The Histone Deacetylase Inhibitor ITF2357 Reduces Production of Pro-Inflammatory Cytokines In Vitro and Systemic Inflammation In Vivo

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We studied inhibition of histone deacetylases (HDACs), which results in the unraveling of chromatin, facilitating increased gene expression. ITF2357, an orally active, synthetic inhibitor of HDACs, was evaluated as an anti-inflammatory agent. In lipopolysaccharide (LPS)-stimulated cultured human peripheral blood mononuclear cells (PBMCs), ITF2357 reduced by 50% the release of tumor necrosis factor-α (TNFα) at 10 to 22 nM, the release of intracellular interleukin (IL)-1α at 12 nM, the secretion of IL-1β at 12.5 to 25 nM, and the production of interferon-γ (IFNγ) at 25 nM. There was no reduction in IL-8 in these same cultures. Using the combination of IL-12 plus IL-18, IFNγ and IL-6 production was reduced by 50% at 12.5 to 25 nM, independent of decreased IL-1 or TNFα. There was no evidence of cell death in LPS-stimulated PBMCs at 100 nM ITF2357, using assays for DNA degradation, annexin V, and caspase-3/7. By Northern blotting of PBMCs, there was a 50% to 90% reduction in LPS-induced steady-state levels of TNFα and IFNγ mRNA but no effect on IL-1β or IL-8 levels. Real-time PCR confirmed the reduction in TNFα RNA by ITF2357. Oral administration of 1.0 to 10 mg/kg ITF2357 to mice reduced LPS-induced serum TNFα and IFNγ by more than 50%. Anti-CD3–induced cytokines were not suppressed by ITF2357 in PBMCs either in vitro or in the circulation in mice. In concanavalin-A–induced hepatitis, 1 or 5 mg/kg of oral ITF2357 significantly reduced liver damage. Thus, low, nonapoptotic concentrations of the HDAC inhibitor ITF2357 reduce pro-inflammatory cytokine production in primary cells in vitro and exhibit anti-inflammatory effects in vivo.

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INTRODUCTION

Several enzymes termed histone deacetylases (HDACs) comprise nuclear repressor complexes that, once recruited to specific sites of euchromatin, maintain nucleosome histones in a state of deacetylation so that DNA remains tightly bound and inaccessible to transcription factors for gene expression. In contrast, inhibition of HDACs results in hyperacetylation of these histones, which allows for the unraveling of DNA sufficient for the binding of transcription factors and the synthesis of mRNA. In general, inhibitors of HDACs result in a nonspecific increase in gene expression, although decreases in some genes has been reported. HDACs also deacetylate nonhistone substrates in the cytosol. At present, more then 40 transcription factors and 30 other proteins are known to be acetylated at critical lysine residues, and their function is thereby regulated (1). Agents that inhibit HDAC activity vary in structure, but few examples of selective inhibitors of specific HDACs are reported in the literature (2).

Investigations of inhibitors of HDACs have focused on increased expression of cell death genes in malignant cells (3). Most tumor cells survive and proliferate by suppressing proapoptotic genes, and this mechanism is thought to contribute to resistance of tumors to cell death. Thus, in malignant cells, inhibition of HDACs often increases the expression of genes, resulting in apoptosis and terminal differentiation (3). In vitro, the concentrations of HDAC inhibitors required for inducing apoptotic cell death are in the micromolar or higher range; in animals, similar concentrations are needed for tumor regression. Nevertheless, HDAC-inhibiting agents, such as butyrate and valproic acid, have been given to humans for several years in large doses with considerable safety. Presently, several inhibitors of HDACs are in clinical trials for the treatment of cancer (4,5). Agents inhibiting HDACs generally lack organ toxicity and spare the bone marrow and gastrointestinal tract from antiproliferative properties (6).

Agents such as trichostatin A (TSA) or suberoylanilide hydroxamic acid (SAHA) induce differentiation and/or apoptosis of transformed cells in vitro and inhibit tumor growth in vivo (4). Treatment of tumor cells in vitro with SAHA causes an accumulation of acetylated histones H3 and H4 in total cellular chromatin by 2 h, which is maintained through 24 h of culture. X-ray crystallographic studies reveal a catalytic site of HDAC with a zinc atom at its pocket; the hydroxamic moiety of HDAC inhibitors, such as SAHA and TSA, binds to the deacetylase and, by chelating the zinc atom, inhibits the enzymatic property of HDACs (7).

An unexpected and seemingly paradoxical property of HDAC inhibition is the reduction in inflammation in models of autoimmune and inflammatory diseases (8-13). In most of these models, inhibition of HDACs is associated with a decrease in cytokines and a decrease in disease severity. In health, the expression of genes coding for many proinflammatory cytokines

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remains silent. Mechanisms of transcriptional repression dominate until overcome by stimulation from extracellular stress signals such as microbial products via the Toll-like receptors, cytokines themselves via their respective receptors, or immunological activation via the T-cell receptor. Although several transcription factors initiate de novo gene expression of proinflammatory cytokines, synthesis and secretion are tightly controlled events. For example, there are 3 pro-inflammatory cytokines, interleukin (IL)-1β (14), IL-18 (15,16), and (most recently) IL-33 (17), each of which are synthesized as inactive precursors and require caspase-1 for cleavage and secretion of the active cytokine. Therefore, any mechanism for cytokine production is a potential target for acetylation by HDAC inhibitors.

Because specific blockade of cytokines such as tumor necrosis factor α (TNFα), IL-1β, IL-6, or IL-12 is effective in diseases such as rheumatoid arthritis (18-20), Crohn's disease (21), psoriasis (22), ankylosing spondylitis (23), and mutations in the NALP3 gene (24-27), novel therapeutic strategies to target these cytokines is an expanding field of research and clinical application. In addition, reducing the production and activity of more than one cytokine may be an advantage because disease-associated cytokines often act synergistically.

Several studies in vitro and in vivo support the concept that inhibition of HDACs is a strategy to reduce the production and activity of proinflammatory cytokines. We have described the anti-inflammatory activities of the HDAC inhibitor SAHA (8). Other studies have increased the scope of the anti-inflammatory activity of HDAC inhibitors. For example, in the *lpr/lpr* mouse, which spontaneously develops lethal nephritis similar to that of lupus erythematosus, administration of the HDAC inhibitor TSA reduced the severity of the disease (9). In a similar study, daily treatment with SAHA resulted in decreased proteinuria, reduced cytokine and nitric oxide production, and reduced damage to the renal tissues (13). In mice with graft-versus-host disease (GvHD), SAHA treatment reduces mortality, cytokine production, and histological markers of disease severity without impairing graft-versus-tumor responses (28). In the present study, we report that an orally active, synthetic HDAC inhibitor, ITF2357, possesses anti-inflammatory activities in vivo and in vitro.

MATERIALS AND METHODS

Materials

Similar to SAHA, ITF2357 is a HDAC inhibitor containing a hydroxamic acid moiety linked to an aromatic ring as described in patent WO 97/43251, US 6034096. The compound was synthesized at Italfarmaco (Cinisello Balsamo, Italy), and its purity was confirmed by high-performance liquid chromatography (HPLC). SAHA, used as an anti-inflammatory agent as described in WO 03/013493, was also synthesized and its purity ascertained by HPLC. One milligram of ITF2357 was added to 1 mL water and heated to 90° C until dissolved. Kept at room temperature, ITF2357 in water is stable and active for several months. One milligram of SAHA was dissolved in 50 µL DMSO and then diluted in warm water to 1 mg/mL. ConA was purchased from Sigma Chemicals (St. Louis, MO, USA); measurement of alanine aminotransferase (ALT) was made using colorimetric kit (Sigma). Lipopolysaccharide (LPS) *Escherichia coli* (055:B5) and salmon sperm DNA were from Sigma (St. Louis, MO, USA or Milan). Monoclonal antibody OKT3 was purchased from Ortho Diagnostic. [^{3}H]thymidine and [α-^{32}P]dCTP were from Amersham (London, UK) and the nick translation kit from Promega (Madison, WI, USA). Human recombinant IL-18, IL-1 receptor antagonist (IL-1Ra), and TNF binding protein (TNF-bp) were purchased from R&D Systems (Minneapolis, MN, USA). Human IL-12 was provided by Peprotech (Rocky Hill, NJ, USA).

Maize HDAC Assays

HD2, HD-1B, and HD-1A from maize and used to assess the histone deacetylase activity of ITF2357 as described in Koelle et al. (29).

Cellular Crude Extract for Total HDAC Activity and Protein Acetylation Determinations

Human peripheral blood mononuclear cells (PBMCs) (see below) were added to 50-mL conical tubes at a concentration of 2.5×10^6 cell/mL in RPMI 1640 medium with 1% FCS and 0.05% DMSO (vol/vol) and incubated at 37° C with the test compounds (constituted in DMSO 0.05%) at the stated concentrations. After 60 min, LPS was added at final concentration of 10 ng/mL and the cells were incubated at 37° C. At the end of incubation times, the cells were centrifuged at 400*g* for 15 min, the supernatant was collected and stored at –80° C until TNFα determination, and the cells were washed twice with ice-cold phosphate buffer.

