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Histone deacetylase inhibitors impair antibacterial defenses of macrophages

THESE

préparée sous la direction du Docteur Thierry Roger,
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Histone deacetylase inhibitors impairs antibacterial defenses of macrophages

Rapport de synthèse

Les déacetylases d'histones (HDACs) déacétylent non seulement les histones, ce qui a généralement pour effet d'augmenter la transcription et l'expression génique, mais également d'autres protéines comme par exemple des protéines de choc thermique (HSP90), la tubuline alpha, certains récepteurs aux stéroïdes ainsi que de nombreux facteurs de transcription (NF-kB p65, Sp1, etc.). Ainsi les HDACs participent au contrôle de nombreux processus cellulaires.

Les inhibiteurs des HDACs (ou HDACi), de part leur capacité à induire la différenciation cellulaire et l'apoptose, sont parmi les anti-cancéreux les plus prometteurs en cours de développement pour dans le traitement des néoplasies solides et hématologiques. Récemment, l'activité anti-inflammatoire et immuno-modulatrice des HDACi a été mise en évidence et exploitée avec succès pour le traitement de pathologies auto-immunes dans des modèles précliniques.

L'effet des HDACi sur la réponse immunitaire innée restant largement inconnu, nous avons entrepris la première étude d'envergure dans ce domaine. Dans un premier article, nous démontrons que les HDACi inhibent l'expression de nombreux gènes (récepteurs aux produits microbiens, cytokines, chimiokines, molécules d'adhésion et co-stimulatrices, facteurs de croissance, etc.) impliqués dans les défenses anti-infectieuses *in vitro*. En accord avec ces données, les HDACi augmentent la mortalité d'animaux infectés dans des modèles de pneumonie et de candidose bénignes. De manière congruente, les HDACi protègent les animaux de mortalité induite par choc toxique et septique en inhibant la réponse inflammatoire exubérante qui caractérise ces pathologies (Roger T. *et al.*, Blood 2011).

Afin de caractériser plus en détails l'influence des HDACi sur la réponse immunitaire innée, nous avons également analysé l'impact de deux HDACi, l'acide valproïque (VPA) et la trichostatin A (TSA), sur les principaux mécanismes de défenses antimicrobiennes des macrophages. Dans un second article (Mombelli et *al.*, Journal of Infectious Diseases 2011), nous rapportons que la VPA et la TSA diminuent la capacité des macrophages à phagocyter et à détruire les bactéries Gram-positives *Staphylococcus aureus* et Gram-négatives *Escherichia coli*. En accord avec ces données, les HDACi inhibent l'expression de molécules impliquées dans la phagocytose comme les récepteurs éboueurs (Msr 1 et CD14) et de type lectine (Dectin 1), ainsi que les récepteurs aux opsonines (intégrines). Par ailleurs, les HDACi interfèrent avec l'expression de différentes sous unités de la NADPH oxydase ($gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, $p40^{phox}$, $p67^{phox}$ et Rac2) et de l'oxyde nitrique (NO) synthétase inductible (iNOS), qui sont responsables de la production de dérivés oxygénés (ROS) et nitrogénés (NO) essentiels à la destruction des microorganismes dans le phagolysosome. En résumé, cette étude décrit des mécanismes par lesquels les HDACi diminuent la capacité d'ingérer et de détruire les bactéries, et ainsi augmentent la susceptibilité aux infections.

Globalement, nos données indiquent que les HDACi sont de puissants anti-inflammatoires qui pourraient favoriser la survenue d'infections chez les patients cancéreux traités avec ces drogues, comme semble par ailleurs le suggérer des études cliniques rapportées dans la littérature. Nous proposons un suivi clinique infectieux strict chez les patients traités avec ces agents.

Histone deacetylase inhibitors impair innate immune responses to Toll-like receptor agonists and to infection

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Regulated by histone acetyltransferases and deacetylases (HDACs), histone acetylation is a key epigenetic mechanism controlling chromatin structure, DNA accessibility, and gene expression. HDAC inhibitors induce growth arrest, differentiation, and apoptosis of tumor cells and are used as anticancer agents. Here we describe the effects of HDAC inhibitors on microbial sensing by macrophages and dendritic cells in vitro and host defenses against infection in vivo. HDAC inhibitors down-regulated the expression of numerous host defense genes, including pattern recognition receptors, kinases, transcription regulators, cytokines, chemokines, growth factors, and costimulatory molecules as assessed by genome-wide microarray analyses or innate immune responses of macrophages and dendritic cells stimulated with Toll-like receptor agonists. HDAC inhibitors induced the expression of Mi-2β and enhanced the DNA-binding activity of the Mi-2/NuRD complex that acts as a transcriptional repressor of macrophage cytokine production. In vivo, HDAC inhibitors increased the susceptibility to bacterial and fungal infections but conferred protection against toxic and septic shock. Thus, these data identify an essential role for HDAC inhibitors in the regulation of the expression of innate immune genes and host defenses against microbial pathogens. (*Blood.* 2011;117(4):1205-1217)

Introduction

The innate immune system plays an essential role in antimicrobial defenses. Detection of microbial pathogens is carried out by sentinel cells of the innate immune system that are located in tissues (macrophages and dendritic cells [DCs]) in close contact with the host's natural environment or that are rapidly recruited to the site of infection (neutrophils). Recognition of invasive pathogens by immune cells relies on their capacity to detect microbial- or pathogen-associated molecular patterns, such as endotoxin, peptidoglycan, lipopeptides, glucans or mannans, flagellin, and nucleic acids. This process involves the coordinated actions of soluble and cellular molecules composing components of the complement system, acute phase proteins, and membrane-associated or intracellular pattern-recognition molecules, including Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors, retinoic acid-inducible gene I like receptors, C-type lectin receptors, and scavenger receptors. Ligand-activated receptors trigger the mitogen-activated protein kinase, nuclear factor-κB (NF-κB), and interferon-related factor (IRF) signal transduction pathways that induce the transcription and production of immune genes, including cytokines that are critical for the activation of innate and adaptive immunity.[1,2]

Chromatin structure plays a central role in regulating gene expression. Acetylation of histones is an essential epigenetic mechanism controlling chromatin structure, DNA accessibility for transcription factors, and gene expression. The net state of acetylation of the ε amino groups of lysine residues of histones is regulated by the opposing actions of histone acetyltransferases and histone deacetylases (HDACs). Acetylation of histones relaxes the chromatin structure promoting gene transcription, whereas deacetylation of histones compacts the chromatin structure favoring gene silencing. HDACs have been classified into 4 subclasses based on their homology with yeast HDACs, their subcellular localization, and their enzymatic activity.[3] Beside histones, nonhistones proteins (such as α-tubulin, heat shock protein 90, steroid receptors, and regulators of nuclear import) are also modified by reversible acetylation.[4,5] Therefore, histone acetyltransferases and HDACs affect diverse biologic functions, principally cell differentiation, growth, and survival.[6-8]

HDACs are at the center of great interest for 2 major reasons. First, dysregulated HDAC expression or activity has been linked to the pathogenesis of cancer and inflammatory and autoimmune diseases. Second, small-molecule inhibitors of class I, II, and IV HDACs have been shown to exhibit anticancer activity with good safety profiles notably in patients with hematologic malignancies. Thus, these drugs are among the most promising anticancer agents under development.[6,8-10] Here we analyzed the impact of HDAC inhibitors on gene expression profiles in macrophages and DCs in vitro and on the host response to bacteria and fungi in vivo. We found that HDAC inhibitors exerted profound inhibitory effects on the host innate immune antimicrobial defense

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response, down-regulating the expression of innate immune receptors, interfering with transcriptome remodeling after stimulation with TLR agonists, and inhibiting the expression of key antimicrobial cytokines and accessory molecules in whole blood, macrophages, DCs, and splenocytes. Consistent with these immunosuppressive effects, HDAC inhibitors enhanced the susceptibility of mice to bacterial and fungal infections. Conversely, HDAC inhibitors protected mice from septic shock.

Methods

Ethics statement

All animal procedures were approved by the Office Vétérinaire du Canton de Vaud (authorizations 876.5, 876.6, and 877.5) and performed according to the institution guidelines for animal experiments.

Mice, cells, and reagents

Eight- to 12-week-old female BALB/c mice (Charles River Laboratories) were housed under specific pathogen–free conditions. Bone marrow derived macrophages (BMDMs), thioglycollate-elicited peritoneal macrophages, and RAW 264.7 macrophages (ATCC TIB-71) were cultured as previously described.[11] Bone marrow–derived DCs (BMDCs) were obtained by culturing bone marrow cells in Iscove modified Dulbecco medium containing 10% fetal calf serum (FCS; Sigma-Aldrich), 50μM 2-mercaptoethanol, and granulocyte-macrophage colony-stimulating factor. Splenocytes were cultured in RPMI medium containing 2mM L-glutamine and 10% FCS and 50μM 2-mercaptoethanol. Human myeloid DCs (moDCs) were produced as described previously.[12] Human whole blood assay was performed as described previously.[13]

Cells were exposed to *Salmonella minnesota* ultra pure lipopolysaccharide (LPS; List Biologicals Laboratories), Pam_3CSK_4 (EMC Microcollections), CpG oligonucleotide (CpG ODN; Invivogen), toxic shock syndrome toxin-1 (Toxin Technology), staphylococcal enterotoxin B, concanavalin A (Sigma-Aldrich), or heat-inactivated *Escherichia coli* O18 (*E coli*), *Staphylococcus aureus*, and *Candida albicans*. Trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), and valproic acid (VPA) were from Sigma-Aldrich. The concentrations of TSA and VPA used in vitro did not affect the viability of BMDMs (> 85% cell recovery after 18 hours). Orfiril (Desitin Pharmaceuticals), a commercial injectable solution of sodium valproate, was used for in vivo experiments.

Microarray analysis and quantitative real-time PCR

For each experimental condition, 2 independent samples were processed in parallel. Low RNA input fluorescent linear amplification kit (Agilent Technologies) was used for cDNA synthesis and cRNA amplification. Experimental samples were labeled with cyanine 5-CTP, whereas a control Universal mouse RNA mixture (Stratagene) was labeled using cyanine 3-CTP (PerkinElmer). Labeled cRNA was hybridized onto high-density oligonucleotide microarrays containing approximately 20 000 60-mer (Mouse Development Oligo Microarray Kit, reference G4120A, and Mouse Oligo Microarray Kit, V2, reference G4121B, Agilent Technologies). Slides were scanned using a Microarray Scanner G2565AA system (Agilent Technologies) at a resolution of 5 μm. For data analysis, local background-subtracted signals were calculated using Feature Extraction software (Agilent Technologies, Version A6.1.1). To ensure spot quality, features and their respective background, which were not uniform in pixel fluorescence intensity distribution in both channels, were flagged (nonuniformity outlier flagging algorithm). Data were imported in GeneSpring, Version 7.0 (Agilent Technologies) and then normalized using both per spot (signal channel divided by the corresponding control channel and generation of $\log_{10}$ ratio) and per chip (to the 50th percentile). The microarray dataset has been deposited in the Gene Expression Omnibus database (GEO; National Center for Biotechnology Information; accession numbers GPL7291, GSE22409).

Real-time polymerase chain reaction (RT-PCR) was performed with a 7500 Fast Real-Time PCR System using the Power SYBR Green PCR Master Mix (Applied Biosystems) and primer pairs (supplemental Table 4, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).[14] Samples were tested in triplicates. A standard made of successive dilutions of a reference cDNA was processed in parallel. Gene-specific expression levels were assessed relative to the expression of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *Hprt* and reported in arbitrary units. In selected experiments, standards consisted of serial dilutions of a plasmid containing the target gene.

Cytokine measurements

Tumor necrosis factor (TNF) and interleukin-6 (IL-6) concentrations were measured by bioassay,[15] IL-12p40, and interferon-γ (IFN-γ) by enzyme-linked immunosorbent assay (ELISA; BD Biosciences), and a broad screening of cytokines and chemokines production was performed using the Luminex technology (Luminex Corporation).[14]

Proliferation

Splenocytes (1.5×10^5) were cultured for 48 hours in 96-well culture plates. Proliferation was monitored by measuring ^{3}H-thymidine incorporation over 18 hours.

Flow cytometry

BMDCs were incubated with 2.4G2 monoclonal antibody (mAb) and mAbs (BD Biosciences) specific for mouse major histocompatibility class II (14-4-4S-fluorescein isothiocyanate), CD11c (HL3-phycoerythrin [PE]), and CD40 (3/23-biotin revealed with CyChrome-conjugated streptavidin; BD Biosciences). moDCs were incubated with the lineage Cocktail 1 (fluorescein isothiocyanate–conjugated mAbs specific for CD3, CD14, CD16, CD19, CD20, and CD56) and mAbs specific for human CD11c (B-ly6-PE-Cy5), HLA-DR (L243-APC) and CD40 (5C3-PE), CD80 (L307.4-PE), CD86 (FUN-1-PE), or CC-chemokine receptor 7 (CCR7; 3D12-PE).[12] Data were analyzed using FlowJo Version 8.5.3 software (TreeStar).

