Histone Deacetylase Inhibitors Promote Mitochondrial Reactive Oxygen Species Production and Bacterial Clearance by Human Macrophages

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Institute for Molecular Bioscience, IMB Centre for Inflammation and Disease Research and Australian Infectious Diseases Research Centre, The University of Queensland, Brisbane, Queensland, Australia

Broad-spectrum histone deacetylase inhibitors (HDACi) are used clinically as anticancer agents, and more isoform-selective HDACi have been sought to modulate other conditions, including chronic inflammatory diseases. Mouse studies suggest that HDACi downregulate immune responses and may compromise host defense. However, their effects on human macrophage antimicrobial responses are largely unknown. Here, we show that overnight pretreatment of human macrophages with HDACi prior to challenge with *Salmonella enterica* serovar Typhimurium or *Escherichia coli* results in significantly reduced intramacrophage bacterial loads, which likely reflect the fact that this treatment regime impairs phagocytosis. In contrast, cotreatment of human macrophages with HDACi at the time of bacterial challenge did not impair phagocytosis; instead, HDACi cotreatment actually promoted clearance of intracellular *S.* Typhimurium and *E. coli*. Mechanistically, treatment of human macrophages with HDACi at the time of bacterial infection enhanced mitochondrial reactive oxygen species generation by these cells. The capacity of HDACi to promote the clearance of intracellular bacteria from human macrophages was abrogated when cells were pretreated with MitoTracker Red CMXRos, which perturbs mitochondrial function. The HDAC6-selective inhibitor tubastatin A promoted bacterial clearance from human macrophages, whereas the class I HDAC inhibitor MS-275, which inhibits HDAC1 to -3, had no effect on intracellular bacterial loads. These data are consistent with HDAC6 and/or related HDACs constraining mitochondrial reactive oxygen species production from human macrophages during bacterial challenge. Our findings suggest that, whereas long-term HDACi treatment regimes may potentially compromise host defense, selective HDAC inhibitors may have applications in treating acute bacterial infections.

Gene expression in eukaryotic cells is controlled by the accessibility of DNA in the form of chromatin wrapped around histone proteins. Histone acetyltransferases (HATs) catalyze acetylation of lysine residues on core histone tails to allow the initiation of transcription, whereas histone deacetylases (HDACs) reverse this process by catalyzing the removal of acetyl groups. Histone deacetylation results in chromatin compaction and the silencing of gene transcription; however, this is a greatly simplified model of gene regulation (1). HDACs are grouped into distinct classes based on sequence homology to yeast proteins. The classical HDACs are subdivided into class I (HDAC1, -2, -3, and -8), class IIa (HDAC4, -5, -7, and -9), class IIb (HDAC6 and -10), and class IV (HDAC11) HDACs. They are dependent on zinc for enzymatic activity and share sequence similarity, and most of these enzymes are inhibited by broad-spectrum HDAC inhibitors (HDACi) such as trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) (2). In contrast, nonclassical class III HDACs, or sirtuins, function through a distinct NAD^+-dependent mechanism. Although commonly used HDACi such as TSA and SAHA target multiple classical HDACs, isoform-selective HDACi for some individual HDACs have now been described. For example, tubacin and tubastatin A preferentially inhibit HDAC6, while MS-275 is most potent against HDAC1 but also inhibits HDAC2 and HDAC3 (2). HDACi have been found to be effective as anticancer agents, at least for some cancers such as cutaneous T-cell lymphoma, by promoting the expression of tumor suppressor genes and by inducing cell differentiation, growth arrest, and/or apoptosis (3–6). Although HDACs are termed histone deacetylases, they also act on numerous nonhistone proteins, such as transcription factors, cell cycle proteins, and protein kinases (7). Hence, HDACi have many effects beyond the regulation of epigenetic states and gene expression.

Beyond their anticancer applications, HDACi have been investigated as therapeutic agents in models of acute and chronic inflammatory diseases (3, 8–10). HDACi are reported to inhibit proinflammatory cytokine secretion via multiple mechanisms, including inhibiting the transcriptional activity of hypoxia-inducible factor 1-alpha (HIF-1α) (8, 11), impairing recruitment of proinflammatory transcription factors to target promoters (12), reducing the stability of mRNAs encoding inflammatory cytokines (13), and upregulating the expression and activity of the transcriptional repressor complex, Mi-2/NuRD (14). Such studies imply that certain HDACs have proinflammatory functions, and indeed this has been reported for a number of HDACs, for example, HDAC4 (15) and HDAC7 (11). As proinflammatory cytokines play important roles in host defense, it is a distinct possibility that HDACi may, in dampening inflammation, also compromise

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Address correspondence to Matthew J. Sweet, m.sweet@imb.uq.edu.au.

host defense. Indeed, this is a major limitation of many existing anti-inflammatory agents, including anti-tumor necrosis factor alpha (TNF-α) therapy used to treat patients with rheumatoid arthritis and inflammatory bowel disease (16–18).

