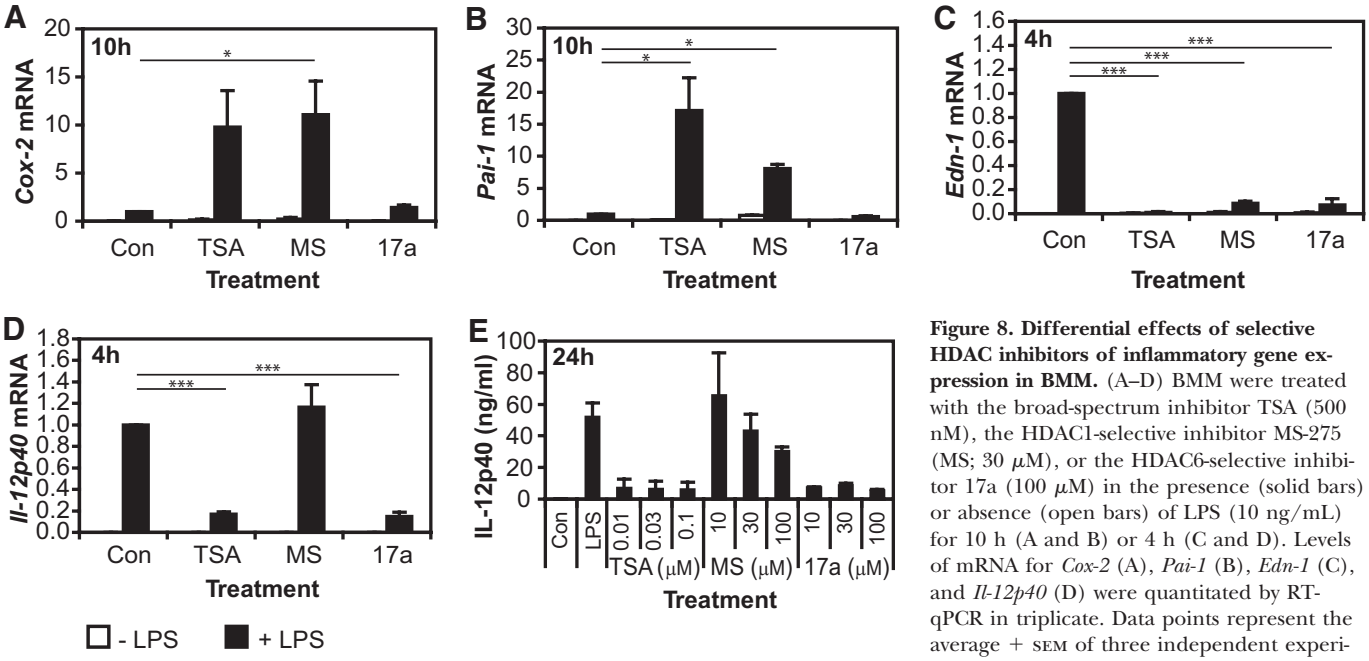


Figure 8. Differential effects of selective HDAC inhibitors of inflammatory gene expression in BMM. (A–D) BMM were treated with the broad-spectrum inhibitor TSA (500 nM), the HDAC1-selective inhibitor MS-275 (MS; 30 μM), or the HDAC6-selective inhibitor 17a (100 μM) in the presence (solid bars) or absence (open bars) of LPS (10 ng/mL) for 10 h (A and B) or 4 h (C and D). Levels of mRNA for *Cox-2* (A), *Pai-1* (B), *Edn-1* (C), and *Il-12p40* (D) were quantitated by RT-qPCR in triplicate. Data points represent the average + SEM of three independent experiments (*, $P<0.05$; ***, $P<0.001$). (E) BMM were treated with varying concentrations of the HDAC inhibitors TSA, MS-275, or 17a in the presence of LPS (10 ng/mL) for 24 h or were left untreated (Con). IL-12p40 release into culture supernatants was assessed by ELISA. Data points are the mean + SD of triplicates, and similar results were apparent in two independent experiments.

PU.1-binding site in the *Ccl7* promoter (data not shown). In total, these data indicate that selective inhibitors have distinct effects on proinflammatory pathways; 17a inhibited expression of *Edn-1* and *IL-12p40* but not *Ccl7*, and MS-275 inhibited expression of *Edn-1* but not *IL-12p40* or *Ccl7*. Similarly, selective HDAC inhibitors had differential effects in amplifying inflammatory responses; MS-275 amplified *Cox-2* and *Pai-1* expression, whilst 17a had no effect.