Electrophoretic mobility shift assay

A total of 2 μg of nuclear extracts was incubated for 15 minutes at room temperature with a radiolabeled consensus NF-κB probe (Santa Cruz Biotechnology) and analyzed by electrophoretic mobility shift assay.[16]

Western blot analyses

Cell lysates were electrophoresed through polyacrylamide gels and transferred onto nitrocellulose membranes.[14] Membranes were incubated with antibodies specific for phosphorylated (phospho)-extracellular signal-regulated kinase (ERK)1/2, total-ERK1/2, phospho-p38, total-p38, phospho-IRF3, (Cell Signaling Technology), c-jun, Mi2b (Santa Cruz Biotechnology), SNF2β/BRG1 (Millipore), IRF7 (Invitrogen), phospho-signal-transducer and activator of transcription protein (STAT1; BD Biosciences), and α-tubulin (Sigma-Aldrich), and then revealed with secondary horseradish peroxidase-conjugated goat antirabbit IgG and the ECL Western blotting analyses system (GE Healthcare). Acid-soluble proteins were extracted and analyzed by Western blotting using antiacetylated histone H3 and H4 antibodies (Cell Signaling Technology).[13]

Chromatin immunoprecipitation

Chromatin immunoprecipitation analysis was performed using the ChIP assay kit (Millipore) using antiacetylated histone H4 (06-866, Upstate Biotechnology) or Mi2b (sc-11378X, Santa Cruz Biotechnology) rabbit polyclonal antisera and normal rabbit IgG (sc-2027, Santa Cruz Biotechnology) as described previously.[13] Immunoprecipitated DNA was amplified by PCR using primers described in supplemental Table 4.

siRNA silencing in RAW 264.7 mouse macrophages

RAW 264.7 macrophages (6×10^4 cells per well) were seeded in 24-well plates and transfected the next day with 187.5 ng of Mi-2b or Brg1 siRNA duplexes or negative control (sequences available in supplemental Table 4) and HiPerFect transfection reagent (QIAGEN) according to manufacturer's instruction. After 3 days, cells were stimulated for 4 hours with 0 to 10 ng/mL LPS. Gene expression was analyzed by real-time PCR. Mi-2b and Brg1 mRNA levels were decreased by 60% in cells transfected with the specific siRNAs.

In vivo models

Klebsiella pneumonia *sepsis*. A total of 10 CFU of a clinical isolate of *K pneumoniae* was injected intranasally into mice treated 15 minutes earlier with a single dose of valproate (Orfiril, 200 mg/kg intraperitoneally).[17]

Systemic candidiasis. Mice were challenged through the tail vein with 1.2×10^5 CFU of a clinical isolate of *C albicans*. Valproate treatment (200 mg/kg intraperitoneally) was administrated 15 minutes before *C albicans* challenge and repeated daily during 24 days.

Pam_3CSK_4-induced shock. Mice were injected with D-galactosamine (2 g/kg intraperitoneally) followed immediately after by Pam_3CSK_4 (1.6 mg/kg intraperitoneally).[11] Valproate injections (200 mg/kg intraperitoneally every 12 hours) were started 2 days before D-galactosamine injection and discontinued 48 hours after Pam_3CSK_4 challenge.

CLP. Animals were pretreated intraperitoneally 1 hour before cecal ligation and puncture (CLP) with valproate (200 mg/kg) and injected subcutaneously every 12 hours with gentamicin (10 mg/kg), clindamycin (30 mg/kg), and buprenorphine (0.1 mg/kg).

Doses of valproate were selected based on previous publications and adjusted to the specific conditions of the sepsis models.

Statistical analysis

Comparisons among treatment groups were performed using the Fisher exact test for categorical data and the Mann-Whitney tests for continuous variables. The Kaplan-Meier method was used for survival, and differences were analyzed by the log-rank sum test. The analyses were performed using Prism software Version 5.03 (GraphPad). All reported *P* values are 2-sided, and values less than .05 were considered to indicate statistical significance. For microarray analyses, statistical significance of differentially expressed genes (2-fold changes) was evaluated by analysis of variance using the Benjamini and Hochberg false discovery rate correction (5%).

Results

Inhibition of HDACs down-regulates the expression of innate immune genes in macrophages

Transcriptome analyses. The transcriptome of BMDMs was examined using the Agilent high-density Mouse Development Oligoarrays, which contained 20 280 unique 60-mer corresponding predominantly to expressed sequence tags and with the Mouse Oligo Microarray Kit (V2) containing 20 156 indexed gene probes. At baseline, TSA (t = 4 hours of incubation), a prototypical broad-spectrum inhibitor of class I, II, and IV HDACs, modified the expression of 1594 macrophage genes (7.7% of the transcriptome), of which 772 (3.6%) were found to be down-regulated and 822 (4.1%) up-regulated. HDAC inhibitors significantly reduced the expression of numerous pattern recognition molecules and immune receptors involved in the sensing of a broad range of microbial products, including bacterial lipopeptides, lipoteichoic acid, peptidoglycan, endotoxin, flagellin, viral nucleic acids, and fungal β-glucan (supplemental Table 1). We then performed time-course analyses of gene expression profiles of BMDMs preincubated for 1 hour with TSA before stimulation for 1, 2, 4, and 20 hours with TLR1/TLR2 (Pam_3CSK_4 lipopeptide) or with TLR4 (LPS) agonists (Figure 1A-B). TSA inhibited the up-regulation of 32% to 58% and 33% to 60% of the genes induced by LPS or Pam_3CSK_4, respectively. Conversely, TSA counter-regulated the down-regulation of 60% to 73% and 51% to 75% of the genes repressed by LPS or Pam_3CSK_4. In contrast, TSA potentiated the effects of LPS and Pam_3CSK_4 on only a small proportion of genes (2%-16%). The genes modulated by TSA included signal transduction, immunoregulation, cytoskeleton and cell structure, metabolism and cell cycle, growth, and apoptosis (complete listing of the genes: Figure 1C-D, supplemental Tables 2-3).

We then focused our analyses on innate immune gene families known to play a critical role in the host antimicrobial defense response. TSA exerted prominent inhibitory effects on LPS- or Pam_3CSK_4-induced genes (Figure 1C-D). Indeed, TSA inhibited LPS- and/or Pam_3CSK_4-induced up-regulation of genes encoding for molecules involved in the sensing of microbial compounds, such as Tlrs, Cd14, Md-2, scavenger receptors (Scarb2), cytosolic microbial sensors (Aim1, Mda-5, Nlrp3, Nod1, Nod2, Eif2ak2/Pkr, Pycard/Asc, Mevf/Pyrin, and RIG-I), c-type lectins (Clecs and Msr1), formyl peptide receptors (Fprs), IgE and IgG Fc receptors, complement and complement receptors (C1qa, C1r, and Cfb/H2-Bf), and adhesion molecules (Icam1, integrins, and Vcam1; Table 1). The effect of TSA on the expression of Tlrs, Cd14, Cd36, and Md-2 was confirmed by real-time PCR (Figure 1E). TSA also inhibited the expression of adaptor molecules (MyD88 and Ticam2), kinases (Iraks, Jaks, Lck, Map3ks, Ripk2, Syk, Tank, Tbk1, and Traf1), phosphatases, and transcription modulators (Atfs, Cebps, Irfs, Junb, Nfkbs, Spic, Stats, and Socs1; Table 1). In addition, TSA down-regulated a wide range of LPS- and/or Pam_3CSK_4-stimulated mediators involved in chemotaxis, inflammation, tissue repair, and antigen processing and presentation. This list of genes included cytokines (Il1a, Il1rn, Il6, Il12a, Il12b, Il15, Il18, Il23a, Il27, Ltb, Tnf, Tnfaip3, Tnfsf4, and Tnfsf9), chemokines (Ccl4, Ccl7-9, Ccl12, Ccl17, Ccl22, Ccl24, Cklfsf3, Cklfsf6, Cklfsf7, Cxcl2, Cxcl5, Cxcl12, Cxcl16, and Cx3cl1), growth factors (Csf2, Edn1, and Tmpo), and their receptors (Il1rl1, Il2rg, Il4ra, Il10rb, Il13ra1, Crlf3, Cxcl12, Cxcl16, Pdfrl, Tnfrsf1a, Tnfrsf14, Ifnar1, Ifnar2, Ifngr2, Csf3r, and Ednrb), cathelicidin antimicrobial peptide (Calmp), matrix metalloproteinases, ubiquitins, proteasome subunits and molecules involved in autophagy (Atg16l), antigen transport (Tap1 and Tap2), and peptide presentation (H-2D, H-2E, and H-2Q; Table 1). Therefore, TSA strongly affected transcriptome remodeling of BMDMs stimulated with microbial products exerting predominantly inhibitory effects, indicating that acetylation of histones or nonhistone proteins is required for optimal transcription of a large number of macrophage genes involved in microbial sensing and host defenses.

Cytokine production. To validate the observations generated from microarray profiling, we quantified the production of cytokines (ie, TNF, IL-6, and IL-12p40) in BMDMs exposed to LPS, Pam_3CSK_4, *E coli*, or *S aureus*. Real-time PCR analyses (Figure 2A), bioassay, and ELISA measurements (Figure 2B-C) confirmed that TSA strongly inhibited TNF, IL-6, and IL-12p40 production in a time- and dose-dependent manner. Yet, TSA did not inhibit LPS-induced Tnf mRNA and protein (Figure 2A-B). Similar results were obtained in thioglycolate-elicited peritoneal macrophages (data not shown).

To confirm the findings obtained with TSA, we tested the effects of 2 other HDAC inhibitors: SAHA, a hydroxamate, and VPA, a short chain fatty acid. Like TSA, SAHA and VPA used at clinically relevant concentrations (4-100nM and 4-100μM) dose-dependently inhibited TNF, IL-6, and IL-12p40 production in BMDMs stimulated with Pam_3CSK_4 (Figure 2D). VPA also markedly reduced (up to 50-fold) the production of 13 of 15 mediators induced by LPS or Pam_3CSK_4 in whole blood (Figure 2 E-F).

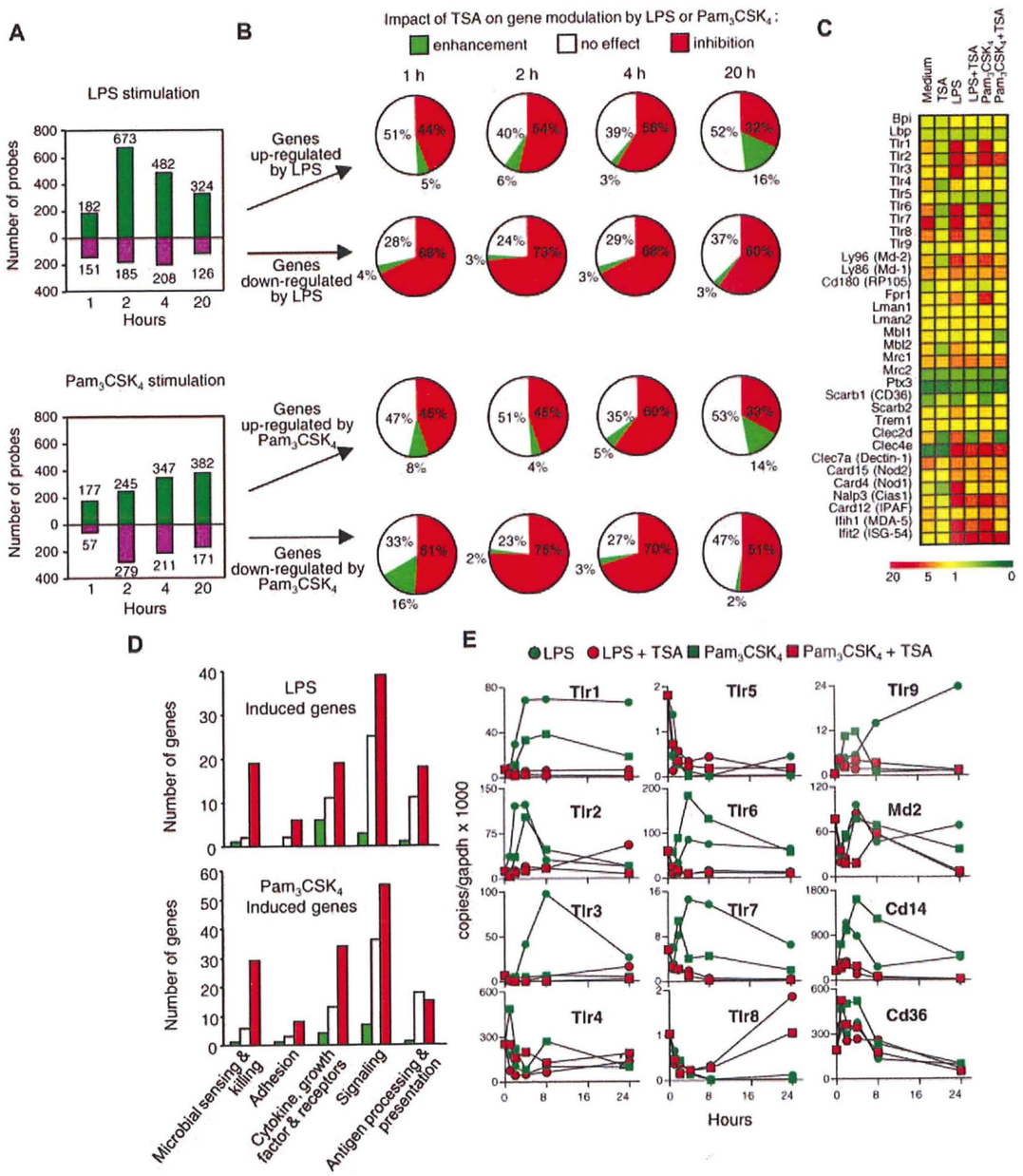


Figure 1. Trichostatin A inhibits the expression of innate immune genes in macrophages. BMDMs were preincubated for 1 hour with or without TSA (100nM) before exposure (C-D, 4 hours) to LPS (100 ng/mL) or Pam_3CSK_4 (100 ng/mL). Transcriptome was analyzed with Agilent Mouse Development Oligoarrays (A-B) or Mouse Oligo Microarray Kit V2 (C-D). (A) Number of genes either up-regulated or down-regulated by LPS and Pam_3CSK_4 without preincubation with TSA (fold change > 2 vs medium). (B) Effect of 1-hour preincubation with TSA on genes (expressed in percentage) either up-regulated or down-regulated by LPS or Pam_3CSK_4 (fold change > 2 vs medium). White represents no change; red, inhibition; and green, increase by TSA compared with stimulation with LPS or Pam_3CSK_4 alone. (C) Heat map of selected pattern recognition molecules (medium, TSA, LPS and Pam_3CSK_4: 4 hours of incubation, LPS + TSA and Pam_3CSK_4 + TSA; 1-hour preincubation with TSA followed by 4-hour incubation with LPS and Pam_3CSK_4). (D) Effect of 1-hour preincubation with TSA (fold changes were calculated vs LPS or Pam_3CSK_4 alone) on a selection of genes up-regulated by LPS or Pam_3CSK_4 and grouped into various categories based on their biologic functions (microbial sensing and killing; adhesion; cytokine, growth factor and receptors; signaling; antigen processing and presentation). White represents no change; red, inhibition; and green, increase by TSA compared with stimulation with LPS or Pam_3CSK_4 alone. (E) Tlr1-9, Md-2, Cd14, and Cd36 mRNA copy number was determined by RT-PCR and expressed relative to that of GAPDH. Data are representative of 2 independent experiments.