Previous studies examining the effects of HDACi on innate immune responses in mouse macrophages reported that treatment with broad-spectrum HDACi, TSA, and valproic acid reduced expression of phagocytic receptors, NADPH oxidase subunits, and inducible nitric oxide synthase (19). Consequently, HDACi impaired the phagocytotic activity of mouse bone marrow-derived macrophages and the production of reactive oxygen species (ROS) and nitric oxide. Furthermore, the ability of mouse macrophages to clear *Escherichia coli* and *Staphylococcus aureus* upon challenge was compromised (19). A genome-wide microarray analysis of gene expression profiles from murine macrophages and dendritic cells stimulated with Toll-like receptor (TLR) agonists also revealed that TSA treatment impaired up to 60% of TLR2 and TLR4 target genes, including pattern recognition receptors, cytokines, chemokines, costimulatory molecules, transcription regulators, and kinases, while only 16% of genes were upregulated (14). However, studies on the impacts of HDACi on host immunity have primarily utilized mouse models or mouse cells, and effects on host defense in humans have not been intensively investigated. Although human and mouse innate immune responses are broadly conserved, some phenotypic differences, divergence in gene repertoires, and differential regulation of orthologous genes have been noted between humans and mice (20–23). Here we report that, while long-term treatment of human macrophages with HDACi impairs the phagocytic capacity of human macrophages, acute treatment with HDACi at the time of bacterial infection actually enhances mitochondrial ROS (mitoROS) production from these cells, which correlates with increased antibacterial responses.

MATERIALS AND METHODS

Materials. TSA purified from *Streptomyces* sp. (Sigma-Aldrich) was dissolved in 100% ethanol, stored at 4°C, and diluted in tissue culture medium to be used at the indicated concentrations. SAHA was synthesized in-house, dissolved in dimethyl sulfoxide (DMSO), stored at −20°C, and diluted in tissue culture medium to be used at 10 μM. Tubastatin A (Sigma-Aldrich) and MS-275 (synthesized in-house) were dissolved in DMSO to 30 mM, stored at −20°C, and used at 20 μM. MitoTracker Red CMXRos (Life Technologies) was stored at −20°C and was dissolved in DMSO to 1 mM before further dilution in tissue culture medium for the treatment of cells. Human recombinant gamma interferon (IFN-γ) (R&D Systems) was stored at a concentration of 2.5 μg/ml at −20°C and was diluted in tissue culture medium to a final concentration of 2.5 ng/ml. Cytochalasin D (Life Technologies) was dissolved in DMSO to 2 mM, stored at −20°C, and used at 10 μM. MitoSOX Red mitochondrial superoxide indicator (Life Technologies) was dissolved in DMSO to 5 mM, stored at −20°C, and used at 5 μM. pHrodo Green *E. coli* BioParticles conjugate (Invitrogen) was dissolved in phosphate-buffered saline (PBS) to 1 mg/ml, sonicated for 5 min, vortexed for 1 min, and then stored at −20°C.

Ethics statement. Before undertaking the studies described, approval for all experiments using primary human cells was obtained from the Medical Research Ethics Committee of the University of Queensland (approval number 2013001519).

Cell isolation and culture. Human monocyte-derived macrophages (HMDM) were prepared from $CD14^+$ monocytes from Red Cross blood donors. In brief, $CD14^+$ monocytes were differentiated into HMDM by culture with 1×10^4 U/ml colony-stimulating factor 1 (CSF-1) for 6 days, as previously described (22, 24). On day 6, HMDM were harvested and replated in the presence of CSF-1 for experimentation. THP-1 cells were cultured as previously described (25), differentiated for 48 h with 30 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich), and rested for 4 h before experimentation. All cells were cultured in an incubator at 37°C with 5% CO_2.

Bacterial culture. The *Salmonella enterica* serovar Typhimurium SLI 344 (26) and *E. coli* MG1655 (27) strains were stored in LB broth containing 50% sterile glycerol at −80°C. The appropriate multiplicity of infection (MOI) for infection assays was calculated by spectrophotometry analysis of bacterial cell density from an isolated colony grown overnight in LB broth. Possible effects of HDACi on *S.* Typhimurium and *E. coli* growth were assessed by spectrophotometric analyses of bacterial cultures grown at 37°C in complete HMDM medium at the indicated time points.

***In vitro* infection assays.** Bacterial infection assays were conducted with HMDM and PMA-differentiated THP-1 macrophages to assess intracellular bacterial loads as previously described (28). In brief, cells were cultured at 4×10^5 cells/well in 1 ml antibiotic-free tissue culture medium at 37°C. Then, they were either primed with HDACi for 18 h prior to bacterial challenge (*S.* Typhimurium or *E. coli*; MOI, 100) or were cotreated with HDACi at the time of infection. At 1 h postinfection, cells were washed with a medium containing 200 μg/ml gentamicin to exclude extracellular bacteria and then maintained in medium containing 20 μg/ml gentamicin. In situations where cells were treated with pharmacological reagents, inhibitors were re-added to the medium containing 20 μg/ml gentamicin and were maintained throughout the incubation period. At appropriate time points, the medium was replaced with 0.01% Triton X-100 in PBS, and cells were lysed by pipetting. Diluted lysates were cultured on agar plates at 37°C overnight, and colony counts were used to assess intracellular bacterial loads. In some experiments, supernatants were harvested at the indicated time points to assess cell death through the release of the metabolic enzyme lactate dehydrogenase (LDH) from dying cells into culture supernatants. LDH release was quantified by measuring LDH in culture supernatants compared to total LDH (LDH present in supernatants from untreated cells following cell lysis with 0.1% Triton X-100), as previously described (25).

Quantification of phagocytosis. HMDM and PMA-differentiated THP-1 macrophages were plated at a density of 5×10^5 cells/well, and on the following day, cells were treated with 10 μM cytochalasin D for 30 min at 37°C to inhibit phagocytosis if required and were then treated with 100 μg pHrodo Green *E. coli* BioParticles conjugate with or without HDACi treatment (either priming or cotreatment). Phagocytosis was assessed at 1.5 h posttreatment with pHrodo Green *E. coli* BioParticles conjugate as measured by flow cytometric quantification of fluorescence using an FACSCanto II flow cytometer (BD Bioscience). FACs data were analyzed using FlowJo 7.6.5 software (Tree Star).