Crude extracts were obtained by suspending the pellet in 200 µL modified lysis buffer (50 mM Tris HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF) together with a cocktail of protease inhibitors available as tablets (Roche, Italy) for 30 min at 4° C. The cells were disrupted by sonication, after which the extract was clarified by centrifugation at 14,000 rpm for 10 min at 4° C. The supernatant was used for determination of total HDAC activity and protein acetylation. Protein content of the extract was determined using the BCA protein assay kit (Pierce, Italy).

Total HDAC Activity Assay

The assay was adapted based on the release of tritiated acetyl residues from a peptide substrate intrinsically labeled with [^{3}H]acetic acid, as described previously (29). The synthetic peptide used in this assay was the N-terminal sequence (SGRGKGGKGLGKGGAKRHRC) of histone H4 (Histone Deacetylase Assay Kit, Upstate Biotechnology, Albany, NY, USA). Radiolabeling of the peptide was done as follows: 100 µg peptide was added to 62.5 µL [^{3}H]acetic acid sodium salt (5.0 mCi/0.5 mL in ethanol, specific activity 5.1 Ci/mole, New England Nuclear, Boston, MA, USA). Thereafter, 5 µL BOP solution (0.24 M BOP and 0.2 M trimethylamine in acetonitrile) was added. The resulting solution was incubated overnight at room temperature with mild agitation, and the radiolabeled peptide solution was loaded onto a Microcon-SCX spin column previously rinsed with 500 µL of 10 mM HCl in methanol. The eluate was separated by centrifugation of the column (2,000*g* for 60 s). The radiolabeled peptide

was eluted with 50 µL HCl 3N in 50% isopropanol. The eluting solution containing the radiolabeled peptide was submitted to 8 cycles of organic solvent extraction (8 × 1 mL of ethylacetate) to separate the remaining free [^{3}H]acetic acid. The resulting aqueous solution was dried by centrifugation under vacuum for 30 min at room temperature and then suspended in 200 µL distilled water, separated into aliquot, and stored at –20° C.

Acetylation of Proteins

Acetylation of proteins was determined by Western blotting of crude cellular extracts. Briefly, the samples (200 µg/lane) were separated by SDS-PAGE (12.5%) and then electrically transferred onto nitrocellulose membranes. The membranes were saturated with 3% nonfat milk in phosphate buffer and incubated with anti-acetyl-lysine monoclonal antibody (Upstate Biotechnology, Albany, NY, USA) according to manufacturer's instructions. Protein bands were then detected using the chemiluminescence detection system ECL Plus (Amersham-Pharmacia Biotech, Italy) onto x-ray film.

Enzymatic Assay for HDAC Inhibitory Activity of Synthetic Compounds

The assay was performed by adding 100 µL substrate (200,000 cpm) with 40 µL buffer (50 mM Tris-HCl, pH 8.0, 750 mM NaCl, 5 mM PMSF, 50% glycerol) and 95 µL distilled water to the crude cellular extract (5 µL). Compounds for testing of HDAC inhibition (50 µL) were added. The mixture was incubated overnight at room temperature and the reaction quenched by adding 50 µL of a solution containing 259 µL HCl 37% and 28 µL acetic acid in 1 mL distilled water. The [^{3}H]acetyl residues released from the substrate were separated by organic extraction by adding 600 µL of ethyl acetate, 200 µL of the organic phase was added to standard scintillation fluid, and radioactivity was measured by a beta-counter. Inhibition of HDACs was expressed as the concentration inhibiting 50% of the control activity (by comparing the radioactivity of the samples containing inhibitors to that of the control containing cellular crude extract alone).

Northern Blot Analysis

For Northern blot hybridization, PBMCs were added to 50-mL conical tubes at a concentration of 50 million cells in 20 mL RPMI with 1% FCS and incubated at 37° C with ITF2357. After 1 h, LPS (10 ng/mL final concentration) was added, the cultures were incubated at 37° C, and at different time points, the tubes were centrifuged at 350*g*. The supernatants were removed and the cell pellets lysed in 750 µL of 4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% *N*-lauroylsarcosine with 0.1 M 2-mercaptoethanol. RNA was prepared as described (30). The methods used for Northern blot analysis have been reported previously (31). The cDNA probes for human TNFα, IL-1β, interferon γ (IFNγ), and IL-8 were obtained from ATCC (Rockville, MD, USA): for TNFα, clone pFC54.t (ATCC no. 53007); for IL-1β, a 1.047-kb fragment cloned in PBR322 (ATCC no. 39925); for IFNγ, a 1.1-kb fragment cloned in *Bam*HI site of pSV529 (ATCC no. 39046); for human β-actin, a 2-kb fragment cloned at the *Eco*RI site of pBluescript SK (ATCC no. 769559); for IL-8, a 1.2-kb fragment cloned at the *Eco*RI site of Bluescript (kindly supplied by N. Polentarutti, Mario Negri Institute, Milan, Italy).

Quantitative PCR

After incubation, cell pellets were lysed in Tri-reagent, and the RNA was sequentially isolated by chloroform extraction and isopropanol precipitation, reconstituted in RNA-free water. The concentration was determined in a GeneQuant UV spectrophotometer (Pharmacia Biotech, Cambridge, UK). To prepare cDNA, 1 to 3 µg total RNA was reverse-transcribed by using random primers at a final concentration of 5.5 mM $MgCl_2$, 50 mM KCl, and 10mM Tris-HCl (pH 8.3), 0.5 mM each of dNTPs, 20 units of RNAse inhibitor, and 50 units of MultiScribe reverse transcriptase (Perkin Elmer). The reaction mixture was incubated at room temperature for 10 min, at 37° C for 60 min, and terminated at 95° C for 5 min. Primers and probes for TNFα and 18S were obtained from Perkin Elmer (Foster City, CA, USA). A ribosomal RNA 18S target probe was added to the mixture as the internal control. TaqMan real-time quantitative PCR was performed by amplifying mixtures containing 100 nM of selected probes, 200 nM of primers, and target cDNA template. Fifty cycles at 94°C for 20 s and 62ºC for 1 min were applied using ABI PRISM 7700 (Applied Biosystems) according to the manufacturer's protocol. Data were calculated in picograms of cytokine mRNA per nanogram of 18S mRNA using a standard curve for each cytokine.

Percentage of Sub-G_1 Cells

Freshly obtained PBMCs were cultured under identical conditions with or without LPS (10 ng/mL) in the presence or absence of ITF2357 (25-100 nM) for 24 h in parallel cultures used for cytokine determinations. Cells were gently removed using a rubber policeman, washed with phosphate-buffered saline (PBS), and resuspended in DNA staining solution containing 50 µg/mL propidium iodide, 0.3% saponin, 1 mM EDTA, 1% sodium azide, 1% FCS, and 20 µg/mL DNase-free RNase I (Sigma) in PBS. The suspension was incubated at 4° C in the dark for 6 h before analyzing a minimum of 10,000 cells per sample using a flow cytometer (FACScalibur, Becton Dickinson). The percentage of sub-G_1 cells was calculated using CellQuest software (Becton Dickinson).

Annexin V Staining

Freshly isolated PBMCs were cultured in RPMI supplemented with 2% FCS in the presence of increasing concentrations of ITF2357 (10 to 1000 nM) with and without LPS (10 ng/mL) at 2.5 million cells per mL in 96 flat-bottom wells. After 24 h, the cells were labeled with annexin-V-FLUOS (Roche) and propidium iodide (PI) following the manufacturer's instructions. The percentage of annexin-V–positive and annexin-V/PI double-positive cells was determined by flow cytometric analysis. Monocyte and lymphocyte populations were identified by forward and side light scatter properties. The identity of monocytes was confirmed by PE-Cy5–conjugated anti-CD14. The percentages of annexin-V–positive and annexin-V/PI double-positive cells were calculated within the specific gates.

Caspase 3/7 Determinations

Monocytes were isolated from freshly obtained unfractionated PBMCs by anti-CD14–labeled magnetic beads (CD14 MicroBeads, Miltenyi Biotec, Bergisch Gladbach, Germany) and incubated at 50,000 cells/well in flat-bottom 96-well plates in the presence of increasing concentrations of ITF2357 for 1 h. Thereafter, LPS

Table 1. Inhibition of Maize HDAC by ITF2357

Compound	HD2	HD1-B	HD1-A
ITF2357	10[a]	7.5	16
ITF2363[b]	1150	420	300
ITF2375[c]	NA	NA	NA

[a]Inhibitory concentration of 50% in nM.
[b]Chemical name 4-aminobenzohydroxamic acid.
[c]Inactive metabolite of ITF2357.
NA indicates no activity.