Table 1. Selection of genes whose up-regulation by LPS or Pam_3CSK_4 is inhibited by TSA in BMDMs

Adhesion molecule	Innate receptor	Signal transduction	Phosphatase	Transcription regulator	Cytokine, chemokine, and growth factor	Cytokine, chemokine, and growth factor receptor	Antigen processing and presentation	Ubiquitination	Others
Alcam	*Aim1*	*Csnk1a1*	*Dusp1 (Mkp-1)*	*Atf3*	*Ccl4*	*Ccrl2*	*Atg16l*	*Ubc*	*Calmp*
Cd47	*Birc (cIAP2)*	*Irak2*	*Dusp4*	*Atf4*	*Ccl7*	*Crlf3*	*Ctsz*	*Ube1l*	*C1qa*
Icam1	*Card4 (Nod1)*	*Irak3*	*Dusp16*	*Atf5*	*Ccl8*	*Csf3r*	*H2-DMb1*	*Ube2c*	*C1r*
Itga4	*Carc15 (Nod2)*	*Jak1*	*Ptpn6*	*Batf*	*Ccl9*	*Ednrb*	*H2-Ea*	*Ube2d3*	*H2-Bf*
Itga5	*Cd14*	*Jak2*	*Ptpn9*	*Bcl3*	*Ccl12*	*Ifnar1*	*H2-Eb1*	*Ube2e2*	*F10*
Itgal	*Cias1 (Nlrp3)*	*Lck*	*Ptpn12*	*Bcl7c*	*Ccl17*	*Ifnar2*	*H2-M3*	*Ube2j1*	*Mmp9*
Itgax	*Clec2d*	*Map3k7ip2*		*Bcl10*	*Ccl22*	*Ifngr2*	*H2-Q10*	*Ube2j2*	*Mmp14*
Itgb3	*Clec4a2*	*Map3k8 (Tpl2)*		*Cebpb*	*Ccl24*	*Il1rl1*	*H2-Q7*	*Ube2l6*	*Mmp25*
Vcam1	*Clec4d*	*Mapkapk2*		*Cebpd*	*Ccrl2*	*Il2rg*	*Psma3*	*Ube2m*	*Nos2*
	Clec4e	*Myd88*		*Cited2*	*Csf2*	*Il4ra*	*Psma4*	*Ube2r2*	*Pde4b*
	Eif2ak2 (Pkr)	*Pias1*		*Hmgb2*	*Cklfsf3*	*Il10rb*	*Psma5*	*Ube2v1*	*Pld2*
	Fcer1g	*Prkr*		*Ikbke*	*Cksfs6*	*Il13ra1*	*Psma6*	*Ubl3*	*Ptger4*
	Fcgr2b	*Ptk2*		*Irf1*	*Cklfsf7*	*Pdfrl*	*Psmb7*	*Ubtd1*	*Ptges*
	Frp1	*Ripk2*		*Irf2*	*Cxcl2*	*Ptafr*	*Psmb8*	*Ufm1*	*Ptgir*
	Fpr-rs2	*Socs1*		*Irf5*	*Cxcl5*	*Tnfrsf1a*	*Psmb9*	*Usp12*	*Ptgs2*
	Ifih1 (Mda5)	*Stk19*		*Irf7*	*Cxcl12*	*Tnfrsf14*	*Psmb10*	*Usp18*	*Sod2*
	Ly96 (Md-2)	*Syk*		*Junb*	*Cxcl16*		*Psme1*	*Usp24*	*Txn1*
	Mefv (Pyrin)	*Tank*		*Klf6*	*Cx3cl1*		*Psme2b*	*Usp42*	*Tnfrsf5 (Cd40)*
	Msr1	*Tbk1*		*Nfkb1*	*Edn1*		*Tap1*		
	Pycard	*Ticam2*		*Nfkb2*	*Il1a*		*Tap2*		
	Scarb2	*Traf1*		*Nfkbib*	*Il1rn*		*Tapbp*		
	Ddx58 (RIG-I)	*Trim30*		*Nfkbie*	*Il6*		*Tapbpl*		
	Tlr1			*Nfkbiz*	*Il12a*				
	Tlr2			*Pias1*	*Il12b*				
	Tlr3			*Rel*	*Il13ra1*				
	Tlr6			*Rela*	*Il15*				
	Tlr7			*Relb*	*Il18*				
	Tlr9			*Spic*	*Il23a*				
				Stat3	*Il27*				
				Stat5a	*Inhba*				
					Ltb				
					Pbef1				
					Tnf				
					Tmpo				
					Tnfaip3 (A20)				
					Tnfsf4 (OX-40L)				
					Tnfsf9 (4-1BBL)				

HDAC inhibitors down-regulated the production of IL-1ra and IL-10, suggesting that the reduced expression of proinflammatory cytokines was not the result of an increased expression of anti-inflammatory cytokines.

Expression of IFNs and IFN-dependent genes. Together with cytokines and chemokines, type I interferons (IFN-α and IFN-β) are central mediators of innate and adaptive immune responses against viral and bacterial infections.[18,19] In response to LPS stimulation, macrophages produce copious amounts of IFN-β that stimulates the transcription of *Ifna*, *Ccl2*, *Ccl8*, *Ccl12*, *Cxcl10*, and *Nos2* (encoding for iNOS), *Irf7*, and *Irf8*.[20,21] LPS, but not Pam_3CSK_4, induced rapid (1 hour) Ifnb mRNA expression and sustained IFN-β secretion by BMDMs, whereas LPS or Pam_3CSK_4 induced early (2 hours and 4 hours, LPS) or late (8 hours and 24 hours, Pam_3CSK_4) Ifna4 mRNA up-regulation (Figure 3A-C). Interestingly, TSA up-regulated and markedly prolonged (up to 24 hours) IFN-β mRNA and protein expression after LPS exposure (Figure 3A-B), confirming microarray data (supplemental Table 2). In contrast, it completely inhibited the up-regulation of Ifna4 mRNA (Figure 3C). TSA also inhibited the expression of LPS-induced Ccl8, Ccl12, Cxcl10, Nos2, and Irf7 mRNA (Figure 3D) and of numerous other LPS-induced IFN-β-dependent genes (supplemental Figure 1). Yet, TSA did not reduce LPS-induced Ccl2 and Irf8 mRNA and late (24 hours) IFN-β-independent Ifna4 mRNA (Pam_3CSK_4) and Ccl5 mRNA (LPS and Pam_3CSK_4). Thus, the massive accumulation of IFN-β induced by TSA in BMDMs did not overcome its inhibition of IFN-β-dependent gene expression. Of interest, TSA dose-dependently suppressed the proliferation of splenocytes induced by LPS, Pam_3CSK_4, CpG ODN, or *E coli* (Figure 3E). It also inhibited the release of IFN-γ by splenocytes exposed to staphylococcal enterotoxin B, toxic shock syndrome toxin-1, or concanavalin A (Figure 3F).

HDAC inhibitors impair the response of DCs

We then examined the effects of HDAC inhibitors on innate immune responses of mouse BMDCs and human moDCs. TSA inhibited the production of cytokines (IL-6 and IL-12p40) and the up-regulation of CD40 by BMDCs stimulated with LPS, Pam_3CSK_4, CpG ODN, *E coli*, *S aureus*, or *C albicans* (Figure 4A-D). Similarly, TSA and VPA inhibited proinflammatory and anti-inflammatory cytokines (TNF, IL-1α, IL-1β, IL-1ra, IL-6, IL-10, IL-12p40, and IL-12p70), chemokines (IP-10/CXCL10, MIP-1β/CCL4, MCP-1/CCL2, and RANTES/CCL5), IFN-γ, G-CSF, and

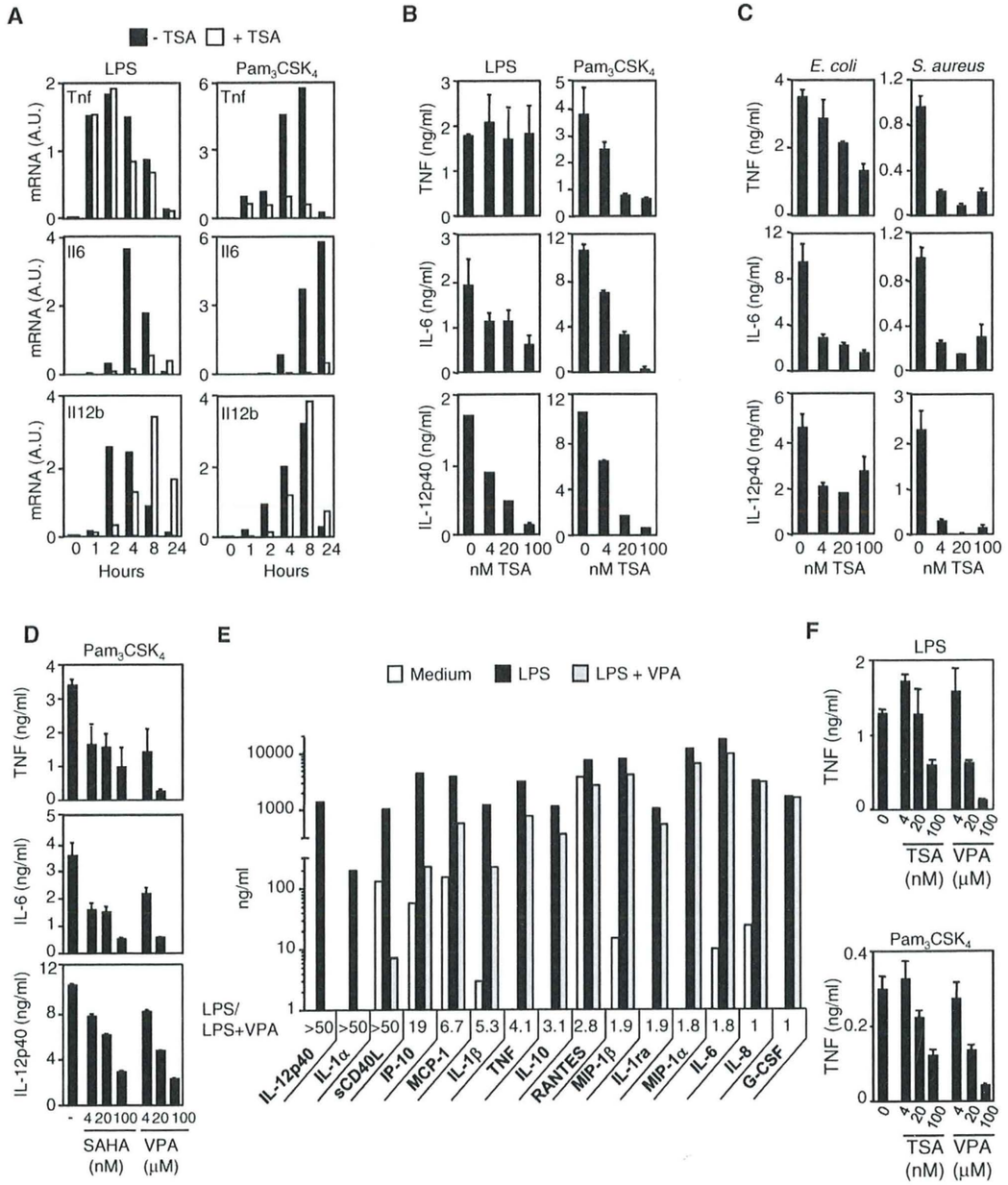


Figure 2. HDAC inhibitors inhibit cytokine release by macrophages exposed to microbial products and bacteria. BMDMs were preincubated for 1 hour with or without TSA (100nM unless specified) before exposure to LPS (100 ng/mL), Pam_3CSK_4 (100 ng/mL), and heat-killed *E coli* or *S aureus* (10^7 CFU/mL). (A-C) TNF, IL-6, and IL-12p40 mRNA (A) and protein (B-C) production by BMDMs. TNF, IL-6, and IL-12p40 mRNA levels were analyzed by RT-PCR, and results are expressed as the ratio of cytokines to GAPDH mRNA levels. Data are representative of 3 independent experiments. Cytokine were quantified in cell culture supernatants collected after 8 hours (TNF) and 18 hours (IL-6 and IL-12p40). Data are mean ± SD of triplicate samples from one experiment representative of 3 independent experiments. A.U. indicates arbitrary units. (D) BMDMs were preincubated for 1 hour with or without SAHA (4, 20, and 100nM) or VPA (4, 20, and 100μM) before exposure to Pam_3CSK_4 (100 ng/mL). TNF, IL-6, and IL-12p40 were quantified in cell culture supernatants collected after 8 hours (TNF) and 18 hours (IL-6 and IL-12p40). Data are mean ± SD of triplicate samples from 1 experiment representative of 3 independent experiments. (E-F) Human whole blood was incubated for 18 hours with VPA (E, 100μM) or TSA together with either LPS (10 ng/mL) or Pam_3CSK_4 (100 ng/mL). Cytokine and chemokine production was assessed by the Luminex technology ("Cytokine measurements"), and LPS/LPS + VPA ratios were calculated (E). TNF was quantified by bioassay. Data are mean ± SD of triplicate samples from one donor and are representative of 2 independent experiments (F).