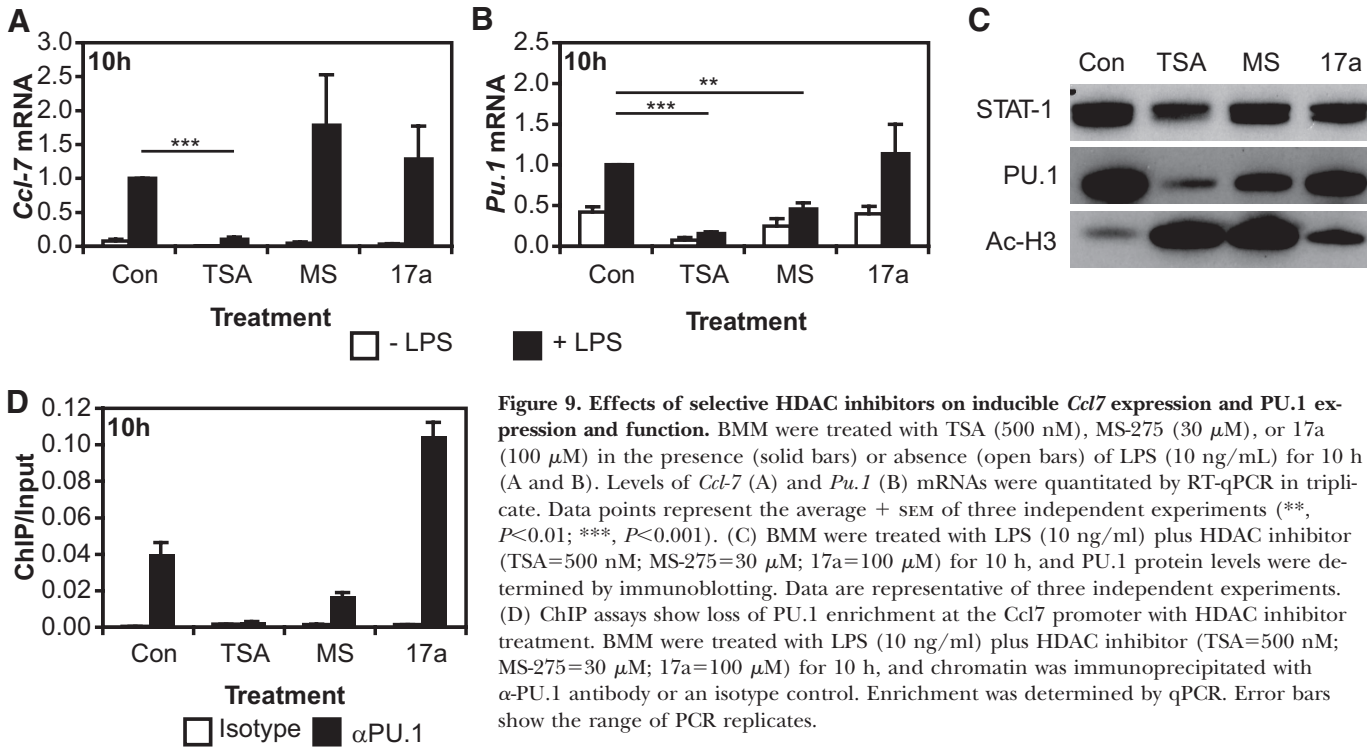


Figure 9. Effects of selective HDAC inhibitors on inducible *Ccl7* expression and PU.1 expression and function. BMM were treated with TSA (500 nM), MS-275 (30 μM), or 17a (100 μM) in the presence (solid bars) or absence (open bars) of LPS (10 ng/mL) for 10 h (A and B). Levels of *Ccl-7* (A) and *Pu.1* (B) mRNAs were quantitated by RT-qPCR in triplicate. Data points represent the average + SEM of three independent experiments (**, $P<0.01$; ***, $P<0.001$). (C) BMM were treated with LPS (10 ng/ml) plus HDAC inhibitor (TSA=500 nM; MS-275=30 μM; 17a=100 μM) for 10 h, and PU.1 protein levels were determined by immunoblotting. Data are representative of three independent experiments. (D) ChIP assays show loss of PU.1 enrichment at the Ccl7 promoter with HDAC inhibitor treatment. BMM were treated with LPS (10 ng/ml) plus HDAC inhibitor (TSA=500 nM; MS-275=30 μM; 17a=100 μM) for 10 h, and chromatin was immunoprecipitated with α-PU.1 antibody or an isotype control. Enrichment was determined by qPCR. Error bars show the range of PCR replicates.