Quantification of mitochondrial ROS. HMDM were cotreated with HDACi and were infected with *S.* Typhimurium, as above. After 6 h of infection, cells were washed twice with PBS, 5 μM MitoSOX Red mitochondrial superoxide indicator (Life Technologies) was added, and cells were incubated for 10 min at 37°C before mitoROS levels were quantified by flow cytometry.

Immunoblotting. Levels of specific proteins were assessed by Western blotting. Briefly, cells were lysed and homogenized in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, and 1% NP-40) supplemented with 1× protease inhibitors (Roche Life Science). Cell lysates were resolved on precast 4% to 20% gradient SDS-PAGE gels (Bio-Rad) or on 4% to 12% Bis-Tris NuPAGE gels (Invitrogen) and were separated by electrophoresis and then transferred onto nitrocellulose membranes (Bio-Rad). Membranes were probed using anti-acetyl histone H3 (Cell Signaling), anti-acetyl-α-tubulin (Cell Signaling), or anti-GAPDH (anti-glyceraldehyde-3-phosphate dehydrogenase) (Trevigen) antibodies and were then developed by chemiluminescence (Amersham ECL Plus; GE Healthcare).

Statistics. For data combined from 3 or more independent experiments, statistical analyses were performed using Prism 6 software (GraphPad) with error bars indicating the standard error of the mean (SEM). For data sets with 3 or more variables, a one-way analysis of variance (ANOVA) was performed followed by Dunnett's multiple comparison test. Data with confidence values of ≥95% ($P < 0.05$) were considered statistically significant.

RESULTS

HDACi reduce intracellular bacterial loads within human macrophages. We first investigated the potential impact of treatment with HDACi on the antimicrobial activity of human macrophages that were infected with either *S.* Typhimurium or *E. coli*. HMDM and PMA-differentiated THP-1 macrophages were treated with the broad-spectrum HDACi SAHA 18 h prior to infection (priming) or at the time of infection (cotreatment) in order to determine whether potential effects on antimicrobial responses were time dependent. Cells were also primed with IFN-γ as a positive control for priming of antimicrobial responses. Priming or cotreating cells with SAHA significantly reduced intracellular bacterial loads of *S.* Typhimurium and *E. coli* at 24 h postinfection in HMDM (Fig. 1A and B) and PMA-differentiated THP-1 macrophages (Fig. 1C and D). As expected, IFN-γ priming also reduced intracellular bacterial loads within HMDM. The SAHA-mediated reduction in intracellular bacterial loads may simply reflect the direct killing of bacteria or the effects on macrophage viability of this compound. To exclude these possibilities, we next assessed bacterial growth in the presence of an even higher concentration of SAHA than that which was used in infection assays as well as the release of LDH in cell supernatants following the infection of human macrophages with *S.* Typhimurium. SAHA treatment did not affect either *S.* Typhimurium growth (Fig. 1E) or the release of LDH from *S.* Typhimurium-infected macrophages that had been primed overnight with SAHA (Fig. 1F). Thus, the SAHA-mediated reduction in intramacrophage bacterial loads was not due to the direct killing of bacteria or to reduced macrophage viability. These data also highlight that human macrophages are resistant to cell death triggered by HDACi.

Priming but not cotreatment of human macrophages with HDACi at the time of infection reduces phagocytosis in human macrophages. Another potential explanation for the HDACi-mediated reduction in intracellular bacteria (Fig. 1A to D) is impaired phagocytic uptake rather than enhanced antimicrobial responses. Since treatment with HDACi was previously shown to impair phagocytosis in mouse macrophages (19), this was a distinct possibility. We therefore examined the effects of SAHA as well as another broad-spectrum HDACi, TSA, on phagocytosis in human macrophages. TSA (1 μM) and SAHA (10 μM) induced approximately equal levels of hyperacetylation of histone H3 protein (a hallmark of HDAC inhibition) (Fig. 2A), and thus these concentrations were used for subsequent experiments. To assess phagocytosis, the uptake of pHrodo Green *E. coli* BioParticles was measured by flow cytometry in HMDM and PMA-differentiated THP-1 macrophages that were primed or cotreated with either TSA or SAHA. The actin polymerization inhibitor cytochalasin D was used as a positive control for inhibition of phagocytosis (29). Priming, but not cotreatment, with either TSA or SAHA reduced phagocytosis by HMDM (Fig. 2B) and PMA-differentiated THP-1 cells (Fig. 2C) as quantified by fluorescence of phagocytosed pHrodo Green *E. coli*. In contrast, priming with IFN-γ had no effect on phagocytosis in these assays. Thus, the effect of IFN-γ priming in reducing intramacrophage bacterial loads (Fig. 1) is consistent with its well-known capacity to boost macrophage antimicrobial responses. Collectively, the above data (Fig. 1 and 2) suggest that cotreatment of human macrophages with HDACi at the time of infection does not impair bacterial uptake but promotes bacterial clearance. To more directly address this, HMDM from matched donors were infected with live *E. coli* or *S.* Typhimurium isolates and were cotreated with either TSA or SAHA.