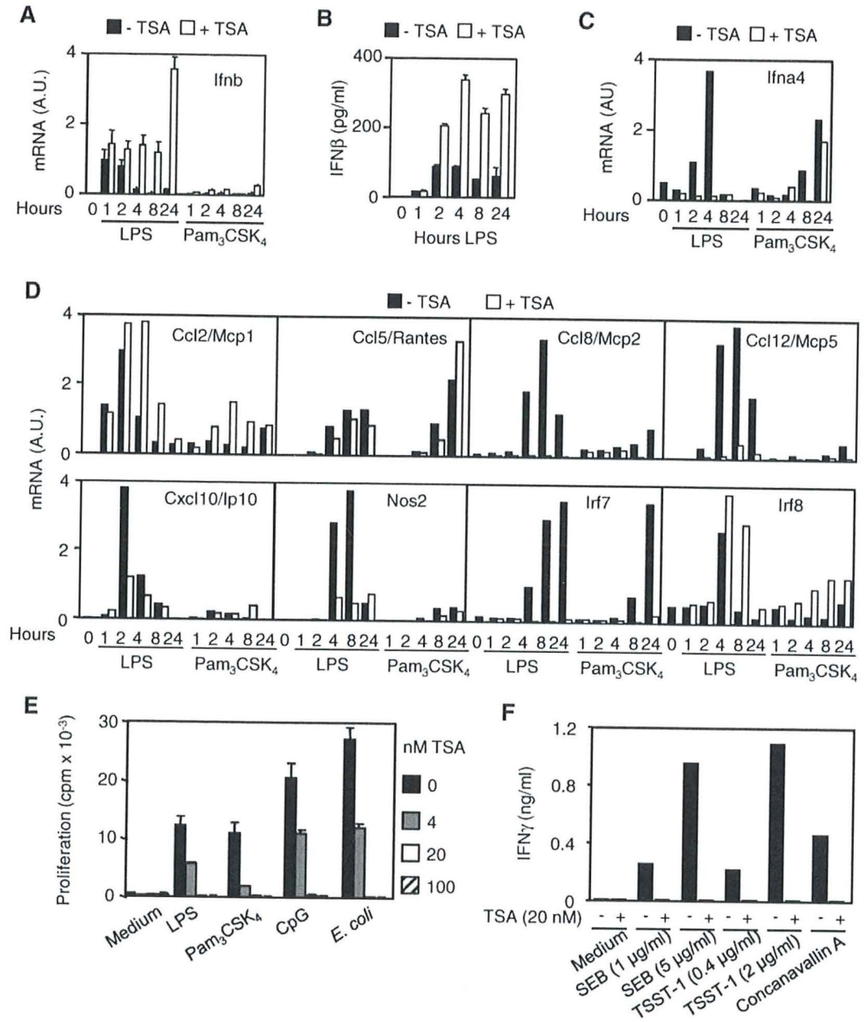


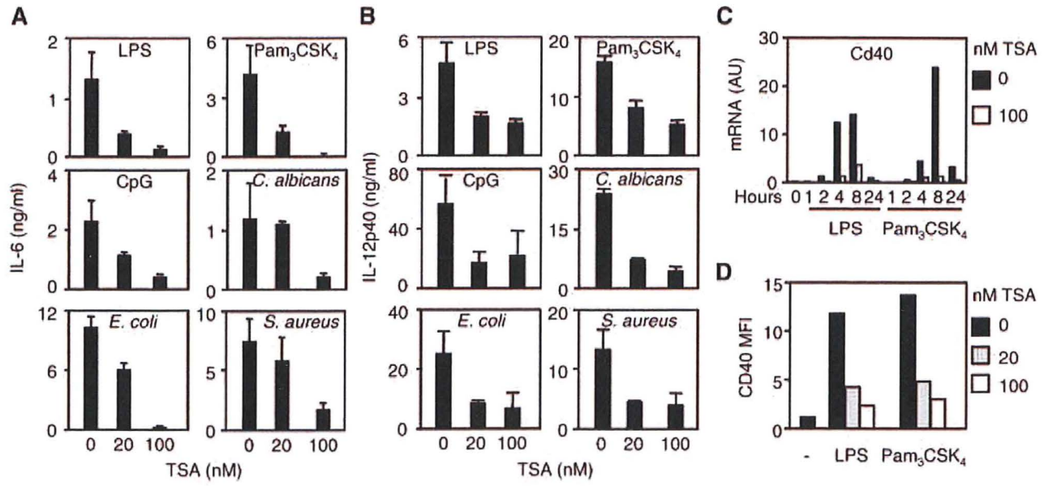
Figure 3. HDAC inhibition enhances the production of IFN-β. (A-D) RNA and cell culture supernatants were collected from BMDMs preincubated for 1 hour with (+) or without (−) TSA (100nM) before exposure to LPS (100 ng/mL) or Pam_3CSK_4 (100 ng/mL). (A-B) IFN-β mRNA and protein expression was quantified by RT-PCR and ELISA. Results are expressed as the ratio of Ifnb mRNA level to that of GAPDH. Data are mean ± SD of triplicate samples from 1 experiment representative of 2 experiments. (C-D) Ifna4 (C), Ccl2, Ccl5, Ccl8, Ccl12, Cxcl10, Nos2, Irf7, and Irf8 (D) mRNA contents were quantified by RT-PCR. Results are expressed as the ratio of mRNA level of the gene of interest to that of GAPDH. Data are representative of 2 independent experiments. A.U. indicates arbitrary units. (E-F) Splenocytes were incubated with TSA and LPS (5 μg/mL), Pam_3CSK_4 (5 μg/mL), CpG ODN (CpG, 0.5μM), *E coli* (5×10^7 CFU/mL), staphylococcal enterotoxin B (SEB, 1-5 μg/mL), toxic shock syndrome toxin-1 (TSST-1, 0.4-2.0 μg/mL), and concanavalin A (5 μg/mL). (E) Proliferation was measured by ^{3}H-thymidine incorporation. Data are mean ± SD of triplicate samples and are representative of 2 independent experiments. (F) IFN-γ production was quantified by ELISA in cell culture supernatants collected after 48 hours. Data are representative of 2 independent experiments.

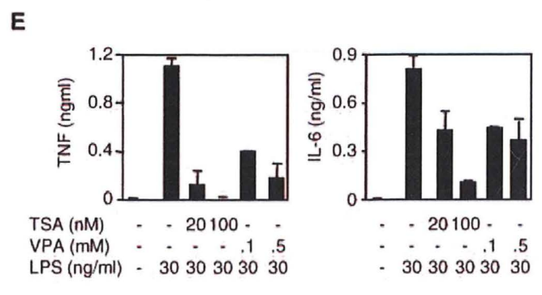
TGF-α production by moDCs in response to LPS stimulation (Figure 4E-F). TSA and VPA also inhibited the up-regulation induced by LPS of the costimulatory molecules CD40, CD80, and CD86 and of CCR7 (Figure 4G). These results suggested that HDAC inhibitors affect biologic activities of DCs, playing a central role in orchestrating the innate and adaptive responses to infection.

HDAC inhibitors increase the expression and the DNA binding of the transcriptional repressor Mi-2β

We then examined whether HDAC inhibitors affected signal transduction pathways in the macrophage (Figure 5). Neither ERK1/2 or p38 phosphorylation nor NF-κB, c-jun, IRF3, or IRF7 nuclear translocation was inhibited by TSA, VPA, or SAHA in BMDMs, peritoneal macrophages, or RAW 264.7 macrophages (Figure 5A-C; and data not shown). Consistent with the fact that it enhanced LPS-induced IFN-β expression (Figure 3B), TSA markedly prolonged STAT1α/β phosphorylation (Figure 5D). Thus, HDAC inhibitors did not inhibit mitogen-activated protein kinases, NF-κB, IRFs, and STAT1 signal transduction pathways induced by microbial products in macrophages.

Given that histone acetylation is associated with active gene transcription, we then analyzed by chromatin immunoprecipitation





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Mediator	Inhibition by TSA and VPA	
	TSA	VPA
IL-1α	+	+
IL-1ra	+	+
IL-1β	+	+
IL-4	-	-
IL-6	+	+
IL-7	-	+
IL-8	-	-
IL-10	+	+
IL-12p40	+	+
IL-12p70	+	+
TNF	+	+
RANTES	+	+
IP-10	+	+
MIP-1α	+	-
MIP-1β	+	+
MCP-1	+	+
G-CSF	+	+
TGFα	+	+
IFNγ	+	+

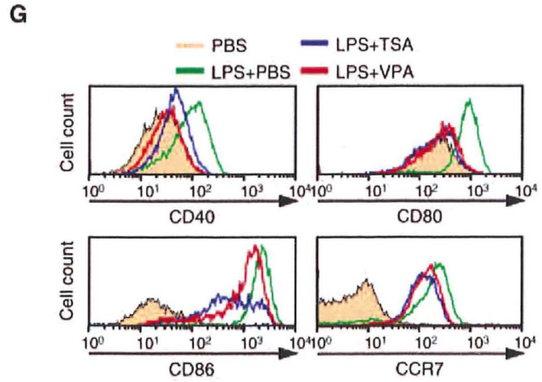

Figure 4. HDAC inhibitors inhibit the response of DCs to microbial stimulation. (A-C) BMDCs were preincubated for 1 hour with TSA before stimulation for 18 hours or the indicated time with LPS (100 ng/mL), Pam_3CSK_4 (100 ng/mL), CpG oligonucleotide (CpG, 0.7μM), and heat-killed *C albicans*, *E coli*, or *S aureus* (10^7 CFU/mL). (A-B) IL-6 and IL-12p40 production. Data are mean ± SD of triplicate samples and are representative of 4 independent experiments. (C) Cd40 mRNA expression quantified by RT-PCR. Results are expressed as the ratio of Cd40 mRNA level to that of GAPDH. Data are mean ± SD of 1 experiment representative of 3 independent experiments. AU indicates arbitrary units. (D) CD40 mean fluorescence intensity (MFI) determined by flow cytometry. Data are representative of 3 independent experiments. (E-G) Human moDCs were preincubated for 1 hour with TSA (E-F, 100nM) or VPA (E-F, 100μM) before exposure to LPS (30 ng/mL) for 18 hours. (E) TNF and IL-6 production. Data are mean ± SD of triplicate samples and are representative of 2 independent experiments. (F) Effect of TSA and VPA on cytokine and chemokine production by 2 independent preparations of moDCs. Mediators were analyzed using the Luminex technology ("Cytokine measurements"). + indicates inhibition (fold change > 2); and −, no effect. (G) CD40, CD80, CD86, and CCR7 expression analyzed by flow cytometry. Data are representative of 2 independent experiments.

the extent of histone H4 acetylation of the Tnf and Il6 promoters in BMDMs stimulated with LPS or Pam_3CSK_4 (Figure 5E). TSA increased histone H4 acetylation of both promoters, suggesting that there was no correlation between the status of histone acetylation and the observed down-regulatory effect of TSA on gene transcription (Figure 2A).