Normal inflammatory responses in HDAC6$^{-/-}$ macrophages

Although 17a was reportedly selective for HDAC6 over HDAC1 and -4 [9], its full selectivity profile against all HDACs has not been assessed. We therefore assessed HDAC-dependent, proinflammatory responses in HDAC6$^{-/-}$ macrophages. As expected, α-tubulin, a known HDAC6 substrate, was hyperacetylated in HDAC6$^{-/-}$ BMM (**Fig. 10A**). In contrast, HDAC6 deficiency did not affect LPS-induced expression of *Edn-1* and *IL-12p40* mRNA or IL-12p40 protein secretion (Fig. 10, B–D). We conclude that the anti-inflammatory effects of 17a are not a result of inhibition of HDAC6 alone and that this compound probably inhibits HDAC-dependent inflammatory genes by targeting one or more related HDAC family members.

DISCUSSION

The impact of HDAC inhibitors on inflammatory diseases is becoming increasingly recognized [5, 12]. When assessed in inflammatory disease models, broad-spectrum inhibitors have been therapeutic in the majority of studies to date, and one might conclude that they will have future clinical applications in the treatment of inflammatory diseases. These compounds cause widespread changes in gene expression and cellular function however, and there is some evidence of contraindications. Although HDAC inhibitors have generally been well tolerated in clinical trials in cancer patients, they do have a number of common side-effects, such as gastrointestinal symptoms and fatigue. Thrombotic events [6] and cardiotoxicity [40] have also been reported.

There is also evidence in animal models to suggest that HDAC inhibitors can cause adverse cardiovascular events. TSA reportedly exacerbated atherosclerosis in a mouse model [20]. In that study, TSA enhanced expression of CD36 and scavenger receptor A, which promote lipid uptake, in both in vitro macrophage cultures and in the aortas of treated mice. Given that elevated levels of COX-2 in macrophages in symptomatic atherosclerotic plaques contributed to PGE_2-mediated plaque destabilization [32, 33], the amplification of *Cox-2* expression in macrophages by broad-spectrum inhibitors (Fig. 1B), as well as the HDAC1-selective inhibitor MS-275 (Fig. 8A), raises similar concerns. This is reinforced further by our demonstration that such inhibitors also increased expression and secretion of PAI-1 (Figs. 1, E and F, 3, B and D, 5B, and 8B), another important driver of cardiovascular disease [34].

One report found that noncytotoxic TSA concentrations amplified inflammatory mediator production from rat microglia [41], and in our study, only cytotoxic concentrations of HDAC inhibitors strongly amplified *Cox-2* and *Pai-1* gene expression in mouse macrophages. This may relate to differences in species, cell type, or the specific inflammatory mediators examined. The cytotoxic concentrations of HDAC inhibitors that we used in our in vitro study are in the range used in several other reports examining HDAC regulation of LPS responses in macrophages [42–44]. Whether concentrations of HDAC inhibitors that are reached in therapeutic regimes in vivo are sufficient to amplify the production of cardiovascular disease mediators that were examined here remains to be determined. It would certainly seem likely, however, given that the mode of action of HDAC inhibitors as anticancer drugs is, at least in part, to kill proliferating tumor cells [40]. Furthermore, a recent report demonstrated that inflammatory mediators actually sensitize human macrophages to HDAC inhibitor-triggered cell death [45]. Thus, in an inflammatory disease setting, even low HDAC inhibitor concentrations in vivo may be sufficient to