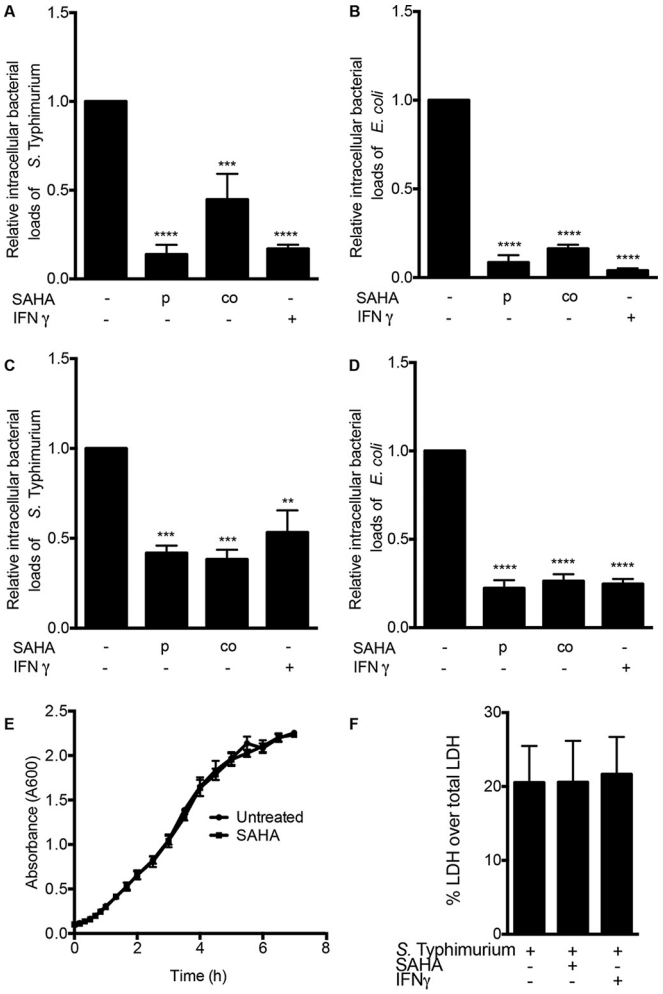

FIG 1 Priming or cotreatment of primary human macrophages with SAHA reduces intracellular bacterial loads of *E. coli* and *S.* Typhimurium. Human macrophages were primed with either 10 μM SAHA or 5 ng/ml IFN-γ for 18 h and were then infected with *S.* Typhimurium or *E. coli* at an MOI of 100. Alternatively, cells were cotreated with 10 μM SAHA at the time of infection. (A to D) Intracellular survival of *S.* Typhimurium (A and C) and *E. coli* (B and D) was assessed at 24 h postinfection in HMDM (A and B) and PMA-differentiated THP-1 macrophages (C and D). Data (mean + SEM) are combined from 3 or 4 independent experiments and are presented as fold change relative to untreated controls. ***, *P* value of <0.001; ****, *P* value of <0.0001 by one-way ANOVA followed by Dunnett's multiple comparison test. p indicates overnight priming with HDACi, and co indicates cotreatment with HDACi at the time of infection. (E) *S.* Typhimurium was cultured with or without 30 μM SAHA for 7 h. Data (mean + SEM) are combined from 3 independent experiments. (F) Cell death was quantified by assessing LDH release into culture supernatants from HMDM primed with 10 μM SAHA or 5 ng/ml IFN-γ for 18 h prior to infection with *S.* Typhimurium for 24 h. Data (mean + SEM) are combined from 4 independent experiments.