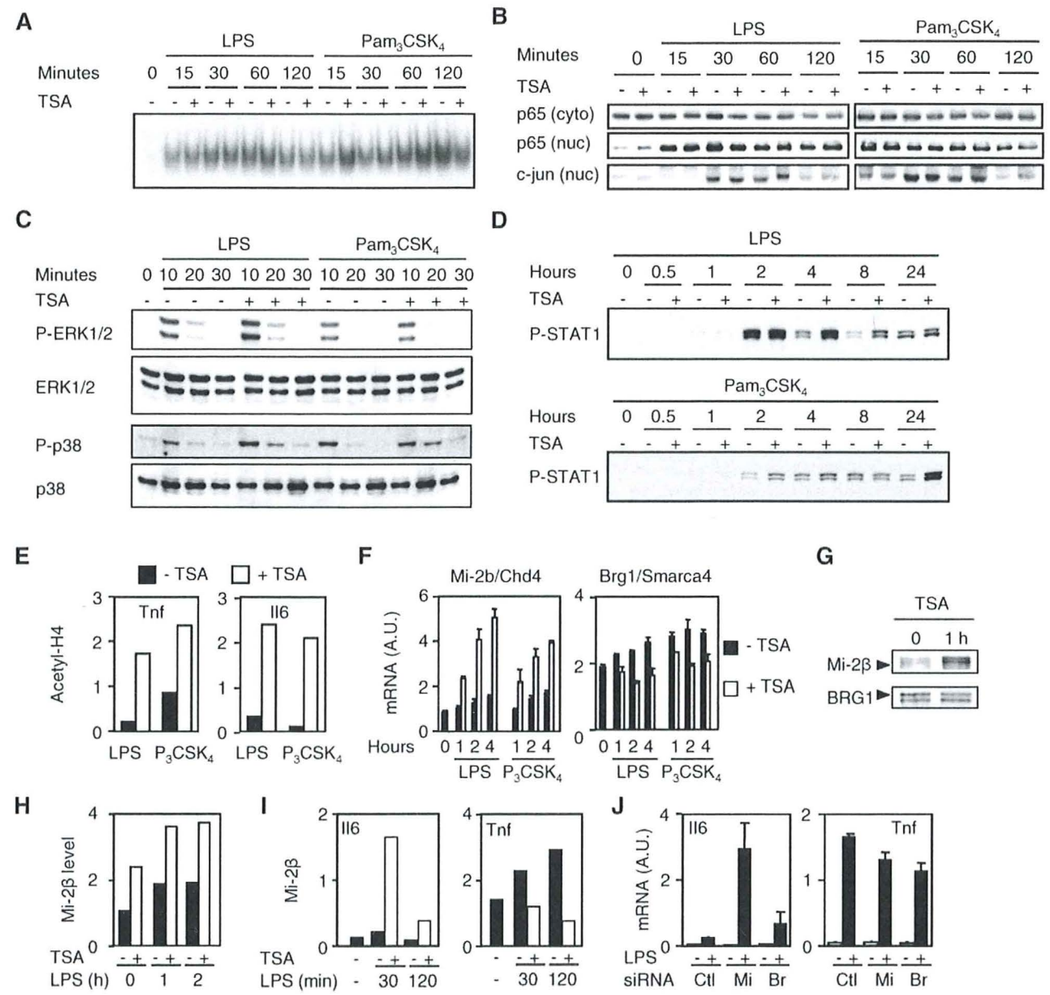

Figure 5. HDAC inhibitors increase Mi-2β expression and recruitment to TSA-sensitive promoter. (A-I) BMDMs were preincubated for 1 hour with (+) or without (−) TSA (100nM unless specified) and exposed to LPS (100 ng/mL) or Pam_3CSK_4 (100 ng/mL) for the indicated time or 1 hour (E). NF-κB DNA binding activity and NF-κB p65, c-jun, phosphorylated (P-), and total ERK1/2 and p38 and P-STAT1 expression were analyzed by electrophoretic mobility shift assay (A) and Western blot (B-D) using nuclear (nuc) and cytosolic (cyto) extracts. The retarded complex detected by electrophoretic mobility shift assay was dose-dependently inhibited by cold wild-type but not mutant NF-κB oligonucleotide, and supershifted using anti-p65 antibody (data not shown). Acetylation of histone H4 (E) and Mi-2β recruitment (I) to Tnf and Il6 promoters were analyzed by chromatin immunoprecipitation. Mi-2β and BRG1 mRNA and protein levels were quantified by real-time PCR (F) and Western blot (G) with densitometric analyses (H). Data are representative of 2 to 5 independent experiments. (J) RAW 264.7 macrophages transfected with control (Ctl), Mi-2β (Mi), or BRG1 (Br) siRNAs. After 3 days, cells were incubated for 4 hours with (+) or without (−) 10 ng/mL of LPS. Il6 and Tnf mRNA levels were analyzed by RT-PCR and results expressed as the ratio of cytokine to GAPDH mRNA levels. Data are representative of triplicate determinations from 1 experiment.

The Mi-2/NuRD and SWI/SNF (also called BAF in mammals) ATP-dependent remodeling complexes play a central role in regulating gene expression.[22] Mi-2β (CHD4) acts as a transcriptional repressor, whereas BRG1 (SMARCA4, the catalytic subunit of the BAF complex) acts as a transcriptional activator of secondary LPS-induced genes in J77.4 macrophages.[23] Given that HDAC inhibitors impaired the expression of secondary (ie, *Il6*) but not primary (ie, *Tnf*) LPS-induced genes in BMDMs, we hypothesized that TSA mediated its effects by affecting the expression of the Mi-2β and BRG1 dyad. Interestingly, TSA markedly upregulated the expression of Mi-2β mRNA in BMDMs exposed to LPS and Pam_3CSK_4 (Figure 5F). In constrast, TSA had a modest inhibitory effect on the expression of BRG1. In line with these findings, TSA increased basal and LPS-induced Mi-2β protein expression (Figure 5G-H). Chromatin immunoprecipitation studies revealed that TSA strongly increased Mi-2β recruitment to the Il6 promoter in BMDMs exposed to LPS, whereas it reduced the binding of Mi-2β to the Tnf promoter (Figure 5I). siRNA-mediated silencing of Mi-2β in RAW 264.7 mouse macrophages greatly enhanced LPS-induced Il6 mRNA expression without a significant effect on Tnf mRNA levels (Figure 5J). Conversely, BRG1 silencing only had a very modest effect on both Il6 and Tnf mRNA expression. Altogether, these data suggest that TSA may inhibit cytokine production via an increased expression of the transcriptional repressor Mi-2β in macrophages.

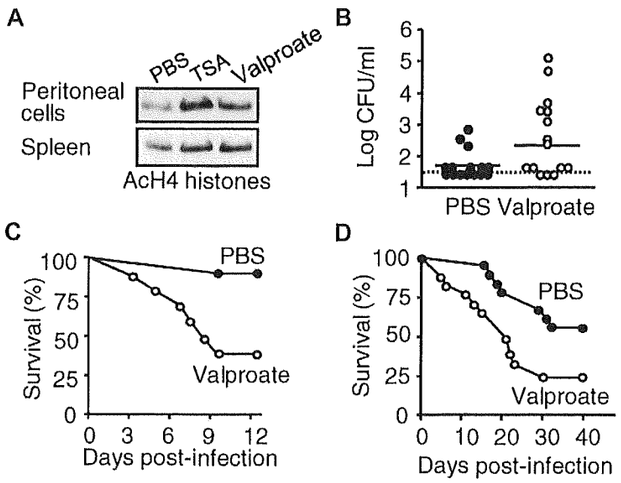

Figure 6. HDAC inhibition increases mortality to nonsevere infection with *K pneumonia* and *C albicans*. BALB/c mice were injected intraperitoneally with valproate (Orfiril, 200 mg/kg) or phosphate-buffered saline (PBS). (A) Peritoneal exudate cells and splenocytes were collected after 1 hour. Histone H4 acetylation (AcH4) was analyzed by Western blotting. (B-C) Mice were infected intranasally with 10 CFU of *K pneumoniae* 15 minutes after valproate. (B) Circulating bacterial counts 3 days after infection and (C) survival (n = 15 mice per treatment groups; $P = .04$ and .0004 for bacterial counts and survival, respectively). The dashed line represents the lower limit of detection. (D) Survival of BALB/c mice injected with 1.2×10^5 CFU of *C albicans* and treated with valproate or PBS daily (n = 18 mice per treatment group; $P = .02$).

HDAC inhibitors impair innate immune responses in vivo

We next investigated the effects of HDAC inhibitors in experimental models of bacterial and fungal sepsis or toxic shock titrated to cause either mild or severe infections or shock. We first verified that valproate enhanced the acetylation of histone H4 in vivo in peritoneal exudate cells and splenocytes (Figure 6A). In an otherwise nonsevere, acute *K pneumoniae* pneumonia model, valproate increased the proportion (53% vs 80%) and magnitude of bloodstream infections (Figure 6B; $P = .04$) and mortality (from 6% to 60%, $P = .0004$; Figure 6C). Consistent with valproate-induced impaired cytokine production by *C albicans*–infected BMDMs and BMDCs (Figure 4; and data not shown), valproate treatment was associated with accelerated (mean time to death: 21.5 days vs > 40 days) and increased mortality (44% vs 75%, $P = .02$) in a model of chronic *Candida* infection (Figure 6D). Thus, inhibition of HDACs impairs host defenses in vivo, increasing the susceptibility to and mortality of bacterial and fungal sepsis.

Severe sepsis and septic shock are characterized by an early overwhelming inflammatory response to microbial invasion, and inhibition of proinflammatory mediators confers protection in sepsis models.[24,25] We therefore tested whether valproate might exert protective effects in models of fulminant toxic shock induced by Pam_3CSK_4 or CLP. Administration of valproate caused a 2- to 3-fold reduction of IL-6 and IL-12p40 circulating levels (Figure 7A) and a notable increase in survival (0%-64%, $P < .001$) in the Pam_3CSK_4 toxic shock model (Figure 7B). Similarly, valproate treatment increased survival from 17% to 42% ($P = .04$) in the CLP model (Figure 7C).

Discussion

These studies identify an essential role for acetylation of histones and nonhistone proteins in the regulation of inflammatory and innate immune gene expression, and in host defensive responses against microbes. Genome-wide microarray analyses revealed a critical role for HDACs in the expression of host defense genes, including pattern-recognition receptors, adaptor molecules, kinases, transcription regulators, complement factors, cytokines, chemokines, and growth factors. HDAC inhibitors exert both immunosuppressive (a predominant effect) and immunostimulatory activities. Located at the forefront of the host defenses against microbial invasion, macrophages and DCs are an important source

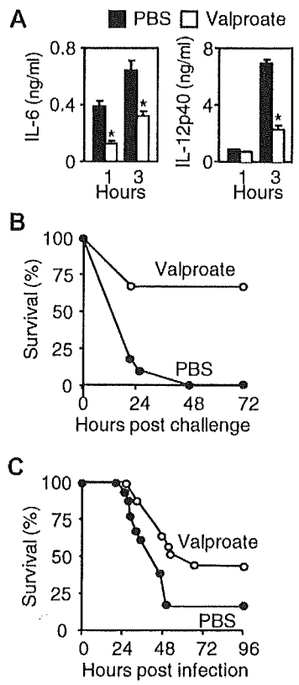

Figure 7. HDAC inhibition protects from lethal toxic shock and severe sepsis. (A-B) BALB/c mice were injected intraperitoneally with valproate (Orfiril, 200 mg/kg started 2 days before and discontinued 2 days after induction of shock) or PBS. Animals were sensitized with D-galactosamine and injected intraperitoneally with Pam_3CSK_4. (A) Plasma levels of IL-6 and IL-12p40 were determined 1 hour and 3 hours after challenge with Pam_3CSK_4. Data are mean ± SD of 8 mice per treatment group. *$P < .001$ for valproate versus PBS. (B) Survival of BALB/c mice subjected to Pam_3CSK_4-induced shock (n = 11 mice per treatment group; $P = .001$). (C) Survival of BALB/c mice subjected to CLP and treated with either valproate or PBS (every 12 hours, starting 30 minutes after surgery; n = 16-18 mice per treatment group). $P = .04$.

of cytokines. Inhibition of HDACs caused a marked reduction of cytokine and chemokine production induced by both cell types after exposure to TLR agonists. Of note, HDAC inhibitors also suppressed the release of IFN-α and IFN-γ by macrophages and splenocytes. Likewise, TSA inhibited IFN-α–stimulated gene activation and late IFN-β induction by Sendai virus and double-stranded RNA, and prevented IFN-α–mediated inhibition of the cytopathic effects of vesicular stomatitis virus.[26,27] Unexpectedly, we observed that HDAC inhibitors sustained LPS-induced IFN-β production in BMDMs. Nevertheless, the expression of numerous IFN-β/STAT1-dependent genes was strongly inhibited by TSA and VPA, indicating that the increased production of IFN-β did not overcome the potent inhibitory effects of HDAC inhibitors.

Phagocytes and professional antigen-presenting cells help bridge innate and adaptive immunity. Macrophages and DCs are key producers of IL-12 and IL-23 promoting the generation of protective Th1 and Th17 responses against intracellular and extracellular pathogens.[28,29] HDAC inhibitors were found to be potent inhibitors of IL-12 and IL-23 production by macrophages and DCs as well as CCR7, which is critical for the migration of DCs to secondary lymphoid organs (present data).[30-32] TSA inhibited the expression of several genes involved in antigen processing and presentation. Moreover, HDAC inhibitors have been reported to inhibit DC-stimulated allogeneic T-cell proliferation and Th1-cell activation[32-34] and to block the differentiation of IL-17–producing T cells.[35] Thus, inhibition of HDACs impacts on several key macrophages and DCs functions likely to affect the production of critical cytokines and the mounting of protective Th1 and Th17 immune responses. Of note, TSA did not inhibit TNF production by BMDMs and peritoneal macrophages stimulated with LPS. In contrast, TSA exerted potent inhibitory effects on whole blood, DCs, and BMDMs stimulated with microbial products other than LPS (Pam_3CSK_4, *E coli*, and *S aureus*). Similar results were obtained using SAHA and VPA. These observations suggest that HDAC inhibitors differentially affect gene expression according to the cell type or the stimulus studied. This could account for the discrepant effects of HDAC inhibitors on TNF production reported in the literature.[30-33,36] Indeed, delaying TSA treatment until 1 hour after the addition of Pam_3CSK_4 to BMDMs, or inhibiting protein synthesis at the time of preincubation with TSA, abrogated TSA-mediated inhibition of Pam_3CSK_4-induced TNF production. Thus, inhibition of stimulus-induced Tnf mRNA expression by TSA requires de novo protein synthesis, which cannot overcome a rapid induction of gene expression in BMDMs stimulated with LPS.