(10 ng/mL) was added for an additional 24-h incubation. The activity of caspase 3/7 was then determined by Apo-ONE Homogeneous Caspase-3/7 Assay (Promega), and the amount of fluorescence was detected by fluorimetric plate reader (Victor-2; Perkin Elmer).

Inhibition of Cytokines from PBMCs In Vitro

Citrated blood was obtained from consenting donors and PBMCs were isolated using centrifugation over Ficoll-Hypaque cushions. After washing, the PBMCs were resuspended in RPMI containing 5% FCS at 5×10^6/mL, added to a 50-mL conical polypropylene tube, and placed at 4° C overnight. The next morning, the PBMCs were resuspended and 100 µL was added to each well of a 96-well flat microtiter plate. PBMCs used after an overnight rest at 4° C are termed "rested PBMCs." For inhibition studies, ITF2357 was added to specific wells and the plates were placed in an incubator for 1 h at 37° C, after which the cells were stimulated with LPS (10 ng/mL, final concentration) or other stimulants in a final volume of 200 µL per well. After 24 h at 37° C, the supernatants were removed and frozen at –80° C until assayed for cytokines.

PBMCs were also prepared from blood obtained as a byproduct of platelet pheresis by the Bon Fils Blood Bank (Aurora, CO, USA; approved by the Colorado Multiple Institutional Review Board). Obtained from the donor within 1 h, this blood was layered over Ficoll-Hypaque and the isolated PBMCs were washed and suspended at 5×10^6/mL in RPMI. PBMCs isolated from this blood were not refrigerated overnight and are termed "freshly obtained PBMCs." Using 48-well flat-bottom plates, 250 µL of freshly prepared PBMCs were added to each well and ITF2357 was added at various concentrations. After 1 h at 37° C, the cells were stimulated with LPS (10 ng/mL) or other stimulants to make a final volume of 500 µL per well. The concentrations of ITF2357 are expressed as nanomoles during the 24-h incubation. Supernatants were removed after a 24-h incubation and frozen at –70° C until assayed.

In the case of intracellular measurements of IL-1α, after the supernatants were removed, 500 µL RPMI containing 0.5% Triton X-100 was added to the wells and the plates were subjected to 3 freeze-thaw cycles. The contents of each well were transferred to Eppendorf tubes and centrifuged at 10,000 rpm in a microfuge at 4° C. The supernatants after centrifugation were assayed for intracellular IL-1α.

Whole Blood Cultures

Whole human blood was also used to study the effect of ITF2357. After signing informed consent (Colorado Multiple Institutional Review Board), venous blood was collected in the morning from fasting healthy subjects into heparinized syringes (final concentration of heparin, 20 units/mL). The blood (250 µL) was mixed with 550 µL RPMI in 4-mL round-bottom polypropylene tubes. ITF2357 or RPMI control was added (100 µL) and incubated for 1 h at 37° C. Thereafter, LPS (100 µL) was added (final concentration 10 ng/mL). The diluted blood (now 1 part in 4) was incubated for 24 h, after which the supernatant plasma-RPMI mixture was aspirated and frozen at –70° C.

Cytokine Assays

Human TNFα, mature IL-1β, and the IL-1β precursor were assayed by ELISA (R&D Systems, Minneapolis, MN, USA). Human IL-1α, IFNγ, IL-1Ra, IL-8, and IL-6 were measured by electrochemiluminescence (ECL) (BioVeris, Gaithersburg, MD, USA). Antibodies for these assays were purchased (R&D Systems) and biotinylated and ruthenylated according to the manufacturer's instructions (BioVeris). Cytokine standards for these assays were obtained from Peprotech (Rocky Hill, NJ, USA). The limit of detection of the ECL assay was 20 pg/mL and the limit of detection for the ELISA was 16 pg/mL. An IL-1α ECL assay was used as previously described (32). Endogenous IL-1Ra was measured in an ECL assay with a sensitivity of 50 pg/mL using biotinylated and ruthenylated antibodies from R&D Systems. Granulocyte-

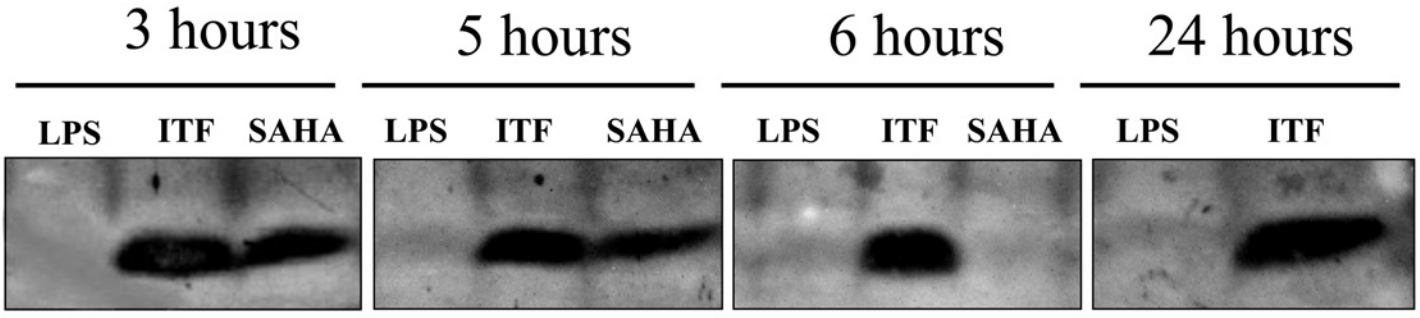

Figure 1. Time course of protein hyperacetylation in LPS-stimulated PBMCs. Resting human PBMCs from a healthy donor were incubated with LPS only (10 ng/mL) or LPS in the presence of ITF2357 (100 nM) or SAHA (100 nM). At the indicated times, the cells were lysed and total protein extracts analyzed by Western blotting using an antibody to acetylated lysines. The results are representative of 1 of 3 donors with similar observations.

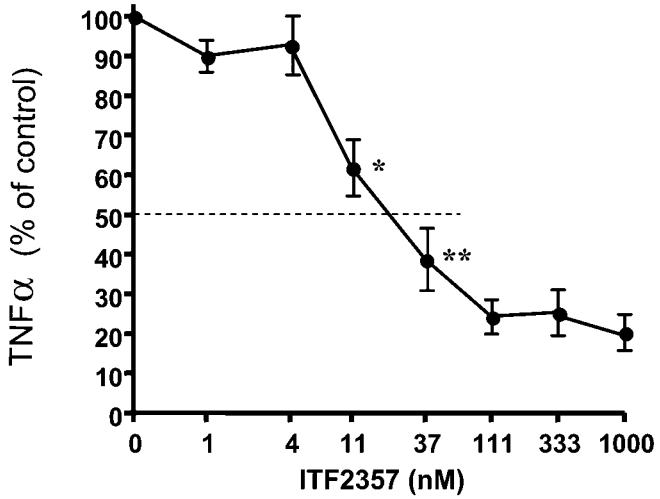

Figure 2. Effect of ITF2357 on the secretion of TNFα in LPS-stimulated PBMCs. After an overnight rest, PBMCs were preincubated with increasing concentrations of ITF2357 for 1 h at 37°C, followed by LPS (10 ng/mL) for an additional 20 h. TNFα released into the supernatant was measured, and the percent reduction from LPS only (set at 100%) is indicated on the vertical axis. The data are presented as mean percent change (± SEM) of 6 donors. The dashed line indicates inhibition of 50% of TNFα induced by LPS only. *$P < 0.05$; **$P < 0.01$ from LPS only.

macrophage colony-stimulating factor (GM-CSF) was measured using an ELISA from R&D Systems. Mouse IL-4, TNFα, and IFNγ were measured by specific ELISA (R&D Systems and Endogen).

Animal Studies

Animal protocols were approved by the University of Colorado Health Sciences Center Animal Review Committee. Mice for LPS induction of serum cytokines were BALB/c, whereas those for anti-CD3-induced cytokines were CD1 obtained from Charles River Laboratory (Calco, Italy). Mice for concanavalin A (Con A)–induced acute hepatitis were BALB C or C57Bl6 obtained from Jackson Laboratories (Bar Harbor, ME, USA). Mice were given 100 µL water or ITF2357 in water by gavage and, after 60 min, were injected intraperitoneally with LPS (30 mg/kg) or intravenously with anti-mouse CD3 (10 µg/mouse; Cymbus Biotechnology, UK) or intravenously with 200 µg/mouse of Con A into a tail vein. Control mice received an intraperitoneal injection of saline or intravenous injection of saline. For LPS, mice were killed by anesthetic overdose, and blood was obtained at 90 min and 6 h. For anti-mouse CD3, mice were killed by anesthetic overdose, and blood was obtained at 90 min. Mice were bled 24 h later for evaluation of serum ALT levels as described previously (33,34).

Statistical Analysis

Comparisons between groups were analyzed by Student 2-tailed *t*-test or with ANOVA for experiments with more than 2 subgroups.