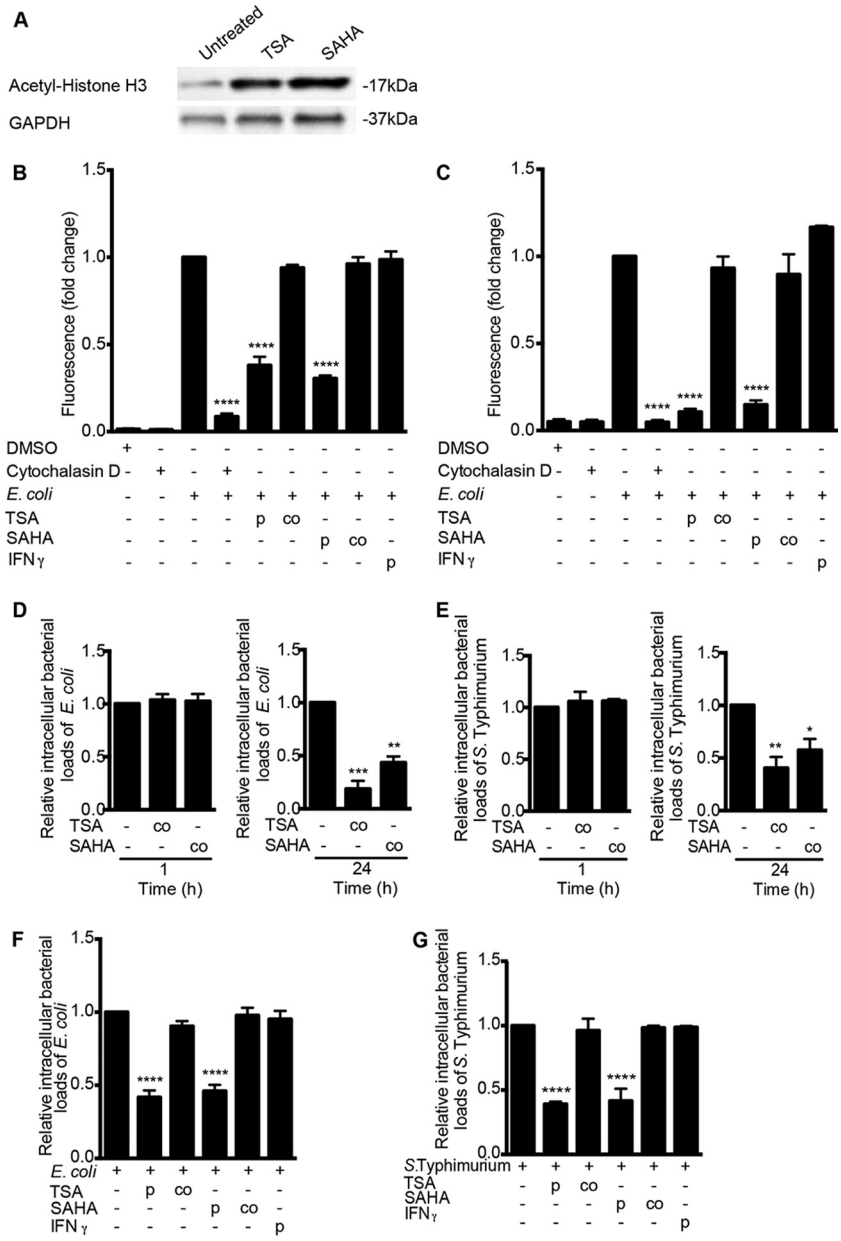

FIG 2 Priming, but not cotreatment, of primary human macrophages with HDAC inhibitors impairs phagocytosis of *E. coli*. (A) HMDM were treated with 1 μM TSA or 10 μM SAHA for 6 h, after which cell lysates were prepared and histone H3 hyperacetylation was assessed by Western blotting. Data show immunoblots from one experiment and are representative of 3 independent experiments. Phagocytic capacity of HMDM (B) and PMA-differentiated THP-1 cells (C) after priming or cotreatment with 1 μM TSA or 10 μM SAHA was measured by uptake of fluorescent pHrodo Green *E. coli* BioParticles. For cytochalasin D treatment (10 μM), cells were treated 30 min prior to treatment with *E. coli* BioParticles. Intracellular bacterial loads of *E. coli* (D) and *S.* Typhimurium (E) in HMDM derived from the same donors as in the phagocytosis assay at 1 and 24 h postinfection are shown after cotreatment with 1 μM TSA or 10 μM SAHA. Bacterial loads of *E. coli* (F) and *S.* Typhimurium (G) in PMA-differentiated THP-1 macrophages at 1 h postinfection, after either cotreatment or overnight priming with 1 μM TSA or 10 μM SAHA, are shown. Data (mean + SEM) (B to G) are combined from 3 or 4 independent experiments and are presented as fold change relative to untreated controls. **, *P* value of <0.01; ***, *P* value of <0.001; ****, *P* value of <0.0001 by one-way ANOVA followed by Dunnett's multiple comparison test. p indicates overnight priming with HDACi, and co indicates cotreatment with HDACi at the time of infection.

Cotreatment of human macrophages with HDACi at the time of infection reduced intracellular bacterial loads at 24 h, whereas these treatments had no effect at 1 h postinfection (Fig. 2D and E). Moreover, a direct comparison of HDACi priming versus cotreatment on bacterial loads at 1 h postinfection in PMA-differentiated THP-1 macrophages also showed that cotreatment with HDACi did not result in a reduction of bacterial loads at this acute time point, whereas priming with HDACi did (Fig. 2F and G). In total, these findings show that overnight priming with HDACi impairs macrophage phagocytosis resulting in reduced intracellular bacterial loads, whereas cotreatment with HDACi does not impair phagocytosis but rather promotes bacterial clearance by human macrophages. We therefore focused subsequent efforts on understanding how cotreatment of human macrophages with

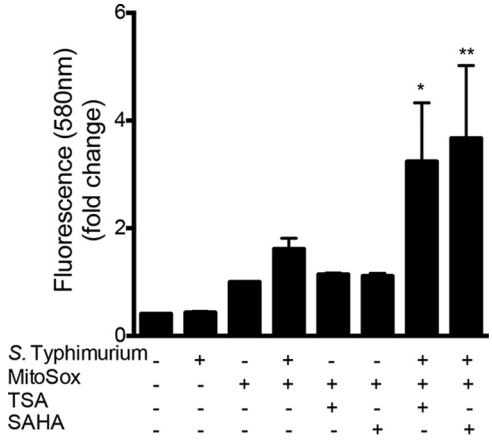

FIG 3 Cotreatment of HMDM with either TSA or SAHA at the time of infection with *S.* Typhimurium enhances mitochondrial ROS generation. HMDM were cotreated with 1 μM TSA or 10 μM SAHA during infection with *S.* Typhimurium at an MOI of 100. At 6 h postinfection, MitoSOX Red was added to cells, and mitochondrial ROS generation (median fluorescence intensity) was measured by flow cytometry. Data (mean + SEM, combined from 3 independent experiments) represent fold change relative to untreated cells stained with MitoSOX Red. *, *P* value of <0.05; **, *P* value of <0.01 by one-way ANOVA followed by Dunnett's multiple comparison test.