HDAC inhibitors have been reported to interfere with the activation of the mitogen-activated protein kinases, IRFs, STAT1, AP-1, or NF-κB signal transduction pathways. However, these findings have been inconsistent and controversial.[26,30,31,36,37] In BMDMs, we did not observe any impact of 3 HDAC inhibitors (TSA, VPA, and SAHA) on ERK1/2 or p38 phosphorylation or on NF-κB, c-jun, IRF3, or IRF7 nuclear translocation induced by LPS or Pam_3CSK_4. Yet, we found that TSA markedly inhibited the recruitment of NF-κB p65 and of RNA polymerase to the Il6 promoter (data not shown). This finding is consistent with the notion that acetylation of the NF-κB subunits themselves or of molecules involved in the NF-κB signal transduction pathway controls the extent, potency, and duration of NF-κB–mediated transcriptional activity.[38]

Transcriptional repression mediated by HDAC inhibitors may also rely on acetylation-dependent recruitment of transcriptional corepressors or changes in chromatin architecture.[38] In line with this hypothesis, we have previously shown that TSA inhibits the expression of the proinflammatory cytokine macrophage migration inhibitory factor via a local deacetylation of chromatin impairing the recruitment of the basal transcriptional machinery to the macrophage migration inhibitory factor promoter.[13,39] In the present study, we provide data suggesting that HDAC inhibitors induce the expression of Mi-2β and the activity of the Mi-2/NuRD complex, which acts as a transcriptional repressor of secondary LPS-induced cytokines, such as IL-6. Although little is known about the in vivo function of Mi-2β, mice deficient in metastasis-associated protein 2, a component of the Mi-2/NuRD complex, develop a lupus-like syndrome characterized by the hypersecretion of cytokines.[40]

A main finding of our study is the fact that HDAC inhibitors impair the host natural defenses against microbial pathogens. Administration of valproate increased the susceptibility of mice to bacterial and fungal infections converting a nonsevere bacterial pneumonia into a highly lethal infection and markedly increasing the mortality of invasive candidiasis. Moreover, we have also observed that HDAC inhibitors reduced the expression of phagocytic receptors and phagocytosis and killing of bacteria by macrophages (M.M., J.L., T.C., T.R., manuscript in preparation). These results clearly demonstrate that the inhibitory effects of HDAC inhibitors on innate immune cells in vitro translate into robust immunosuppressive effects in vivo that negatively impact on the susceptibility to and outcome of infections. Several arguments led us to believe that these observations may have clinical implications. A vast amount of data indicate that interfering with critical mediators of innate or adaptive immunity (eg, TNF or IL-1) increases the risk of infections.[41] Indeed, treatment of patients with TNF antagonists has been associated with an overall increased risk of bacterial (tuberculosis, nontuberculous mycobacteriosis, listeriosis, and salmonellosis) and fungal (candidiasis) infections.[42,43] HDAC inhibitors are currently used for the treatment of hematologic malignancies and solid tumors often in combination with other cancer or immune suppressive therapies increasing the risk of infection. In phase 1 and 2 clinical trials, patients treated with valproate, SAHA, MS-275, and ITF2357 have developed severe infections, even without neutropenia, that could be the result of the immune-suppressive activities of these HDAC inhibitors.[44-49] These data suggest that monitoring of infections may be warranted in clinical trials of HDAC inhibitors.

Taken advantage of their broad anti-inflammatory and immunomodulatory properties targeting several key mediators implicated in the pathogenesis of septic shock (such as TLRs, MyD88, signal transducing molecules, and proinflammatory cytokines), we reasoned that HDAC inhibitors may prove to be beneficial as adjunctive therapy for septic shock. Consistent with this assumption, valproate exhibited remarkable protective effects in a toxic shock model induced by Pam_3CSK_4 and in the CLP peritoneal sepsis model. In a recent fascinating article, Xu et al detected histones in the circulation of septic patients and baboons and showed that these proteins played a pathogenic role in sepsis.[50] Purified histones H3 and H4 were found to be toxic for endothelial cells, to cause microvascular thrombosis, and to be lethal when injected intravenously into mice. Notably, antihistone H4 antibodies rescued mice from lethal shock induced by LPS, TNF, or CLP. Given that histones appear to act as noxious danger-associated endogenous molecular patterns, one may wonder whether the acetylation status of extracellular histones correlates with toxicity. If so, deacetylation of histones released by necrotic and apoptic cells could exert detoxifying and cytoprotective effects.

Taken together, the present data identify protein acetylation as a key mechanism regulating the expression of important innate

immune genes critically implicated in the sensing of and host responses to microbial pathogens. Inhibition of HDACs impairs essential biologic functions of innate immune cells, reducing their capacity to induce a proinflammatory response, to engulf and kill pathogens, and to mount an adaptive response increasing the susceptibility to infection. On the other hand, the broad anti-inflammatory and immune-suppressive properties of HDAC inhibitors were found to be beneficial as adjunctive therapy for septic shock. Thus, these results suggest that the use of HDAC inhibitors as anticancer agents may increase the risk of infection and sepsis, whereas they may offer new therapeutic options for the management of patients with septic shock.

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Authorship

Contribution: T.R. conceived and supervised the studies, performed experiments, and wrote the paper; J.L., G.G., X.C.D., M.M., A.-L.C., M.K.R., and I.M. carried out in vitro experiments; D.L.R. performed in vivo experiments; T.K., P.F., and J.S. performed microarray analyses; and T.C. discussed the study results and wrote the paper.

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Histone Deacetylase Inhibitors Impair Antibacterial Defenses of Macrophages

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Histone deacetylases (HDACs) control gene expression by deacetylating histones and nonhistone proteins. HDAC inhibitors (HDACi) are powerful anticancer drugs that exert anti-inflammatory and immunomodulatory activities. We recently reported a proof-of-concept study demonstrating that HDACi increase susceptibility to bacterial infections in vivo. Yet, still little is known about the effects of HDACi on antimicrobial innate immune defenses. Here we show that HDACi belonging to different chemical classes inhibit at multiple levels the response of macrophages to bacterial infection. HDACi reduce the phagocytosis and the killing of *Escherichia coli* and *Staphylococcus aureus* by macrophages. In line with these findings, HDACi decrease the expression of phagocytic receptors and inhibit bacteria-induced production of reactive oxygen and nitrogen species by macrophages. Consistently, HDACi impair the expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits and inducible nitric oxide synthase. These data indicate that HDACi have a strong impact on critical antimicrobial defense mechanisms in macrophages.

The innate immune system plays a crucial role in host defenses against invasive microorganisms. Professional phagocytes are key sentinel cells of the innate immune system. Pathogen recognition relies on the capacity of phagocytes to sense microbial molecular motifs (eg, lipopolysaccharide, peptidoglycan, lipopeptides, mannans, glucans, flagellin, and nucleic acids) via pattern-recognition receptors comprising Toll-like receptors (TLRs), nucleotide-binding oligomerization domainlike receptors (NLRs), retinoic acid–inducible gene I (RIG-I)–like receptors (RLRs), C-type lectin receptors (CLRs), and scavenger receptors [1]. The engagement of phagocytic receptors, either through a direct interaction with microbial motifs or through the recognition of opsonized infectious agents, stimulates the engulfment and the delivery of the pathogen to the phagosome. Phagosome maturation by fission and fusion with endosomes and lysosomes generates the phagolysosome that provides a powerful microbicidal microenvironment, usually resulting in efficient microbial killing [2, 3]. The release of proinflammatory cytokines during an infection stimulates the production of powerful phagocyte activating molecules like interferon γ (IFNγ).

Reversible acetylation of the ε amino groups of lysine residues from histones and nonhistone proteins (such as α-tubulin, steroid receptors, HSP90, and regulators of nuclear import and transcription) is controlled by histone acetyltransferases and histone deacetylases (HDACs). Generally, acetylated histones are associated with active gene transcription, whereas deacetylated histones are associated with transcription repression [4–6]. The 18 mammal HDACs have been classified into class I (HDAC1–3 and 8), class IIa (HDAC4, 5, 7 and 9), class IIb (HDAC6 and 10), class III (SIRT1–7), and class IV (HDAC11) HDACs [4, 7]. Small-molecule inhibitors of class I, II, and IV HDACs were originally identified

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for their potential to induce cellular differentiation, growth arrest, and apoptosis of transformed cells. HDACi targeting class I and II HDACs have been reported to counteract cancer development by reducing tumor angiogenesis, metastasis, and invasion and antitumor immunity [4–6].

Besides their anticancer properties, HDACi exert immunomodulatory activities that have been exploited for the treatment of inflammatory and autoimmune disease [8]. Recently, we reported that HDACi interfere with the response of innate immune cells stimulated with TLR agonists and increase the mortality of mice to microbial sepsis [9]. Yet, whether HDACi impair the phagocytosis and the killing of bacteria by phagocytes remains unknown. To more deeply characterize the influence of HDACi on innate immune responses, we investigated whether HDACi have an impact on key antibacterial defense mechanisms of macrophages. We report that HDACi reduce the expression of phagocytic and opsonophagocytic receptors and inhibit the phagocytosis of *Escherichia coli* and *Staphylococcus aureus*, 2 of the most common infectious agents, by macrophages. Moreover, HDACi impair the generation of reactive oxygen and nitrogen species by macrophages infected with bacteria, resulting in a marked reduction of bacterial killing.

MATERIALS AND METHODS

Cells and Reagents

Animal procedures were approved by the Office Vétérinaire du Canton de Vaud (authorizations n° 876.6) and performed according to institution guidelines for animal experiments. We purchased 8- to 10-week-old female BALB/c mice from Charles River Laboratories . We obtained mouse bone marrow–derived macrophages (BMDMs) and thioglycolate-elicited macrophages as previously described [10, 11]. RAW 264.7 macrophages were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 2 mmol/L glutamine and 10% fetal calf serum (FCS) [12]. *E. coli* O18:K1:H7 (*E. coli*) and *S. aureus* AW7 (*S. aureus*) are clinical isolates obtained from septic patients hospitalized at the Centre Hospitalier Universitaire Vaudois. We purchased trichostatin A (TSA) and valproic acid (VPA) from Sigma-Aldrich, *Salmonella minnesota* Ultra Pure lipopolysaccharide (LPS) from List Biologicals Laboratories , and IFNγ from R&D Systems. The concentrations of TSA (dissolved in ethanol) and VPA (dissolved in phosphate-buffered saline [PBS]) used in this study were selected based on previous publications [13–18] and did not affect the viability (Trypan blue staining and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide [MTT] Cell Proliferation and Viability Assay) of BMDMs (≥ 85% cell recovery after 18 hours of culture with 20–40 nmol/L TSA and 1–2 mmol/L VPA with or without bacteria; n = 6–9 determinations; $P > .5$ for all conditions). Ethanol and PBS vehicle controls were performed in each experiment. For simplicity only 1 set of data is presented in each (Figures 2, 4, 5, and 6).

Assay for Bacterial Uptake and Bacterial Killing

E. coli and *S. aureus* were grown overnight at 37°C in tryptic soy broth (BD Biosciences), washed in PBS, and adjusted to 10^7 colony-forming units (CFU)/mL in RPMI medium containing 10% FCS. BMDMs (4×10^5 cells in 24-well cell-culture plates, Costar) were treated with TSA or VPA for 18 hours. Medium was changed and cells were incubated for 1 hour with bacteria at a multiplicity of infection of 20 bacteria per macrophage. Nonadherent bacteria were removed by washing with PBS. Extracellular bacteria were killed by a 30-minute exposure to either 100 μg/mL of gentamicin (*E. coli*) or 10 μg/mL of lysostaphin (*S. aureus*). We washed and lysed BMDMs. We plated serial dilutions of cell lysates on agar plates and enumerated colonies to calculate the number of phagocytosed bacteria. In parallel wells, BMDMs were treated as previously except that, after 30 minutes of incubation with antibiotics, cells were washed and incubated for a further 24 hours in culture medium containing 20 μg/mL gentamicin or 10 μg/mL lysostaphin. Bacteria were enumerated and results expressed as percent changes in bacterial counts using the following formula: (count after 24 hours/count after 1 hour) × 100. Of note, neither TSA nor VPA at the concentrations used in these assays were toxic for bacteria.

RNA Analysis by Quantitative Real-Time Polymerase Chain Reaction

RNA was isolated using the RNeasy kit (Qiagen). Reverse transcription was performed using the ImProm II RT System kit (Promega). Quantitative real-time polymerase chain reaction (PCR) was performed with a 7500 Fast Real-Time PCR System using the Power SYBR Green PCR Master Mix (Applied Biosystems) and primer pairs (Supplementary Table 1) as previously described [19]. We tested samples in triplicate. For each measurement, we processed in parallel a standard made of successive dilutions of a reference complementary DNA. The relative expression levels of NADPH oxidase subunits and inducible nitric oxide synthase (iNOS) were reported to the relative expression of glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and expressed in arbitrary units (AU). The expression of phagocytic receptors and TLRs was calculated with the comparative Ct (ΔΔCt) method . The expression of the target gene was first normalized to the endogenous control (Gapdh) and then to that of a calibrator (ie, data obtained from cells cultured with vehicle set at 1). Importantly, the Ct values of Gapdh were not affected by TSA or VPA (19.24 ± 0.13, 19.10 ± 0.03, 19.30 ± 0.48, 19.21 ± 0.06, and 19.10 ± 0.15 in BMDMs cultured for 18 hours with medium, 20 nmol/L TSA, 40 nmol/L TSA, 1 mmol/L VPA, and 2 mmol/L VPA, respectively; $n = 6$ determinations; $P > .05$ for all conditions compared with cells cultured in medium). In selected experiments, results were validated using *Hprt* as an endogenous control.

Flow Cytometric Analysis

BMDMs cultured for 18 hours with TSA (40 nmol/L) and VPA (2 mmol/L) were incubated 30 minutes at 4°C in PBS containing 5% FCS, 5 mM ethylenediaminetetraacetic acid (EDTA), 2.4G2 monoclonal antibody, and monoclonal antibodies specific for macrophage scavenger receptor 1 (Msr1/CD204), CD11c, CD14, and major histocompatibility complex II (MHC-II) [20]. Acquisition and analysis were performed using a FACSCalibur (BD Biosciences) and FlowJo 8.5.3 software (FlowJow).