RESULTS

Inhibition of HDAC Activity by ITF2357

As shown in Table 1, ITF2357 inhibits the activity of maize HDAC preparations HD2, HD-1B, and HD-1A using the methods

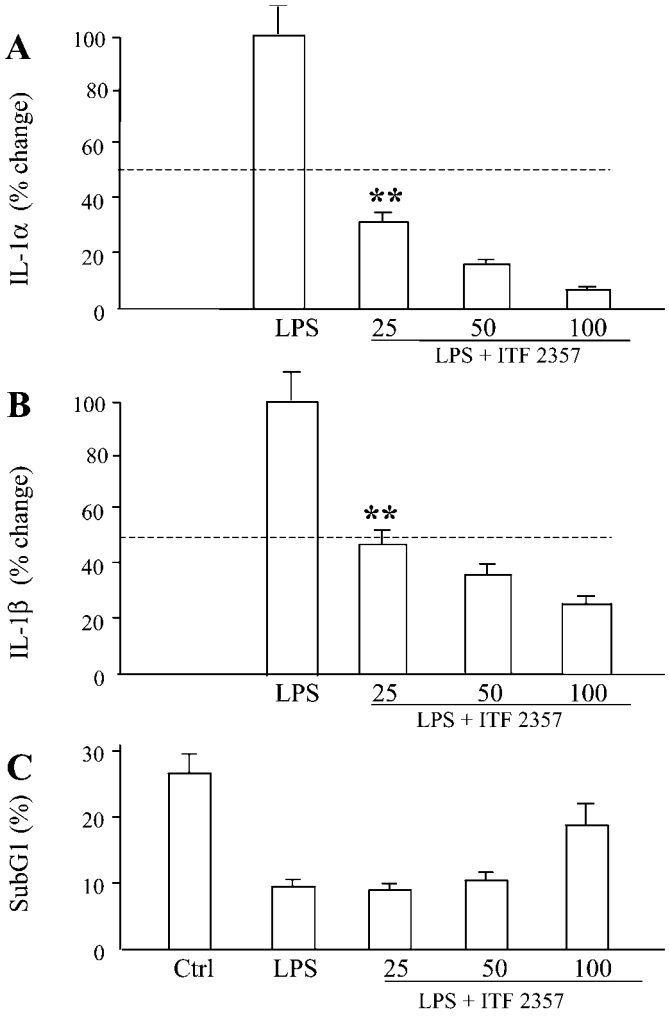

Figure 3. Effect of ITF2357 on intracellular IL-1α and secreted IL-1β from LPS-stimulated PBMCs. Freshly obtained PBMCs were preincubated for 1 h with increasing nanomolar concentrations of ITF2357 and then stimulated with LPS (10 ng/mL) for 24 hours. (A) Levels of intracellular IL-1α are shown as the percent change from LPS only (set at 100%) as indicated on the vertical axis. (B) Levels of secreted IL-1β are shown as the percent change from LPS only. The data are the mean percent change ± SEM of 6 donors. The dashed line indicates inhibition of 50%. (C) FACS analysis of percent of total cell PI staining expressed at sub-G_1 cell cycle. Mean ± SEM of 4 donors are shown. Cells in sub-G_1 cycle are from the same cultures shown in B. *$P < 0.05$; **$P < 0.01$ from LPS only.

described in Koelle et al. (29). The IC_{50} varied from 7.5 to 16 nM. Analogs of ITF2357, inactive as cytokine inhibitors, were also tested for HDAC inhibition (Table 1).

Because HDAC inhibition results in hyperacetylation of histones, levels of hyperacetylated proteins were measured using an antibody to acetylated lysines in total cellular extracts of LPS-stimulated PBMCs in the presence of ITF2357 or SAHA. Rested PBMCs were preincubated with either inhibitor for 1 h at 37° C and then stimulated with LPS. After 3, 6, and 24 h, extracts of the cell pellets were made and the acetylated lysines were determined in total cellular extracts. Figure 1 depicts the effect of these two HDAC inhibitors on acetylated total proteins. LPS alone did

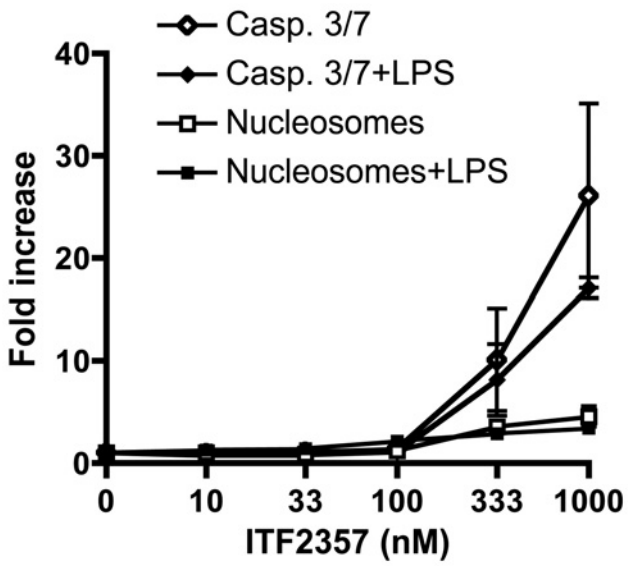

Figure 4. Caspase 3/7 activation and nucleosome formation. Purified monocytes were incubated with and without ITF2357 (as shown under the horizontal axis). After 1 h, LPS (10 ng/mL) was added to some wells. After 24 h, caspase activation and nucleosome formation were assayed. The data are expressed as fold increase (mean ± SEM, *n* = 3 donors) over monocytes without ITF2357.

not result in detectable hyperacetylation at any time point. After 3 h of incubation with LPS in the presence of either SAHA or ITF2357, hyperacetylation is clearly present. However, a greater duration of hyperacetylation up to 24 h was observed in cells exposed to ITF2357, whereas SAHA-induced hyperacetylation was absent after 6 hours. In this study, only 1 band of hyperacetylated proteins was observed and corresponded to the molecular weight range of histone H4.

Inhibition of LPS-Induced TNFα in PBMCs Incubated with ITF2357

As shown in Figure 2, TNFα production was inhibited in a dose-dependent fashion with 50% reduction at approximately 10 to 22 nM. By comparison, IC_{50} for TNFα in these cultures by SAHA was at 400 nM (data not shown), but similar to that previously reported (8). IL-1β levels in the supernatants of these cultures were reduced by 50% at a concentration of 10 to 20 nM of ITF2357 (*n* = 6, Figure 3B).

Effect of ITF2357 on IL-1α and IL-1β Production

Unlike IL-1β, TNFα, and other cytokines in LPS-stimulated human PBMCs, IL-1α remains nearly entirely as an intracellular cytokine (35) and therefore, total cellular IL-1α was measured following extraction with Triton X (see "Methods"). As shown in Figure 3A, ITF2357 reduced the total intracellular IL-1α synthesis

A

	RPMI		+ LPS	
ITF2357 [nM]	% Ann. V	% PI	% Ann. V	% PI
0	9.62 ± 1.45	7.83 ± 0.82	5.38 ± 1.56	2.86 ± 0.02
10	9.91 ± 2.26	6.26 ± 0.71	6.83 ± 1.35	3.61 ± 1.06
30	9.40 ± 1.42	8.49 ± 1.44	6.55 ± 1.71	3.00 ± 0.59
100	11.54 ± 1.98	10.26 ± 1.73	6.58 ± 1.46	3.02 ± 0.63
300	14.65 ± 2.55	14.32 ± 2.10	9.31 ± 1.80	5.05 ± 0.80
1000	17.40 ± 2.73	17.13 ± 3.24	17.07 ± 5.95	12.60 ± 1.93

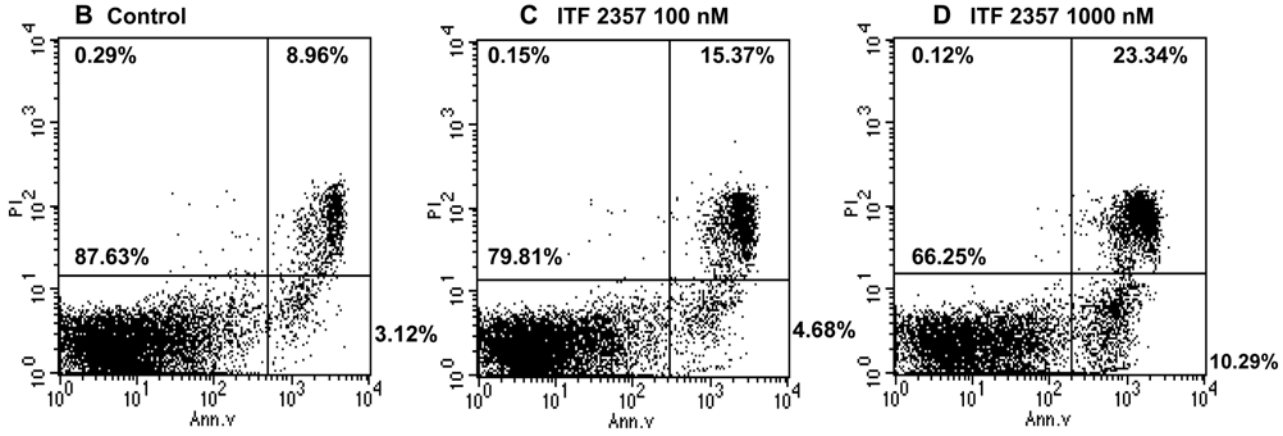

Figure 5. Effect ITF2357 on apoptosis and necrosis in PBMCs. (A) PBMCs in RPMI were incubated with increasing concentrations of ITF2357 for 1 h followed by the addition of LPS (10 ng/mL) to appropriate wells. After 24 h at 37° C, the percentage of annexin-V– and PI-positive PBMCs was determined. The data are the mean ± SEM of 5 separate experiments/donors. (B) FACS analysis of PI and annexin V staining in control PBMCs. (C) PI and annexin V staining in PBMCs incubated with 100 nM ITF2357. (D) PI and annexin V staining in PBMCs incubated with 1000 nM ITF2357. The data in B, C, and D are from 1 representative experiment.