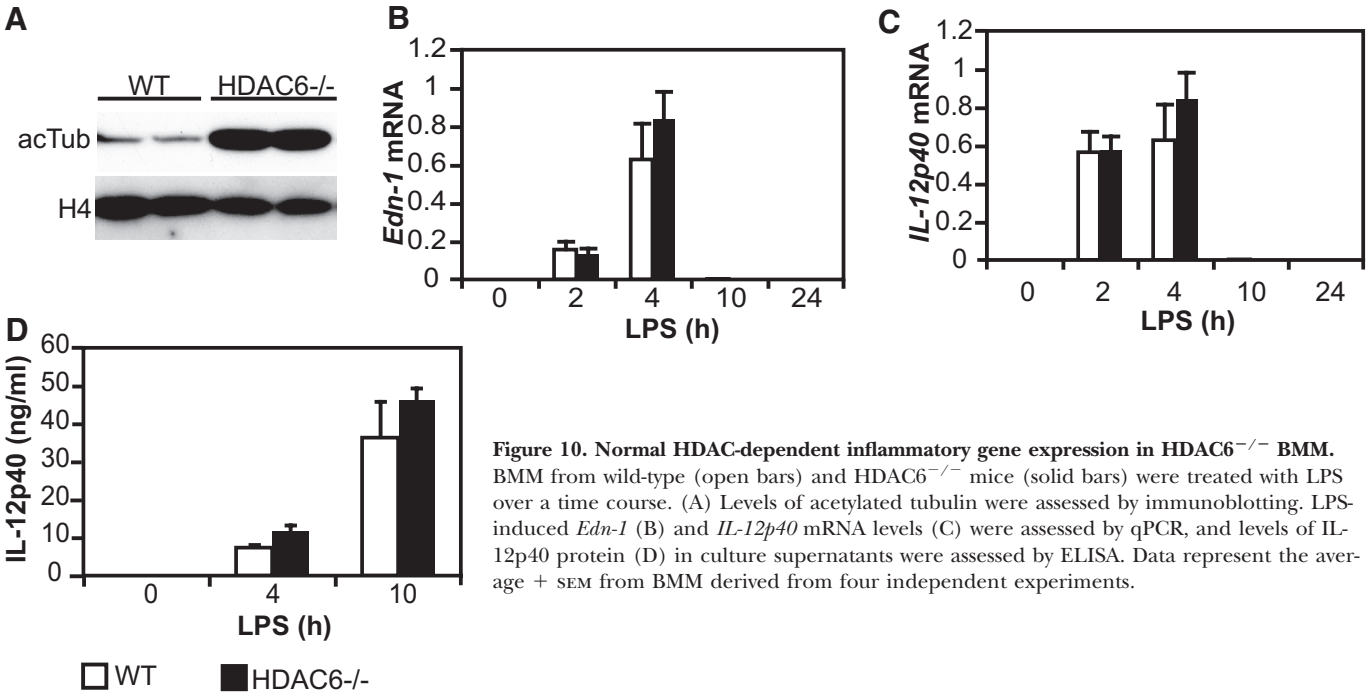


Figure 10. Normal HDAC-dependent inflammatory gene expression in HDAC6$^{-/-}$ BMM. BMM from wild-type (open bars) and HDAC6$^{-/-}$ mice (solid bars) were treated with LPS over a time course. (A) Levels of acetylated tubulin were assessed by immunoblotting. LPS-induced *Edn-1* (B) and *IL-12p40* mRNA levels (C) were assessed by qPCR, and levels of IL-12p40 protein (D) in culture supernatants were assessed by ELISA. Data represent the average + SEM from BMM derived from four independent experiments.

elicit concomitant production of cardiovascular disease mediators and macrophage cell death. Further assessment of the effects of HDAC inhibitors in cardiovascular disease models is clearly warranted, particularly for broad-spectrum and class I HDAC inhibitors that amplified the production of cardiovascular mediators examined in this study.

We have some understanding of the specific HDACs that mediate anti-inflammatory effects. For example, HDAC1 negatively regulates the *Cox-2* promoter [28], consistent with the amplifying effect of MS-275 on the expression of this gene, which we observed here (Fig. 8A). Another class I HDAC, HDAC2, was required for the anti-inflammatory action of glucocorticoids [22], and loss of HDAC2 function is associated with glucocorticoid insensitivity and disease exacerbation in chronic obstructive pulmonary disease [21]. Thus, at least some class I HDACs have anti-inflammatory functions, and targeting these HDACs could have undesirable consequences. Indeed, HDAC inhibitors blocked the anti-inflammatory effects of glucocorticoids on macrophage inflammatory responses [46], thus highlighting this concern. Nonetheless, class I HDAC inhibitors may also have beneficial effects in at least some inflammatory diseases. MS-275, for example, effectively inhibited the inducible expression of *Edn-1* in macrophages (Fig. 8C) and was efficacious in prophylactic and therapeutic regimes in rodent arthritis models [14]. It is possible that part of the efficacy of HDAC inhibitors in rheumatoid arthritis models relates to apoptosis of cells that contribute to disease pathology, for example, synovial fibroblasts [47] and macrophages [45]. In this respect, class I HDAC inhibitors would be predicted to be efficacious.

The observation that the pro- and anti-inflammatory effects of TSA could be separated over a concentration range (Fig. 4) implied that some HDACs have pro- but not anti-inflammatory functions. Furthermore, given that the suppressive effects of the various HDAC inhibitors on proinflammatory gene expression varied (TSA inhibited *Ccl7*, *Edn-1*, and *IL-12p40*; MS-275 inhibited only *Edn-1*; and 17a inhibited *Edn-1* and *IL-12p40* but not *Ccl7*), there are likely to be multiple mechanisms through which individual HDACs contribute to the induction of proinflammatory genes. Our inhibitor studies pointed toward HDAC6 as one candidate, but we excluded this possibility using HDAC6-deficient BMM. It should be noted that the selectivity profile for compound 17a has only been examined in a limited way [9], and we speculate that 17a suppresses inflammatory responses by acting on another class II HDAC; a full analysis of the selectivity profile of 17a should reveal candidate proinflammatory HDACs. Nevertheless, our data suggest that it should be possible to block the production of EDN-1, as well as IL-12 and presumably, other inflammatory mediators, with selective HDAC inhibitors that do not target class I HDACs.