Analysis of Oxidative Burst Using the Dichlorofluorescein Diacetate Fluorescence Assay

BMDMs (4×10^5 cells in 24-well cell-culture plates) were cultured as previously described [21] and incubated for 18 hours with TSA and VPA. Dichlorofluorescein diacetate (DCFDA) (20 μmol/L, Sigma-Aldrich) was added to the cultures followed 15 minutes later by bacteria (5×10^8 CFU/mL). After 30 minutes, cell fluorescence was measured by flow cytometry.

Western Blot Analysis

Cell lysates were electrophoresed through polyacrylamide gels and transferred onto nitrocellulose membranes as previously described [12]. Membranes were incubated with antibodies directed against iNOS (BD Biosciences), $p47^{phox}$ (Santa Cruz) and tubulin (Sigma). After washing, membranes were incubated with *horseradish peroxidase* (HRP)–conjugated secondary antibody (Pierce). Signals were revealed using the ECL Western blotting analysis system (GE Healthcare).

Nitrite/Nitrate Measurements

BMDMs (10^5 cells in 96-well cell-culture plates) were preincubated for 1 hour with TSA and VPA and stimulated with LPS (100 ng/mL), IFNγ (100 U/mL), *E. coli* (10^8 CFU/mL), and *S. aureus* (10^8 CFU/mL). Cell-culture supernatants were collected after 24 hours. The concentrations of nitrite/nitrate were measured using the Griess reagent.

Statistics

Statistical analyses were performed using PRISM (GraphPad Software). Comparisons between the different groups were performed by analysis of variance and appropriate post hoc analyses. *P* values are 2-sided, and values of $< .05$ were considered to indicate statistical significance.

RESULTS

HDACi Inhibit Bacterial Phagocytosis by Macrophages

We recently reported that HDACi impair host defenses to bacterial infection in vivo [9]. Whether HDACi impact the phagocytosis and the killing of bacteria is currently unknown. To fill this gap, we first analyzed the phagocytosis of gram-negative (*E. coli*) and gram-positive (*S. aureus*) bacteria by BMDMs pretreated with 2 chemically unrelated HDACi: TSA, a hydroxamate widely used as a prototypical broad-spectrum HDACi, and VPA, a clinically relevant short fatty acid. The dose and duration of treatment with TSA and VPA were in the range of those used in cancer preclinical studies or measured in patients enrolled in cancer clinical trials (VPA) (see for example [13–18]). We evaluated phagocytosis after 1 hour of contact between bacteria and macrophages. As shown in Figure 1, HDACi dose dependently reduced the number of *E. coli* (2- to 4-fold; $P < .05$) and *S. aureus* (1.5- to 2-fold; $P < .05$) phagocytosed by BMDMs.

HDACi Impair the Expression of Phagocytic Receptors

Macrophages express phagocytic scavenger receptors, including macrophage scavenger receptor 1 (Msr1/SR-AI/CD204), CD14, CD36, and C-type lectins such as Dectin-1 (encoded by *Clec7a*) that mediate the recognition of microbial ligands expressed at the surface of pathogens and initiate phagocytosis. Macrophages also express opsonic phagocytic receptors of the integrin family ($integrin_{\alpha X}$/Itgax/CD11c, $integrin_{\beta 2}$/Itgb2/CD18, and $integrin_{\alpha 5}$/Itga5/CD49e) that facilitate the uptake of microorganisms coated with opsonins like the mannose-binding lectin, complement subcomponents, growth arrest specific 6, ficolins, and pentraxins [2, 22]. TSA and VPA reduced 2- to 10-fold Msr1,

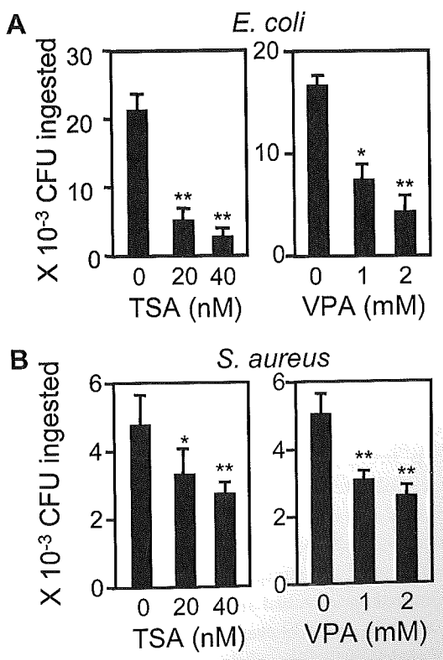

Figure 1. Histone deacetylase inhibitors impair the phagocytosis of *Escherichia coli* (*A*) and *Staphylococcus aureus* (*B*). Mouse bone marrow–derived macrophages (BMDMs) were incubated for 18 hours with increasing concentrations of trichostatin A (TSA) and valproic acid (VPA) before the addition of 10^7 colony-forming units (CFU) of *E. coli* or 1.5×10^7 CFU of *S. aureus*. The number of bacteria ingested by BMDMs was determined 1 hour later. Data are presented as mean ± standard deviation (SD) of quadruplicate samples from 1 experiment representative of 2–3 experiments. *, $.05 < P < .005$; **, $P < .005$.

CD14, Dectin-1 and Itgax messenger RNA (mRNA) levels in BMDMs (Figure 2*A*). TSA inhibited Itgb2 expression more efficiently than did VPA (2.4-fold with 40 nM TSA vs 1.4-fold with 2 mM VPA), whereas HDACi did not affect CD36, Itga5, or Itga6 expression. Flow cytometry analyses confirmed that TSA and VPA inhibited the expression of Msr1, CD11c and CD14 by BMDMs (Figure 2*B*). As a control of nonspecific broad inhibitory effects of HDACi, MHC-II expression was not affected by HDACi. All together, these data suggest that reduced expression of phagocytic receptors may contribute to impair the phagocytosis of *E. coli* and *S. aureus* in macrophages treated with HDACi.

HDACi Inhibit Bacterial Killing

TLRs play crucial roles in the sensing of invasive microorganisms and in transmitting signals involved in the maturation of phagosomes [23]. Interestingly, we observed that HDACi strongly reduced baseline expression of TLR1–7 and TLR9 in BMDMs (Figure 2*C*). Pathogen delivery to phagolysosomes usually results in effective microbial killing [2, 24]. In agreement, < 5% of *E. coli* and 25% of *S. aureus* phagocytosed by BMDMs were recovered 24 hours later in macrophages ($P < .001$; Figure 3). TSA and VPA reduced 5-fold and 3-fold *E. coli* and *S. aureus* killing, respectively (ie, increasing bacteria recovery to 25% and 75% of the ingested inoculum; $P < .05$). Thus, HDACi inhibit both the phagocytosis (Figure 1) and the killing (Figure 3) of bacteria by macrophages, in agreement with the observation that HDACi increased the susceptibility of mice to microbial infection [9].

HDACi Interfere With the Generation of Reactive Oxygen Species

In response to microbial challenge, macrophages produce highly toxic reactive oxygen species (ROS) that contribute to pathogen destruction [25]. The generation of ROS in BMDMs was analyzed by flow cytometry using the cell permeable nonfluorescent dye 2′,7′-dichlorofluorescein diacetate (DCFDA) that is transformed on oxidation into the highly fluorescent DCF. *E. coli* and *S. aureus* strongly increased DCF fluorescence in BMDMs, which was inhibited 2- to 3-fold by TSA or VPA (Figure 4*A* and 4*B*). HDACi also inhibited the production of ROS in BMDMs stimulated with phorbol myristate acetate (>10-fold reduction, data not shown) indicating that HDACi inhibit the oxidative burst induced by microbial and nonmicrobial stimuli.

In macrophages, ROS are generated during the respiratory burst through the action of the phagocytic NADPH oxidase, an enzymatic complex composed of 2 membrane-associated subunits (gp91^{phox}/NOX2 and p22^{phox}), 3 cytosolic subunits (p47^{phox}, p40^{phox}, and p67^{phox}), and the Rac2 regulatory subunit [26, 27]. Cytokines, particularly IFNγ, and microbial products released during an infection prime and amplify macrophage respiratory burst through the induction of NADPH oxidase subunits [21]. Real-time PCR and Western blot analyses revealed that TSA and VPA dose-dependently inhibited baseline

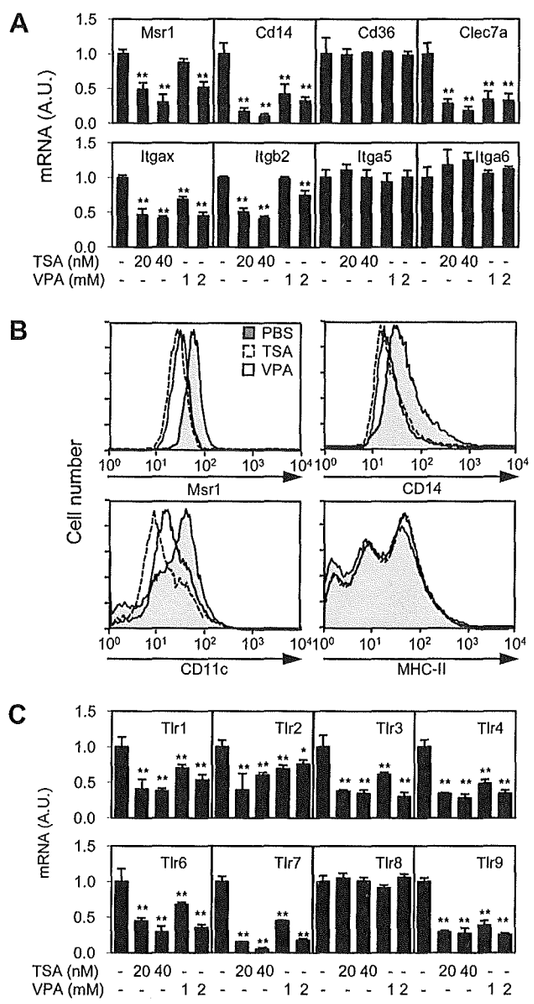

Figure 2. Histone deacetylase inhibitors inhibit the expression of phagocytic and Toll-like receptors. *A*, Real-time polymerase chain reaction (PCR) analysis of Msr1, CD14, CD36, Clec7a, Itgax, Itgb2, Itga5, and Itga6 messenger RNA expression in mouse bone marrow–derived macrophages (BMDMs) incubated for 8 hours with increasing concentrations of trichostatin A (TSA) and valproic acid (VPA). Data are presented as mean ± standard deviation (SD) of triplicate samples from 1 experiment and are representative of 2 independent experiments. *B*, Flow cytometry analysis of Msr1, CD14, CD11c, and major histocompatibility complex II (MHC-II) expression by BMDMs incubated for 18 hours with medium (*gray area*), TSA (*dashed line*), and VPA (*solid line*). Results are representative of 2 independent experiments. *C*, Real-time PCR analysis of Toll-like receptors in BMDMs incubated for 8 hours with increasing concentrations of TSA and VPA. Data are presented as mean ± standard deviation (SD) of triplicate samples from 1 experiment and are representative of 2 independent experiments. AU, arbitrary units; *, $.05 < P < .005$; **, $P < .005$.

expression of NADPH oxidase subunits and potently inhibited the upregulation of the catalytic gp91^{phox} and regulatory p47^{phox}

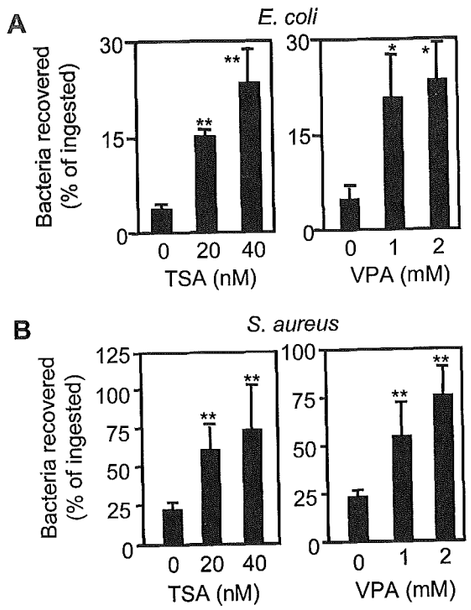

Figure 3. Histone deacetylase inhibitors impair the killing of *Escherichia coli* and *Staphylococcus aureus*. Mouse bone marrow–derived macrophages (BMDMs) were cultured for 18 hours with increasing concentrations of trichostatin A (TSA) and valproic acid (VPA) before the addition of 10^7 colony-forming units (CFU) of *E. coli* (*A*) or 1.5×10^7 CFU of *S. aureus* (*B*). The number of bacteria recovered from macrophages after 24 hours was divided by the number of bacteria recovered after 1 hour and expressed in percentage using the formula (count after 24 hours/count after 1 hour) × 100. Data are presented as mean ± standard deviation (SD) of quadruplicate samples from 1 experiment representative of 2–3 experiments. *, $.05 < P < .005$; **, $P < .005$.