A

	RPMI only		+ LPS	
ITF2357 [nM]	% Ann. V	% PI	% Ann. V	% PI
0	6.23 ± 1.11	11.01 ± 1.49	4.89 ± 1.37	8.71 ± 1.72
10	7.10 ± 0.97	9.65 ± 1.18	5.65 ± 1.96	8.49 ± 2.09
30	8.33 ± 1.39	13.08 ± 4.66	6.88 ± 2.49	10.52 ± 5.33
100	9.00 ± 1.68	12.57± 5.53	4.86 ± 1.22	13.21 ± 9.10
300	9.37 ± 1.37	27.83±12.42	6.47 ± 0.25	24.28 ±12.64
1000	13.24 ± 4.30	79.04 ± 2.79	13.98 ± 3.67	78.53 ± 2.43

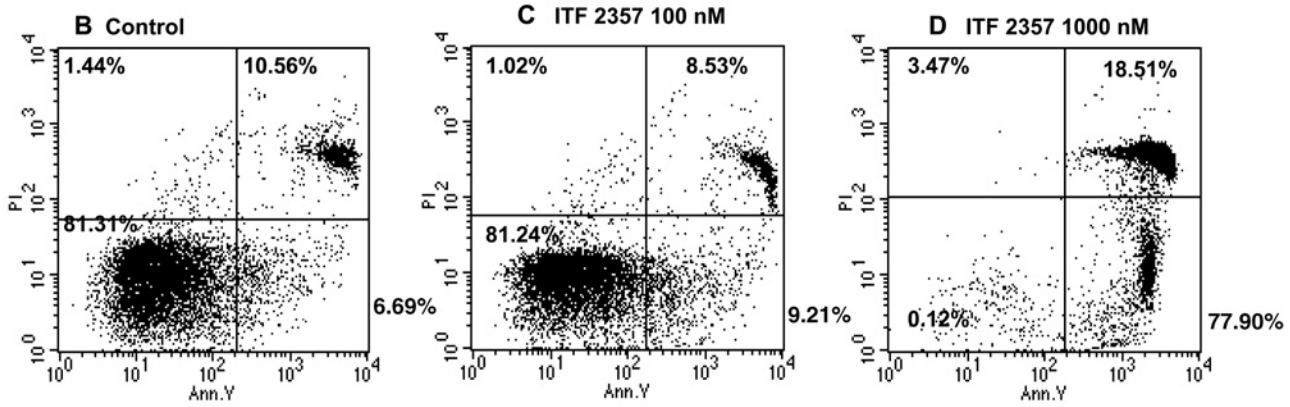

Figure 6. Effect of ITF2357 on apoptosis and necrosis in monocytes. Purified monocytes in RPMI were incubated with increasing concentrations of ITF2357 for 1 h followed by the addition of LPS (10 ng/mL) to appropriate wells. After 24 h at 37° C, the percentage of annexin-V– and PI-positive cells was determined. (A) The data are the mean ± SEM of 3 separate experiments/donors. (B) FACS analysis of PI and annexin V staining in control monocytes. (C) PI and annexin V staining in monocytes incubated with 100 nM ITF2357. (D) PI and annexin V staining in monocytes incubated with 1000 nM ITF2357. The data in B, C, and D are from 1 representative experiment.

in freshly obtained PBMCs with 50% inhibition at concentrations below 25 nM. LPS-induced secretion of IL-1β into the supernatant was also measured in these same cultures (Figure 3B) with 50% inhibition at 25 nM. The decrease in IL-1β secretion in the presence of SAHA has been previously reported (8) and, similar to SAHA, ITF2357 did not affect the intracellular levels of the IL-1β precursor under these conditions (data not shown). IL-8 production in these cultures was also not affected (data not shown), similar to observations with SAHA (8). In addition, neither ITF2357 nor SAHA affected the synthesis of IL-1Ra (data not shown).

Lack of Cell Death at Concentrations of ITF2357 Inhibiting Cytokines

As depicted in Figure 3C, after 24 h of incubation under the same conditions as shown in Figure 3A and B, the percentage of cells in sub-G_1 was not increased compared with cells cultured under control conditions (no LPS and no ITF2357). The lower percentage of cells in sub-G_1 with stimulation by LPS reflects a protective role for LPS in cell death. As shown in Figure 4, there was no increase of caspase 3 or 7 activity in monocytes at concentrations from 10 to 100 nM and no increase in annexin-V staining or PI uptake in PBMCs (Figure 5) or isolated monocytes (Figure 6) under similar conditions in the presence or absence of LPS. At ITF2357 concentrations of 300 nM or greater, one can observe a small increase in parameters of apoptosis. However, concentrations of ITF2357 below 100 nM, which inhibit more than 50% of TNFα, IL-1α, and IL-1β secretion, have no effect on cell death.

Effect of ITF2357 on IFNγ Production

As shown in Figure 7, LPS-induced IFNγ from freshly stimulated PBMCs was reduced by ITF2357 with 50% inhibition at 100 nM and 80% reduction at 200 nM. However, unlike the monocyte source of LPS-induced TNFα, IL-1, IL-8, or IL-1Ra, the source of IFNγ in PBMC cultures is primarily derived from T cells. Therefore, PBMCs were stimulated with the combination of IL-12 plus IL-18 for IFNγ production. The combination of these cytokines is commonly used to induce IFNγ in T cell and natural killer cells, as neither cytokine alone induces IFNγ (36). As shown in Figure 8, left panel, IFNγ was reduced by 50% at a concentration of ITF2357 of 25 nM. In parallel experiments, the role of IL-1 and TNF in IFNγ production stimulated by the combination of IL-12/IL-18 was assessed. To block IL-1 activity, IL-1Ra was used, and to block TNF activity, TNF-bp was added. There was no effect on IFNγ production when either cytokine was blocked with these specific antagonists. Moreover,

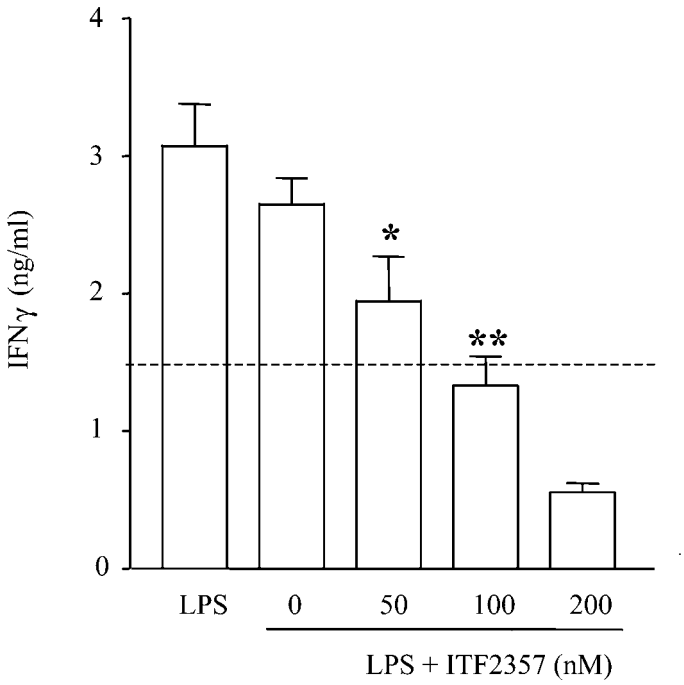

Figure 7. Effect of ITF2357 on LPS-induced IFNγ production. Freshly obtained PBMCs were preincubated for 1 h with increasing concentrations of ITF2357 (horizontal axis) and stimulated with LPS (10 ng/mL) for 24 h. Levels of secreted IFNγ are shown in ng/mL on the vertical axis. The data are presented as the mean percent change ± SEM of 4 donors and the dashed line indicates inhibition of 50%. *P < 0.05; **P < 0.01 from LPS only.

blocking either cytokine did not affect the dose-response to ITF2357 in these cultures (Figure 8, middle and right panels).