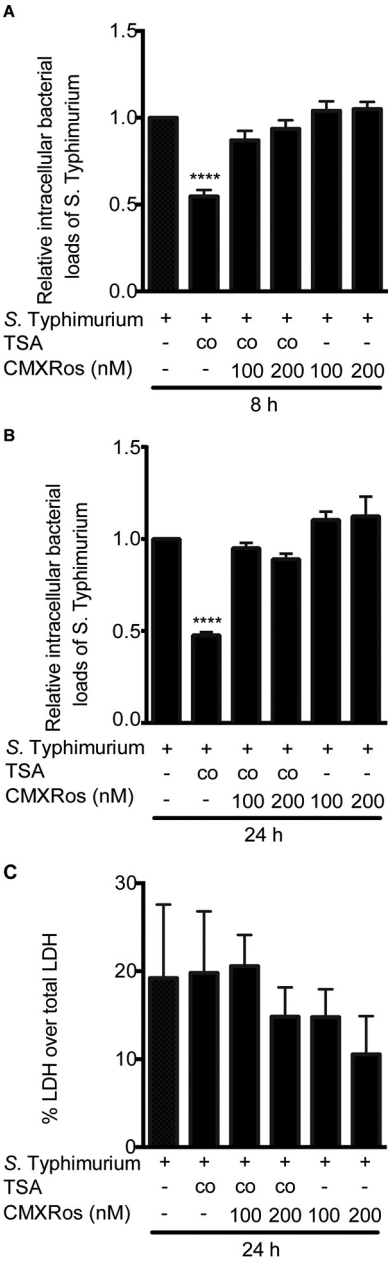

FIG 4 Treatment with MitoTracker Red CMXRos prevents HDACi-mediated clearance of *S.* Typhimurium from HMDM. HMDM were cotreated with 1 μM TSA in the presence or absence of 100 or 200 nM MitoTracker CMXRos during infection with *S.* Typhimurium at an MOI of 100. Intracellular survival was assessed at 8 h (A) and 24 h (B) postinfection. Data (mean + SEM) are combined from 3 independent experiments. ****, *P* value of <0.0001 by one-way ANOVA followed by Dunnett's multiple comparison test. (C) Cell death was assessed by quantifying LDH release from 24-h culture supernatants. Data (mean + SEM) are combined from 4 independent experiments.

HDACi promotes antimicrobial responses. These studies concentrated on the professional intramacrophage pathogen *S.* Typhimurium, given the clinical significance of devising strategies to clear intracellular reservoirs of this pathogen.

HDACi promote mitochondrial ROS release from human macrophages. Previous studies reported that treatment of breast cancer cells and human monocyte lymphoma U937 cells with various HDACi, including TSA, resulted in a loss of mitochondrial membrane potential and enhanced mitoROS production (30, 31). In addition, mitoROS was previously shown to play an important role in the clearance of *S.* Typhimurium, at least in mouse macrophages (32). Thus, we examined whether HDACi enhanced mitoROS production by HMDM upon bacterial challenge with *S.* Typhimurium. Generation of mitoROS in HMDM cotreated with TSA or SAHA during infection with *S.* Typhimurium was quantified by flow cytometric detection of MitoSOX Red fluorescence. *S.* Typhimurium infection, which triggers multiple TLRs, resulted in a modest increase in mitoROS production from HMDM at 6 h postinfection (Fig. 3), which is in accordance with a previous report that TLR activation triggers mitoROS production in mouse macrophages at this time point (32). Whereas HDACi alone did not promote mitoROS production from these cells, TSA and SAHA significantly enhanced *S.* Typhimurium-induced mitoROS production. Thus, cotreatment of human macrophages with HDACi during bacterial infection with *S.* Typhimurium increases mitoROS levels. To determine whether enhanced mitoROS production was likely to be causally related to HDACi-mediated bacterial clearance, we used a pharmacological approach. The mitochondrial stain MitoTracker Red CMXRos is reported to bind irreversibly to the polarized mitochondrial membrane (33, 34) and is thus predicted to impair the generation of mitoROS. We found that CMXRos blocked HDACi-mediated bacterial clearance (Fig. 4A and B) without having pronounced effects on macrophage viability (Fig. 4C). Although CMXRos-mediated inhibition of mitoROS production was not assessed because this stain interferes with this assay due to spectral overlap, these data implicate enhanced mitoROS production as a likely mechanism accounting for HDACi-mediated clearance of intracellular bacteria.

A specific inhibitor of HDAC6 reduces *S.* Typhimurium survival in human macrophages. A recent study showed that the HDAC6 inhibitor tubastatin A improved survival and reduced bacterial loads in the blood in a mouse cecal ligation and puncture