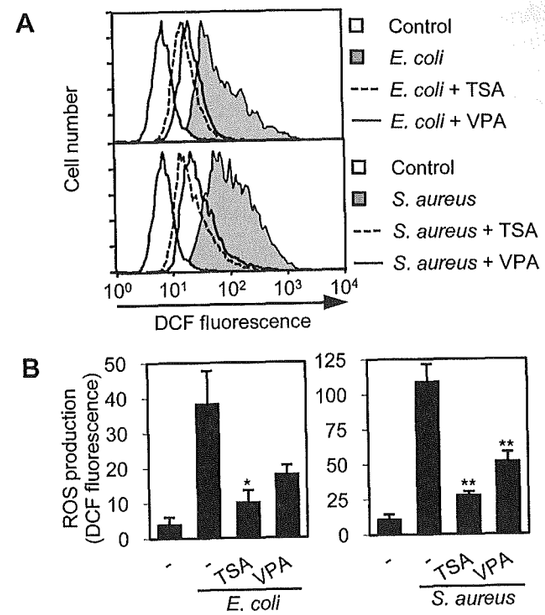

Figure 4. Histone deacetylase inhibitors interfere with the generation of reactive oxygen species. Mouse bone marrow–derived macrophages (BMDMs) were incubated with trichostatin A (TSA; 40 nmol/L) and valproic acid (VPA; 2 mmol/L) and exposed to *Escherichia coli* or *Staphylococcus aureus* as described in Material and Methods. *A*, The generation of reactive oxygen species (ROS) was quantified by flow cytometry by measuring dichlorofluorescein (DCF) diacetate oxidation into fluorescent DCF. *B*, Data are means ± standard deviation (SD) of 2 independent determinations. $P = .03$ and .008 for *E. coli* and *S. aureus* vs control. *, $P = .05$; **, $.05 < P < .005$ vs *E. coli*– and *S. aureus*–treated cells.

subunits in LPS+IFNγ–stimulated macrophages (Figure 5). All together, these data provide compelling evidence that HDACi inhibit ROS production in macrophages.

HDACi Inhibit Nitric Oxide Production and *iNos* Gene Expression

Nitric oxide (NO) is produced during the nitrosative burst by iNOS and represents an important antimicrobial effector mechanism [25, 27]. TSA and VPA dose-dependently inhibited the production of NO by BMDMs stimulated with LPS+IFNγ (50%–80% reduction; $P < .05$), in line with previous work showing that HDACi inhibit cytokine-induced NO release [28, 29]. More relevant for microbial infection, TSA and to a lesser extent VPA inhibited NO production induced by *E. coli* and *S. aureus* (50%–60% reduction using 20 nmol/L and 40 nmol/L TSA and 30%–35% reduction using 2 mmol/L VPA; $P < .05$) (Figure 6*A*). In agreement with these findings, real-time PCR and Western blot analyses demonstrated that TSA and VPA inhibited iNOS mRNA and protein expression in BMDMs (Figure 6*B* and 6*C*). Similar results were obtained using thioglycolate-elicited peritoneal and RAW 264.7 macrophages (data not shown). Taken together, these results suggest that HDACi impair NO production by BMDMs in response to bacterial challenge by interfering with iNOS expression.

DISCUSSION

In this study, we report for the first time to our knowledge that HDACi inhibit the phagocytosis and the killing of bacteria, the expression of phagocytic receptors, and the generation of oxidative and nitrosative bursts induced by bacteria in macrophages. These data extend our previous work demonstrating that HDACi interfere with cytokine production by macrophages and impair host defenses to bacterial infection [9].

The inhibition of *E. coli* and *S. aureus* phagocytosis by HDACi was associated with a reduced expression of phagocytic receptors, among which is Msr1 (scavenger receptor A1). Msr1 binds a wide range of microbial ligands and mediates

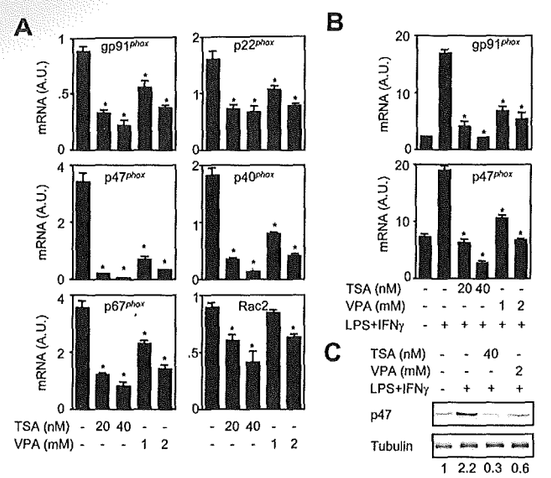

Figure 5. Histone deacetylase inhibitors inhibit nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits expression. *A*, Real-time polymerase chain reaction (PCR) analysis of gp91, p22, p47, p67, p40, and Rac2 messenger RNA expression in mouse bone marrow–derived macrophages (BMDMs) cultured for 8 hours with trichostatin A (TSA) and valproic acid (VPA). Results are expressed as the ratio of the gene of interest to that of glyceraldehyde-3-phosphate dehydrogenase. Real-time PCR (*B*) and Western blot (*C*) analyses of gp91 and p47 expression in BMDMs cultured for 1 hour with TSA and VPA and then stimulated for 6 hours with lipopolysaccharide (LPS; 100 ng/mL) + interferon γ (IFNγ; 100 U/mL). Data are presented as means ± standard deviation (SD) of triplicate samples from 1 experiment and are representative of 2 independent experiments (*A, B*). Abbreviation: AU, arbitary units.*, $P < .005$ vs control (*A*) and LPS+IFNγ (*B*).

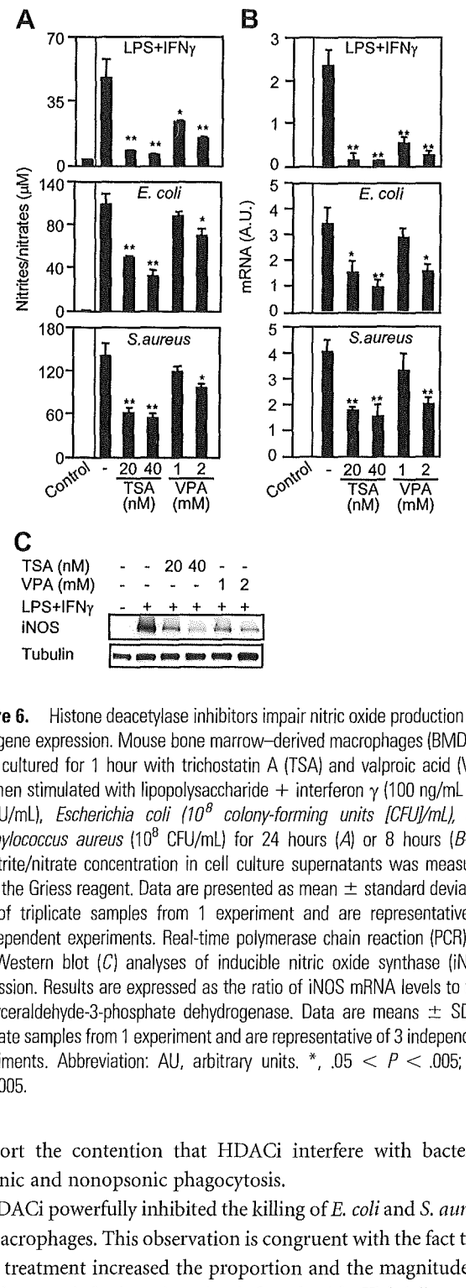

Figure 6. Histone deacetylase inhibitors impair nitric oxide production and *iNos* gene expression. Mouse bone marrow–derived macrophages (BMDMs) were cultured for 1 hour with trichostatin A (TSA) and valproic acid (VPA) and then stimulated with lipopolysaccharide + interferon γ (100 ng/mL and 100 U/mL), *Escherichia coli* (10^8 *colony-forming units [CFU]/mL*), and *Staphylococcus aureus* (10^8 CFU/mL) for 24 hours (*A*) or 8 hours (*B–C*). *A*, Nitrite/nitrate concentration in cell culture supernatants was measured using the Griess reagent. Data are presented as mean ± standard deviation (SD) of triplicate samples from 1 experiment and are representative of 4 independent experiments. Real-time polymerase chain reaction (PCR) (*B*) and Western blot (*C*) analyses of inducible nitric oxide synthase (iNOS) expression. Results are expressed as the ratio of iNOS mRNA levels to that of glyceraldehyde-3-phosphate dehydrogenase. Data are means ± SD of triplicate samples from 1 experiment and are representative of 3 independent experiments. Abbreviation: AU, arbitrary units. *, $.05 < P < .005$; **, $P < .005$.

nonopsonic phagocytosis of *E. coli* and *S. aureus* [30]. Moreover, *Msr1*$^{-/-}$ mice are more susceptible than wild-type mice to *S. aureus* infection [31]. Thus, HDACi-mediated inhibition of Msr1 expression may well help impair bacterial phagocytosis, although HDACi may target other phagocytic receptors such as the mannose receptor, MARCO, or CD14. Of note, HDACi decreased Dectin-1 expression in BMDMs. Considering that Dectin-1 is a major receptor involved in the recognition of β-glucan, we speculate that HDACi may affect the phagocytosis of yeast. In line with this hypothesis, we observed that VPA increases mortality of mice infected with *Candida albicans* [9].

The αx and β2 integrin subunits contribute to the structure of complement receptor (CR) 3 and CR4 that mediate the recognition of opsonized microorganisms by phagocytes. β2 integrins play an important role in antimicrobial defenses as suggested by the observation that patients with leukocyte adhesion deficiency type I (LADI) syndrome (ie, patients deficient in functional β2 integrin) have defects in phagocytosis and are prone to bacterial infections [32]. All together, inhibition of the expression of αx and β2 integrins and scavenger and lectin receptors by HDACi support the contention that HDACi interfere with bacterial opsonic and nonopsonic phagocytosis.

HDACi powerfully inhibited the killing of *E. coli* and *S. aureus* by macrophages. This observation is congruent with the fact that VPA treatment increased the proportion and the magnitude of bloodstream infections in mice infected with *Klebsiella pneumoniae* [9]. Reactive oxygen and nitrogen species are among the most deleterious components produced by phagocytes and implicated in the destruction of microorganisms [2, 24]. Deficiency in members of the NADPH oxidase complex or in iNOS, which control the generation of superoxide ($O_2^{-\circ}$) and nitric oxide (NO°) radicals, impair the killing of *E. coli* and *S. aureus* by innate immune cells and compromise mouse survival [33–36]. Moreover, germ-line mutation in 1 of the components of NADPH oxidase complex results in chronic granulomatous disease characterized by recurrent bacterial and fungal infections and reduced life expectancy [25]. Inhibition of NADPH oxidase subunit and iNOS expression and of reactive oxygen and nitrogen species generation by HDACi likely represents an effective mechanism by which these drugs impair the killing of bacteria.

In agreement with the notion that phagocytosis is coupled with a proinflammatory cytokine response and with the observation that HDACi inhibit TLR expression, HDACi strongly impaired the secretion of cytokines and chemokines (TNF, IL-6, IL-12p40, and MIP-2α, data not shown) by macrophages infected with *E. coli* and *S. aureus*. These data expand on recent studies showing that HDACi inhibit cytokine production induced by cytokines and purified microbial products in innate immune cells [14, 15, 28, 37, 38]. Considering that proinflammatory mediators released during the course of an infection coordinate the development of innate and adaptive immunity, one may expect that HDACi interfere with the generation of pathogen-specific adaptive immune response.

HDACi have been reported to interfere with signaling pathways controlling the expression of genes particularly relevant for this study. Indeed, HDACi down-regulate the expression of PU.1 transcription factor [37, 39], which regulates constitutive expression of HDACi-target genes encoding for integrins, scavenger receptors, TLR4, CD14, and p40, p47, and p67 NADPH oxidase subunits [20, 40]. Moreover, HDACi have been reported to interfere with the activation of mitogen-activated protein kinases, NF-κB, and AP-1, which control inflammatory and antimicrobial host responses [15, 41]. HDACi also impair gene expression through chromatin modifications or aceytlation-dependent recruitment of transcriptional repressors, albeit these mechanisms are less well characterized. For example, TSA inhibits the expression of the proinflammatory cytokine macrophage migration inhibitory factor through a local deacetylation of migration inhibitory factor-promoter–associated histones impairing the recruitment of the basal transcriptional machinery [42, 43]. Finally, we have recently shown that TSA inhibits macrophage response to LPS stimulation by inducing the expression of Mi-2β and the activity of the Mi-2/NuRD transcriptional repressor complex [9].

HDACi have been used to treat inflammatory diseases in mouse models [8]. Abundant preclinical and clinical studies indicate that interfering with critical mediators of innate or adaptive immunity increases the risk of infections. Thus, one may question whether HDACi might affect natural host defenses in patients, as could be anticipated from the powerful immunomodulatory and anti-inflammatory activities of HDACi in vivo [8] and the increased susceptibility to bacterial and fungal infections of mice treated with HDACi [9]. Patients treated with HDACi (suberoylanilide hydroxamic acid, MS-275, valproate, and ITF2357) in phase I and II clinical trials have developed episodes of severe infection even in the absence of treatment-induced neutropenia [44–49], suggesting the need to monitor the immune status and susceptibility to infection of patients treated with HDACi, especially immunosuppressed cancer patients [4–6, 50].

In summary, this study demonstrates that HDACi impair the capacity of macrophages to ingest and destroy gram-positive and gram-negative bacteria. The fact that HDACi impede the expression of phagocytic receptors, the generation of oxygen- and nitrogen-reactive species, and the release of proinflammatory cytokines provide a plausible mechanism whereby HDACi negatively impact critical antimicrobial functions of innate immune cells and increase the susceptibility of mice to bacterial and fungal infection [9].

Supplementary Data

Supplementary data are available at The Journal of infections Diseases online (http://www.oxfordjournals.org/our_journals/jid/).

Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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