Effect of ITF2357 on Anti-CD3-Induced IFNγ and IL-2 in PBMCs

Anti-CD3 was used to trigger T cells via the T-cell receptor and mimic the activation of T cells during antigen presentation. ITF2357 was added to PBMCs, which had been rested overnight, and then challenged with LPS or anti-CD3 for the remaining 20 h. As shown in Figure 9B, there was no reduction in IFNγ using anti-CD3 as a stimulant at concentrations as high as 100 nM, whereas the same PBMC preparations stimulated with LPS revealed a near-complete suppression of IFNγ (Figure 9A). In the same cultures, anti-CD3–induced IL-2 was also unaffected by ITF2357 (data not shown).

IL-6 Production

We sought to examine IL-6 production in freshly cultured PBMCs stimulated by the combination of IL-12 plus IL-18. As shown in Figure 10, significant IL-6 production was observed in PBMCs of 2 donors cultured at 5 million cells per milliliter compared with 2.5 million cells. The doubling of cell concentration may provide conditions for greater cell–cell contact. Under the condition of 5 million cells per milliliter, ITF2357 at 50 nM reduced IL-6 production by more than 70%. As shown in Figure 11, 50% inhibition was observed between 25 and 50 nM. Also shown in Figure 11, inhibition of IL-6 production by ITF2357 in PBMCs stimulated with the combination of IL-12 plus IL-18 was unaffected by blocking either IL-1 or TNF (Figure 11, middle and right panels).

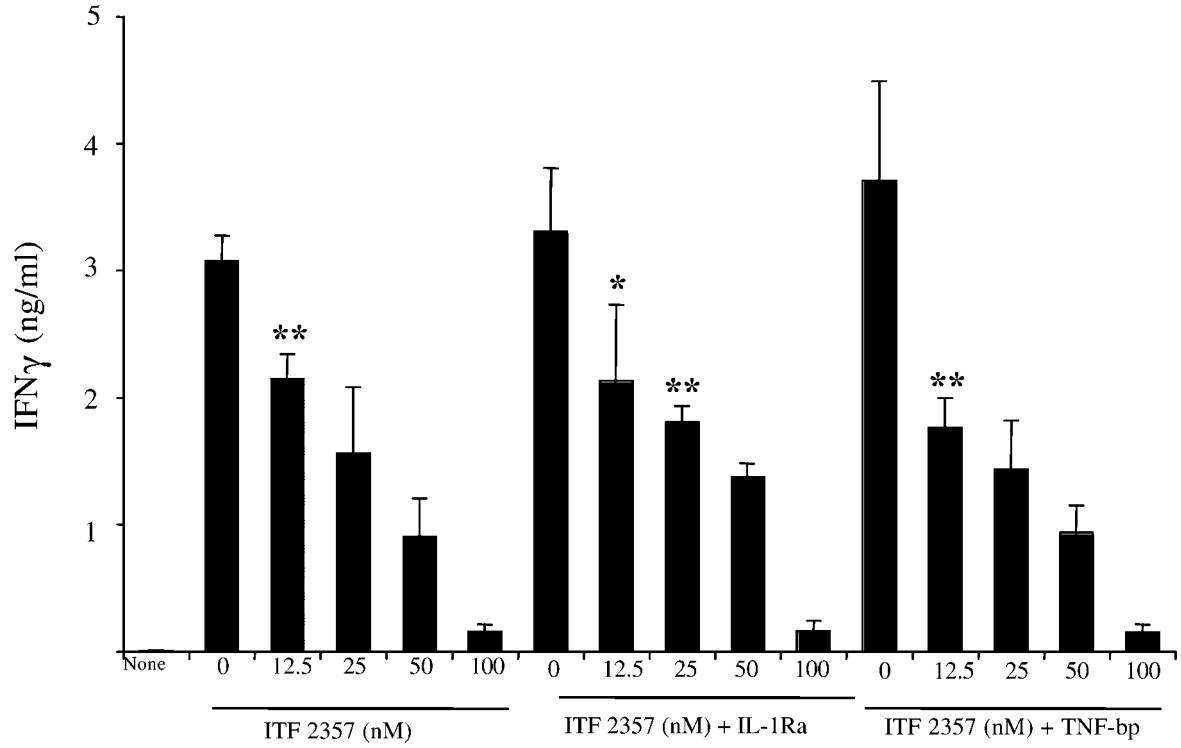

Figure 8. Suppression of IL-18/IL-12–induced IFNγ by ITF2357. Freshly cultured PBMCs were pretreated with increasing concentrations of ITF2357 (shown under horizontal axis) for 1 h as described in Figure 7. The cells were stimulated with the combination of IL-18 (10 ng/mL) plus IL-12 (1 ng/mL) (left panel). Matching cultures were similarly stimulated in the presence of IL-1Ra (10 μg/mL, middle panel) or TNF-bp (1 μg/mL, right panel). IFNγ was measured in the supernatants after 24 hours. Data are mean ± SEM of IFNγ in ng/mL of 4 donors. *P < 0.05; **P < 0.01 compared with no ITF2357.

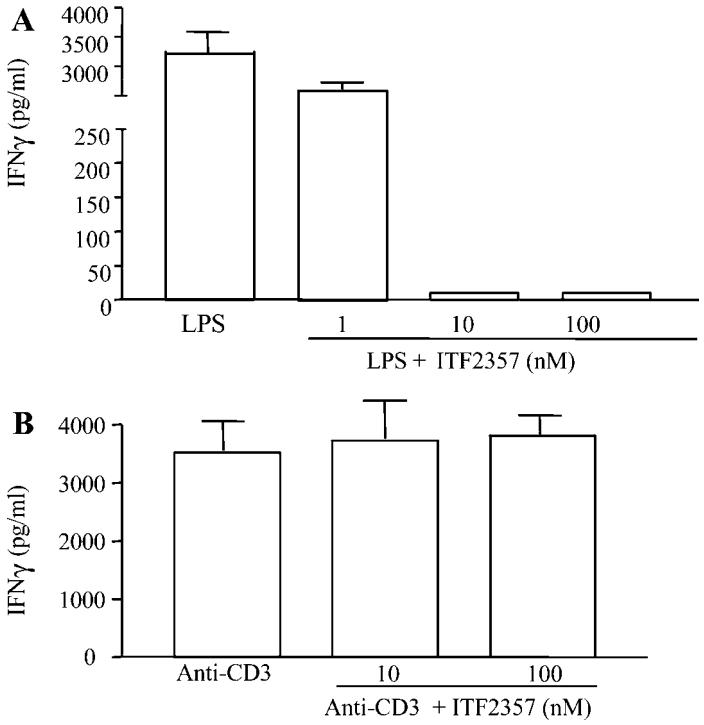

Figure 9. Stimulus-dependent suppression of IFNγ by ITF2357. (A) LPS-induced IFNγ from resting PBMCs stimulated with LPS (10 ng/mL). Resting PBMCs were preincubated with ITF2357 as indicated for 1 h at 37° C before the addition of LPS. Mean ± SEM of IFNγ in pg/mL of 4 donors. (B) After preincubation with ITF2357, the cells from the same donors were stimulated with anti-CD3 (500 ng/mL). After 24 h, IFNγ was measured in the supernatants. Mean ± SEM of IFNγ in pg/mL of 3 donors are shown.

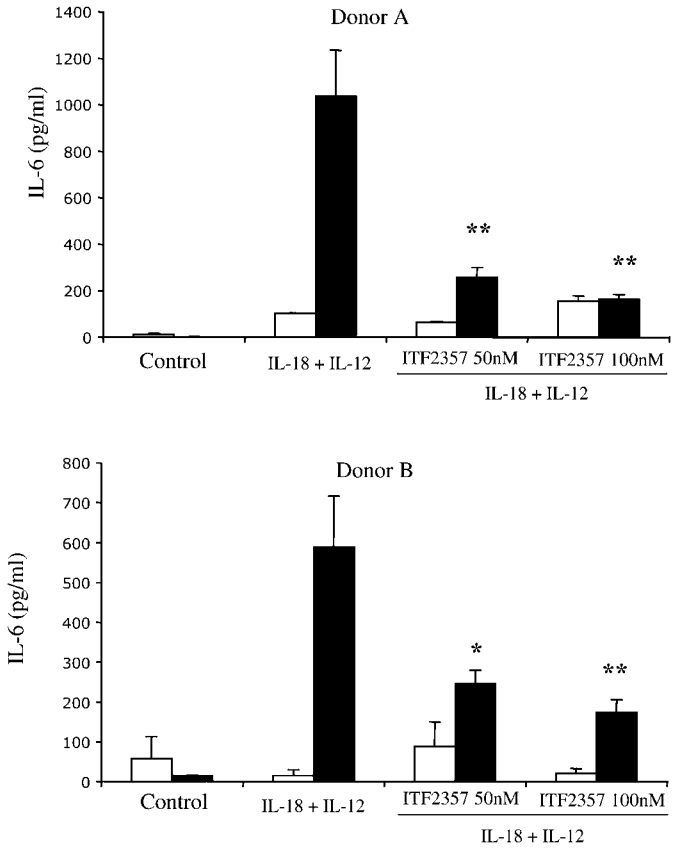

Figure 10. Effect of ITF2357 on IL-6 production. Freshly cultured PBMCs were preincubated with ITF2357 for 1 h and then stimulated with the combination of IL-18 (10 ng/mL) and IL-12 (1 ng/mL) as in Figure 8. Open bars indicate PBMCs at a concentration of 2.5 million cells/mL, and solid bars represent PBMCs at 5 million cells/mL. Mean ± SEM of IL-6 measured in the supernatant of triplicate wells after 24 h are shown for 2 donors. *$P < 0.05$; **$P < 0.01$ compared with no ITF2357.