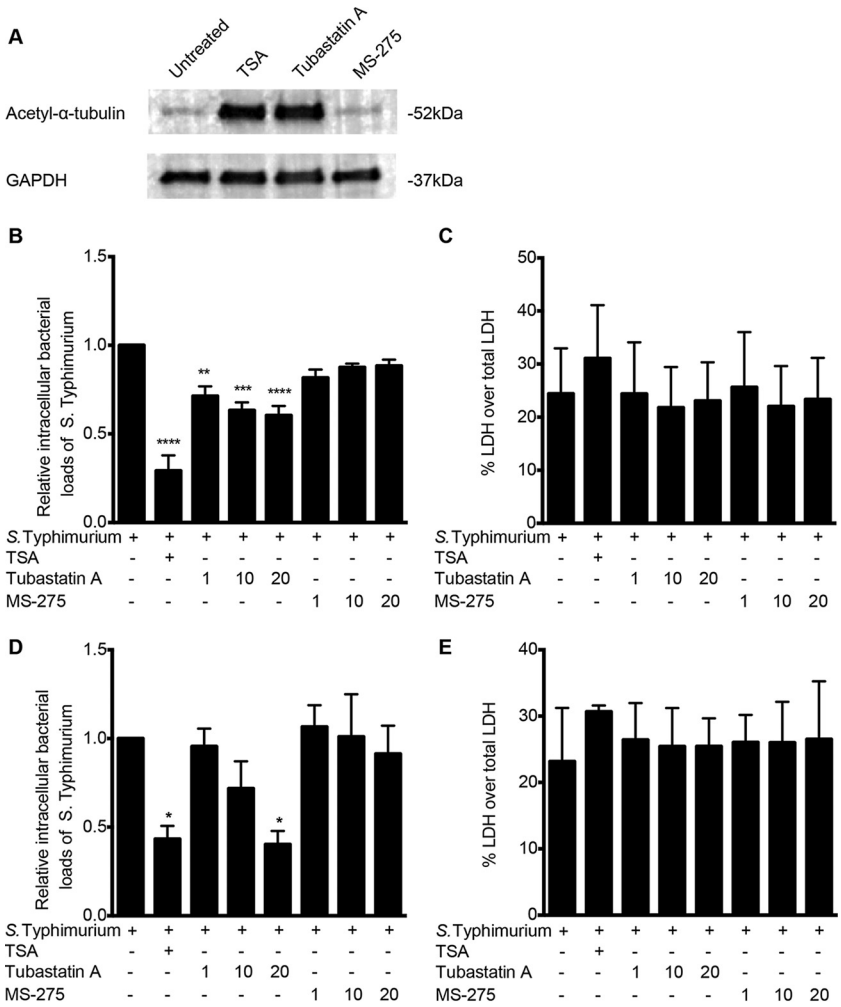

FIG 5 Cotreatment of human macrophages with tubastatin A at the time of infection with *S.* Typhimurium enhances bacterial clearance. (A) α-Tubulin hyperacetylation was assessed in HMDM treated with 1 μM TSA, 20 μM tubastatin A, or 20 μM MS-275 for 6 h. Data show immunoblots from one experiment and are representative of 3 independent experiments. Intracellular bacterial loads of *S.* Typhimurium in HMDM (B) and PMA-differentiated THP-1 (D) were determined in cells cotreated with either 1 μM TSA, 1 to 20 μM tubastatin A, or 1 to 20 μM MS-275 during infection (MOI, 100). Intracellular survival was assessed at 24 h postinfection. Data (mean + SEM) are combined from 4 independent experiments. Cell death in HMDM (C) and PMA-differentiated THP-1 (E) was assessed by quantifying LDH release from 24-h culture supernatants. Data (mean + SEM) are combined from 3 independent experiments.

model of sepsis, whereas this effect was not observed with the class I HDACi MS-275 (35). To determine whether HDACi-mediated antimicrobial responses in human macrophages are likely linked to HDAC6 inhibition, we treated HMDM and PMA-differentiated THP-1 macrophages with the HDAC6-selective inhibitor tubastatin A, and for comparison, we also used MS-275, which inhibits the class I HDACs, HDAC1 to -3 (2). As expected, treatment of HMDM with TSA (1 μM) or tubastatin A (20 μM), but not with MS-275 (20 μM), resulted in hyperacetylation of α-tubulin (Fig. 5A), which is a hallmark of HDAC6 inhibition (36). Cotreatment with tubastatin A significantly reduced intracellular bacterial loads of *S.* Typhimurium in HMDM (Fig. 5B) and PMA-differentiated THP-1 macrophages (Fig. 5D), with 20 μM tubastatin A having similar efficacy to 1 μM TSA in THP-1 cells. In contrast, cotreatment with MS-275 had no effect on intracellular bacterial loads. Neither tubastatin A nor MS-275 affected cell viability of *S.* Typhimurium-infected macrophages as assessed by the release of LDH (Fig. 5C and E). These data therefore suggest that HDAC6, but not HDAC1, HDAC2, or HDAC3, constrains infection-induced mitoROS production and that selective inhibition of HDAC6 may promote mitoROS and boost clearance of intracellular bacteria by human macrophages. However, the fact that tubastatin A had effects similar to those of TSA on tubulin hyperacetylation (Fig. 5A), and yet was less effective than TSA in promoting bacterial clearance from HMDM (Fig. 5B), suggests that multiple HDACs may be involved in constraining human macrophage antimicrobial responses.

DISCUSSION

HDACi are known to exert direct antimicrobial effects against some pathogens, such as *Plasmodium* species (37), and to reactivate latent HIV reservoirs to enable viral clearance (38, 39). As such, HDACi are being pursued as potential therapeutic agents in these infectious disease contexts. Although some mouse studies have suggested that HDACi may compromise host defense against bacterial and fungal pathogens (14, 19), very few studies have

examined the regulation of human macrophage antimicrobial responses by HDACi. Given some differences in innate immune pathways between human and mouse, particularly in relation to host defense (20, 22), we examined the effects of HDACi on human macrophage responses to bacterial challenge in this study.

Cotreatment of primary human macrophages or PMA-differentiated THP-1 macrophages with HDACi reduced intracellular loads of *E. coli* and *S.* Typhimurium at 24 h postinfection (Fig. 1A to D) without affecting macrophage viability (Fig. 5C and E), macrophage phagocytic activity (Fig. 2B to G), or bacterial growth (Fig. 1E). Our findings are thus consistent with HDACs constraining human macrophage host defense responses upon bacterial challenge, such that HDACi promote the clearance of intracellular bacteria. Similar conclusions were recently reached using *Anaplasma phagocytophilum* (40) and *Mycobacterium* infection models in THP-1 cells (41). In our studies, we found that cotreatment with broad-spectrum HDACi enhances *S.* Typhimurium-inducible mitoROS levels in HMDM (Fig. 3), while treatment of these cells with MitoTracker Red CMXRos, which is predicted to inhibit mitoROS production, abrogates the ability of HDACi to promote bacterial clearance (Fig. 4). Enhanced antimicrobial responses were also observed in human macrophages upon cotreatment with the HDAC6-selective inhibitor, tubastatin A, but not with the HDAC1-selective inhibitor MS-275, also known as Entinostat (Fig. 5). Collectively, these data are consistent with inhibition of HDAC6 and/or related HDACs, enhancing human macrophage antimicrobial responses likely via enhanced mitoROS production.