HDACs may also promote inflammatory responses indirectly by suppressing the expression of anti-inflammatory cytokines. This has been reported for HDAC11, which acts as a negative regulator of IL-10 expression [48]. We find that in macrophages, however, HDAC inhibitors target the early induction of *IL-12p40* and *Edn-1*, so it seems unlikely that this pathway is responsible for the effects that we observe here.

In addition to an understanding of the HDACs that promote inflammatory responses, knowledge of the components of TLR pathways that these HDACs target, is also required. There are certainly several transcription factors that are regulated by acetylation/deacetylation and activated by TLR ligands (e.g., IFN regulatory factor family members, NF-κB) [1]. There is also the possibility that signaling molecules upstream of these transcription factors are modulated by acetylation. For example, it was shown recently that LPS triggers acetylation of MKP-1 in macrophages. This resulted in an increased association between MKP-1 and MAPK p38, as well as reduced phosphorylation of this MAPK [26]. Thus, HDAC inhibitors may suppress inflammatory responses, at least in part, by increasing MKP-1 acetylation and reducing p38 activation. Whether other TLR signaling components or TLRs themselves are acetylated remains to be determined.

Our studies with selective inhibitors provide some insights into the regulation of individual inflammatory genes by HDACs. Although the effects of MS-275 and compound 17a on inflammatory gene expression overlapped with the effects of broad-spectrum inhibitors, neither affected the mRNA expression of *Ccl-7*. We demonstrated previously that the inhibition of basal and LPS-induced *Ccl-2* and *Ccl-7* mRNA expression by TSA correlated with down-regulated expression of the *Pu.1* transcription factor [23]. Previous studies showed that TSA down-regulated the expression of *Pu.1*, as well as its target genes, in murine cell lines [49] and that decreased histone H4 acetylation at the *Pu.1* locus correlated with expression of this gene [50]. On the basis of inhibitor studies, our data would suggest that HDAC6 is not required for constitutive *Pu.1* expression in macrophages and that broad-spectrum inhibitors probably inhibit *Pu.1* and *Ccl-7* expression by targeting other HDACs. Indeed, we have found that *Pu.1* mRNA expression is normal in HDAC6$^{-/-}$ BMM (M. R. Andrews and M. J. Sweet, unpublished data). MS-275 did reduce PU.1 mRNA and protein expression somewhat (Fig. 9, B and C), so it is possible that HDAC1 does partially contribute to *Pu.1* expression in macrophages, but we postulate that other HDACs are also involved. Despite the partial reduction in PU.1 protein expression and association with the *Ccl7* promoter (Fig. 9, C and D), MS-275 did not affect LPS-inducible *Ccl7* mRNA expression (Fig. 9A). We hypothesize that the residual PU.1 is sufficient to drive LPS-inducible *Ccl7* expression, although it is also possible that HDAC inhibitors act independently of PU.1 to inhibit this LPS-inducible response.

In summary, this work has identified further potential complications of broad-spectrum HDAC inhibitors that might limit their anti-inflammatory applications. These effects were also apparent with a HDAC1-selective inhibitor but not with a compound that was reportedly selective for HDAC6. LPS-induced *Edn-1* and *IL-12p40* mRNA levels were not impaired in HDAC6$^{-/-}$ macrophages, however, and we speculate that related HDAC enzymes promote inflammatory responses in macrophages. The characterization of the role of individual

HDACs in driving inflammatory responses may ultimately lead to the development of more selective HDAC inhibitors for anti-inflammatory applications.

AUTHORSHIP

M. J. S., D. F. P., D. A. H., and M. A. H. designed the project, with important intellectual input from K. M. I., K. S., M. R. A., and P. M. M. J. S. wrote the manuscript with key input from M. A. H., M. R. A., K. M. I., K. S., L. I. L., D. P. F., and D. A. H. M. A. H., M. R. A., L. I. L., K. S., K. M. I., and E. L. contributed significant experimental data to the manuscript. R. C. R. and G. T. L. synthesized essential chemical reagents, and G. M., C. C., and P. M. provided essential cellular reagents for the study. K. S. and K. M. I. contributed to the supervision of staff and students involved in the project. M. J. S. and D. P. F. provided funding and supervised the project.

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