Other In Vitro Effects of ITF2357 on Cytokine Production

PBMCs stimulated with either LPS or anti-CD3 were used to assess the effect of ITF2357 on production of GM-CSF. In cells from 2 subjects, ITF2357 at 100 nM reduced LPS-induced GM-CSF by 99% and 89%, respectively. However, when stimulated with anti-CD3, there was no reduction in GM-CSF by ITF2357 at 37 or 100 nM (data not shown).

Whole blood cultures were also studied for production of cytokines. Heparinized whole blood diluted in RPMI (1 part blood to 3 parts RPMI) was pretreated with ITF2357 at 50 and 100 nM for 1 h followed by stimulation with LPS (10 ng/mL). Cytokines were measured in the supernatants. After 24 h, the reduction in TNFα was 66% ± 12% and 76% ± 21%, $n = 4$, respectively. In the same cultures, IL-1β production was reduced by 53% ± 10% and 68% ± 11%, respectively, and that of IFNγ by 71% ± 23% and 82% ± 16%, respectively.

IL-1Ra was also examined under conditions of LPS-stimulated whole blood cultures. For comparison, the same whole blood cultures were also used to measure the effect of ITF2357 on TNFα release. Whole blood production of LPS-induced TNFα was reduced by approximately 50% at 100 nM. In the same cultures, there was no effect of 100 nM of ITF2357 on IL-1Ra production (data not shown).

Steady-State Northern Blot Analysis of LPS-Induced Cytokine mRNA Levels

Resting PBMCs were incubated with ITF2357 (50 nM), LPS (10 ng/mL), or LPS in the presence of ITF2357 (50 nM). After 3.5, 7, and 24 h, total RNA was harvested, electrophoresed, and hybridized with specific cDNA probes. Densitometry was performed on the radiograph. As shown in Figure 12 (lane 1), resting PBMCs did not express mRNA for TNFα, IL-1β, IFNγ, or IL-8 at each time point and ITF2357 (lane 2) similarly had no effect. LPS (lane 3) induced TNFα mRNA, which reached maximal levels at 7 h but in the presence of ITF2357 (lane 4) was significantly reduced. The level of TNFα in the supernatant of these cells was reduced by 54% at 7 hours. After 24 h, there was still a marked reduction in steady-state TNFα mRNA levels, and the inhibition of TNFα protein secreted from these cells was 85%. In contrast, there was no change on steady-state levels of IL-1β mRNA by ITF2357 and a small increase at 24 h. These observations are consistent with the absence of a reduction in the intracellular levels of IL-1β protein. However, there was a reduction (52%) in the supernatant level of IL-1β at 24 h. IFNγ mRNA levels were absent after 3.5 h but reached maximal levels after 24 h. There was a dramatic

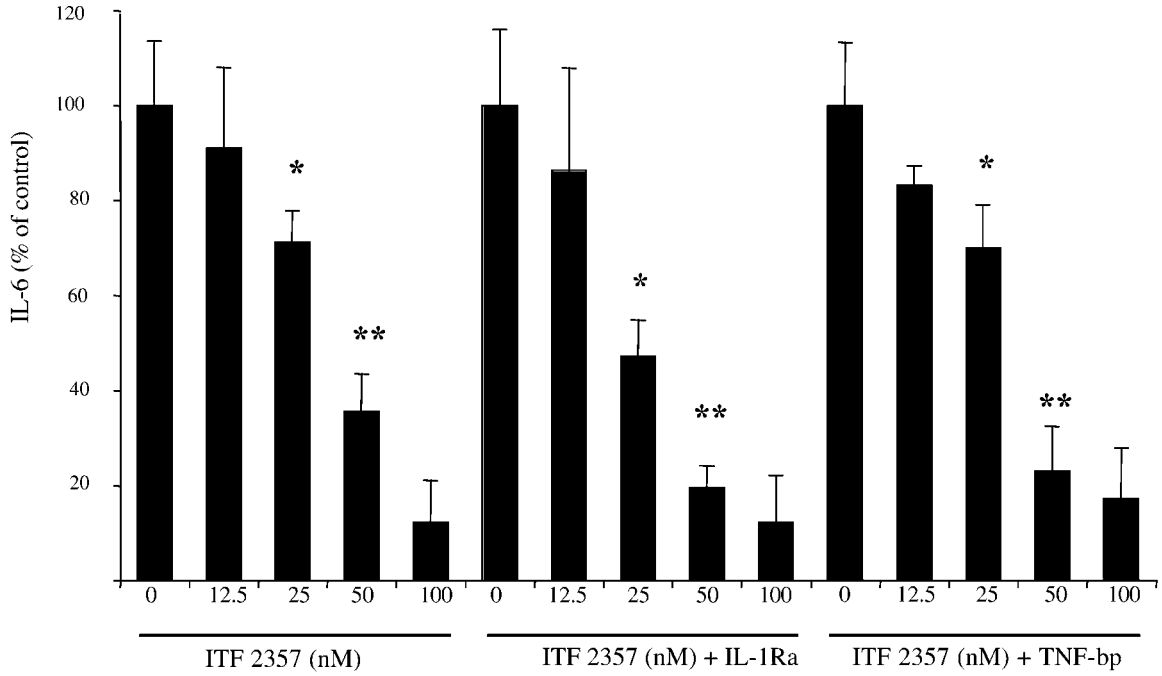

Figure 11. Inhibition of IL-6 by ITF2357 is independent of IL-1β and TNFα. Left panel depicts the dose-response inhibition of IL-18/IL-12–induced IL-6 by increasing concentrations of ITF2357. Freshly isolated PBMCs were preincubated with ITF2357 for 1 h. The combination of IL-18/IL-12 was added for an additional 20 h, and IL-6 was measured in the supernatants. Matching cultures were similarly stimulated in the presence of IL-1Ra (10 μg/mL, middle panel) or TNF-bp (1 μg/mL, right panel). Data are mean ± SEM of IL-6 in ng/mL of 4 donors. *P < 0.05; *P < 0.01 compared with no ITF2357.

reduction in IFNγ mRNA levels by ITF2357 at 7 and 24 h. At 24 h, there was a 94% reduction in IFNγ protein levels in the supernatant. Neither IL-8 mRNA nor protein was affected by ITF2357.

Real-Time PCR

In addition to the above studies using Northern blot analysis, real-time PCR was employed to examine the effect of ITF2357 on LPS-induced steady-state mRNA levels. Resting PBMCs from 3 donors (A, B, and C) were used. Total mRNA was harvested after 4 h and in parallel cultures, cytokines were measured in the supernatants after 24 h. Copy number (pg/ng of 18S RNA) of each cytokine mRNA was established using standard curves. For each donor, copy numbers for unstimulated PBMCs were less than 5 pg/ng 18S RNA, and there were no significant changes in the presence of 50 nM of ITF2357, consistent with the results by Northern blot analysis. LPS induced TNFα mRNA levels 8-, 10-, and 25-fold greater than in unstimulated PBMCs. As shown in Figure 13, the decrease in the presence of ITF2357 was 80%, 52%, and 89%, respectively. The inhibition of TNFα secreted after 24 h was 62%, 53%, and 67%, respectively. LPS induced increases in IL-1β mRNA of 15-, 20-, and 47-fold greater than in unstimulated cells for donors A, B and C, respectively, but were unaffected by ITF2357.