Previous studies with mouse macrophages have shown that mitochondria are recruited to phagosomes downstream of TLR1, TLR2, and TLR4 activation, leading to the release of mitoROS and to the enhanced killing of *S.* Typhimurium (32). Inhibition of HDAC activity has also been reported to increase the generation of mitoROS in other cellular contexts. For example, treatment of the human breast cancer cell lines MDA-MB-231 and MCF-7 with TSA led to cell death, and this was dependent on mitoROS induction through impairment of the mitochondrial respiratory chain (31). The HDACi spiruchostatins A and B were also shown to stimulate the production of mitoROS, contributing to the apoptosis of human monocytic lymphoma U937 cells (30). Although we found that HDACi boosted *S.* Typhimurium-inducible mitoROS generation (Fig. 3), these agents did not enhance cell death in human macrophages challenged with this pathogen (Fig. 1F and 5C and E). Thus, human macrophages appear to be somewhat resistant to mitoROS-initiated cell death. However, our finding that treatment with the MitoTracker Red CMXRos stain blocked the ability of broad-spectrum HDACi to reduce bacterial loads within HMDM (Fig. 4) is consistent with a causal role of HDACi-induced mitoROS in the clearance of intracellular bacteria. Due to its cationic lipophilic structure, this stain passively distributes into the mitochondrial matrix to cause monovalent substitution of thiols, preventing changes in the mitochondrial membrane potential (33, 34), which is linked to the generation of mitoROS (42). However, further mechanistic studies are required to definitively link the effects of HDACi on mitoROS production to bacterial clearance. Enhanced antimicrobial activity was also observed upon cotreatment with the selective HDAC6 inhibitor tubastatin A but not with MS-275 (Fig. 5B and D), which inhibits HDAC1 to -3. This finding is in keeping with a previous study that showed that an HDAC6 inhibitor improved survival and reduced bacteremia in a murine sepsis model (35). Moreover, previous studies have linked HDAC6 to the control of mitochondrial function (43, 44), and a very recent study reported that HDAC6 inhibition disrupted mitochondrial membrane potential and enhanced ROS production in melanoma cells (45). Collectively, these studies suggest that HDAC6 may have a regulatory role in controlling mitoROS production in infected macrophages such that inhibition of HDAC6 increases mitoROS concentrations to facilitate bacterial clearance. In contrast, on the basis of inhibitor studies (Fig. 5B and D), HDAC1 to -3 (class I HDACs) are unlikely to exert such effects.

Prior studies have reported that priming of mouse bone marrow-derived macrophages with TSA or another broad-spectrum HDACi, valproic acid, reduced the uptake of *E. coli* and *Staphylococcus aureus* and impaired the expression of phagocytic and opsonophagocytic receptors in these cells (19). Further, the authors also showed that priming with HDACi reduced cellular ROS production at 30 min post bacterial challenge as well as inducible nitric oxide production, leading to impaired bacterial killing by mouse macrophages. Similarly, mouse macrophages and dendritic cells pretreated with SAHA, valproic acid, or TSA had impaired antimicrobial responses to *E. coli*, *S. aureus*, and *Candida albicans*, and *in vivo* pretreatment with HDACi increased susceptibility to bacterial and fungal infections in mouse models (14). These effects were linked to the downregulation of host defense genes such as pattern recognition receptors and costimulatory molecules as well as to the repression of macrophage cytokine production through the Mi-2/NuRD complex (14). We similarly found that overnight pretreatment of human macrophages with broad-spectrum HDACi impaired phagocytic responses (Fig. 2B, C, F, and G); however, we did not investigate whether HDACi priming suppressed specific host defense genes in these cells. Overall, these findings may raise some concerns about potential applications for broad-spectrum HDACi, particularly for long-term treatment regimes. However, clinical evidence linking HDACi to infectious disease susceptibility is rather limited so far. Indeed, systemic-onset juvenile idiopathic arthritis patients treated for 12 weeks with the HDACi givinostat concomitantly with glucocorticoids were not reported to display enhanced susceptibility to infections (46). Moreover, it is quite possible that the anti-inflammatory effects of HDACi may actually reduce the immunopathology that is associated with many infectious diseases.

Nonetheless, concerns remain about the potential for broad-spectrum HDACi to increase susceptibility to infectious diseases by reducing phagocytosis and/or inflammation-mediated immune cell recruitment. This highlights the need for studies with selective HDACi, which may offer new opportunities to promote antibacterial responses without compromising host defense. Indeed, a very recent study showed that the HDAC6-selective inhibitor tubastatin A promotes bacterial clearance, reduces inflammatory cytokine production, and improves survival in a mouse cecal ligation puncture model of sepsis (35). This exciting finding raises the possibility that pharmacological agents targeting HDAC6 may have dual activities as anti-infective agents in promoting innate immune-mediated bacterial clearance and in reducing immunopathology caused by excessive inflammation. In this regard, bacterial and viral infections are well known to exacerbate inflammation and increase disease severity in various chronic conditions, particularly respiratory diseases (47). Moreover, in mouse models, allergic airway disease is associated with impaired host defense responses against bacterial pathogens such as *Haemophilus influ-*

enzae (48). The potential to reduce inflammation and boost host defense with HDACi may thus be particularly attractive in the context of new therapies for inflammatory diseases of the airways. However, the fact that loss of HDAC2 expression and function has been associated with excessive inflammation and corticosteroid resistance in chronic lung diseases (49) again highlights the need for studies with selective HDACi in this context. Future studies assessing the efficacy of selective inhibitors of HDAC6, as well as other HDACs, in models of infection-mediated exacerbation of inflammatory diseases are thus clearly warranted. More specifically, short-term HDACi treatment may be predicted to reduce infection-induced exacerbations of inflammatory diseases.

In summary, the findings herein reveal that HDACs constrain infection-induced mitoROS production from human macrophages and that inhibition of these enzymes provides a means of enhancing macrophage antimicrobial responses. HDAC-selective inhibitors may provide new prospects as anti-infective agents, and since broad-spectrum HDACi are already approved for clinical use in cancer, there is clear potential for rapid translation of other HDACi into the clinic.

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