Effect of ITF2357 on Circulating TNFα and IFNγ During Endotoxemia

The ability of ITF2357 to affect circulating levels of TNFα was assessed in vivo following LPS challenge. Mice received increasing oral doses of ITF2357 by gavage 1 h before an intraperitoneal injection of LPS; blood was obtained 90 min after LPS. As shown in Figure 14A, LPS-induced TNFα increased to ~4-5 ng/mL in mice treated with a control injection of saline. ITF2357 pretreatment induced a progressive decrease in circulating TNFα beginning at 0.01 mg/kg. At 0.1 mg/kg, the inhibitor reduced TNFα by ~60% (P < 0.05) and at 1.0 mg/kg by 53% (P < 0.001). In another group of mice treated similarly, serum was obtained 6 h after LPS administration and IFNγ was determined. At 10 mg/kg, oral ITF2357 reduced circulating IFNγ by 60%, and at 50 mg/kg, the level of IFNγ was reduced by 95% (Figure 14B). In another experiment, anti-CD3 was injected intraperitoneally in mice pretreated with oral ITF2357. TNFα was not affected (Figure 14C) at all the tested doses, whereas dexamethasone (10 mg/kg) reduced serum TNFα by 60%, consistent with the lack of effect seen in vitro when anti-CD3 was used as the stimulant for cytokines.

Con A Model of Acute Hepatitis

Mice were given 100 μL water or ITF2357 (5 mg/kg) by gavage and, after 1 h, injected intravenously with 200 μg/mouse of ConA. Control mice received an intravenous injection of saline. Mice were bled 24 h later for evaluation of serum ALT levels as described previously (33,34). As shown in Figure 15, ALT levels were reduced by more than 80% by ITF2357 pretreatment. In another experiment, a comparison was made between 1 and 10 mg/kg of oral ITF2357. As shown in Figure 16, a dose of 1 mg/kg ITF2357 was as effective as a dose of 10 mg/kg in reducing ConA hepatitis as measured by ALT levels.

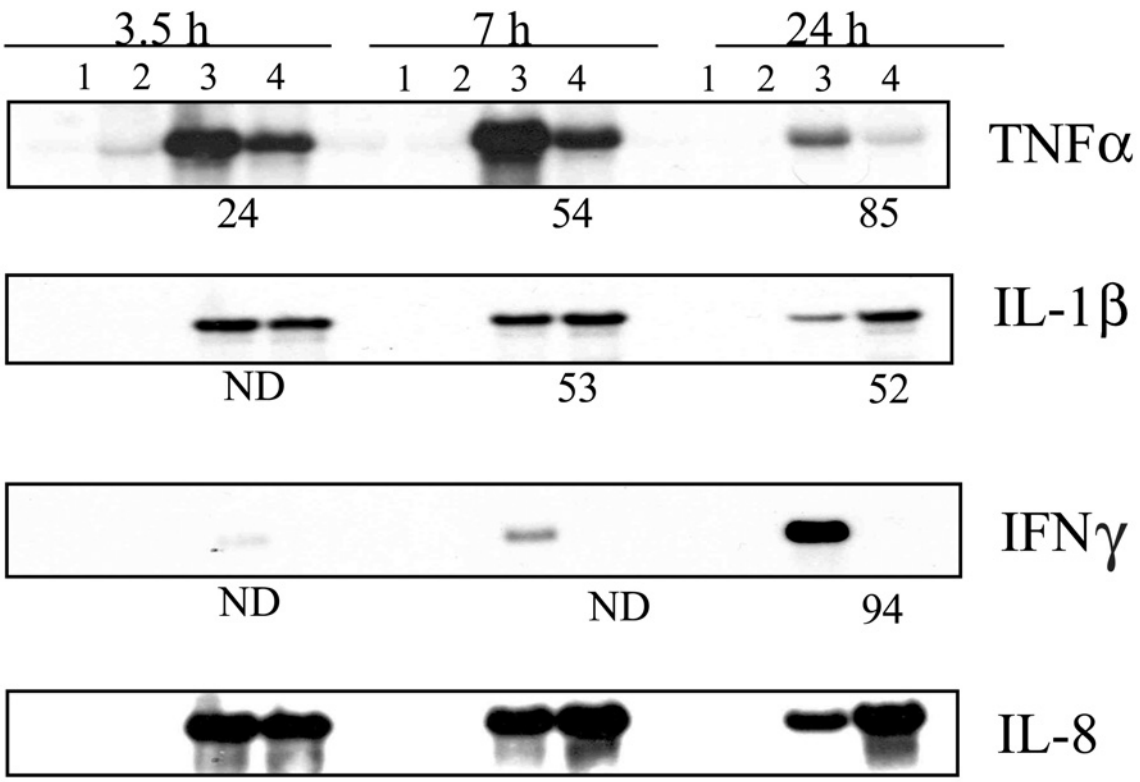

Figure 12. Steady-state mRNA levels in PBMCs. Northern blot analysis of specific cytokine mRNA extracted from resting PBMCs at times indicated. Lane 1, unstimulated PBMCs; lane 2, ITF2357 only (50 nM); lane 3, LPS (10 ng/mL); lane 4, LPS in the presence of ITF2357 (50 nM). In addition, LPS-induced TNFα, IL-1β, IFNγ, and IL-8 were measured in the supernatant by specific ELISA, and the percent inhibition of LPS-induced cytokines by ITF2357 is indicated under the respective bands and time points are indicated. ND = not determined. Shown are the results of a single donor. Similar results were obtained in a second donor.

DISCUSSION

These studies establish that ITF2357 is an inhibitor of HDAC activity but also is an anti-inflammatory agent in vitro and in vivo. Enzymatically, ITF2357 inhibits different histone deacetylases from maize, and biochemical analysis revealed that, similar to SAHA, ITF2357 hyperacetylates histones in human PBMCs. However, compared with SAHA in the same cells, ITF2357 sustains the hyperacetylation for more than 24 h, whereas the effect of SAHA is absent after 6 hours. The present work expands the novel finding that HDAC inhibition reduces cytokine production, particularly cytokines relevant to autoimmune/inflammatory diseases. Of particular importance is the effect of nanomolar concentrations of ITF2357 on TNFα and IFNγ gene expression and synthesis as well as the secretion of IL-1β in PBMCs in vitro. A single oral administration of ITF2357 at doses of 5 mg/kg or less was anti-inflammatory following LPS or intravenous ConA in mice. The implications of these studies are that inhibition of HDAC by low doses of ITF2357 may be effective in the treatment of certain autoimmune and autoinflammatory diseases, particularly those currently treated with TNFα or IL-1β blockade. Similar to SAHA, higher doses of ITF2357 are needed for antitumor effects in mice (unpublished observations) compared with those reducing inflammation in vivo.

Inhibitors of HDAC increase as well as decrease an equal number of genes in the same cells (37). Although the general property of inhibitors of HDAC is one of increasing gene expression of pro-apoptotic genes in cancer cells (38), this property requires micromolar concentrations in vitro and comparable concentrations in vivo. At the same nanomolar concentrations of ITF2357 used to suppress cytokines in LPS-stimulated PBMCs, there was no evidence of cell death by ITF2357 as assessed by 3 methods: the percentage of cells entering sub-G_1, annexin V staining, or caspase 3/7 activation. A 50% or greater inhibition of cytokines was consistently observed at 50 nM or less. Two methods were also used to demonstrate the reduction in steady-state mRNA levels for TNFα upon LPS-stimulation in the presence of ITF2357, Northern blotting and quantitative real-time PCR. We observed marked reductions in steady-state TNFα mRNA, which coincided with reductions in TNFα production.

In the present study using ITF2357 as well as the study on SAHA (8), the secretion of mature IL-1β from LPS-stimulated PBMCs is consistently reduced by more than 80% at concentrations not affecting cell death. However, there are no changes in steady-state IL-1β mRNA levels by either PCR or Northern blot analysis, and intracellular levels of the inactive IL-1β precursor were unaffected by ITF2357. We conclude that inhibition of HDAC by ITF2357 reduces the processing and secretion of the IL-1β precursor.

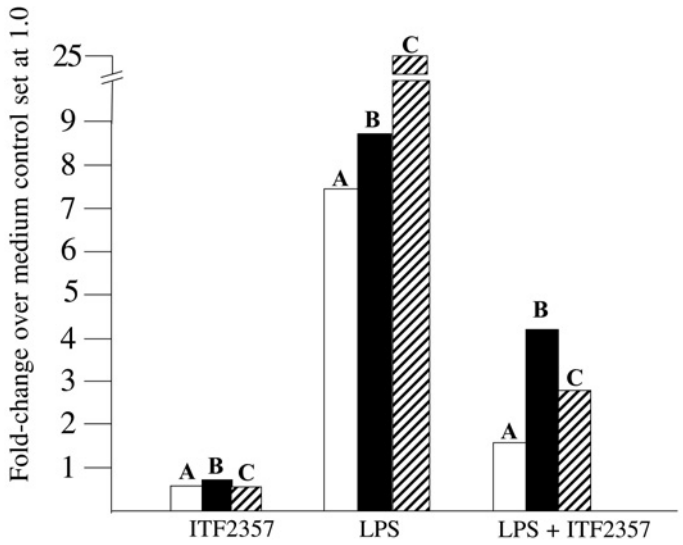

Figure 13. Real-time PCR of steady-state TNFα mRNA. Real-time PCR was performed on RNA extracted from resting PBMCs 4 h after stimulation with LPS (10 ng/mL) in the presence of 50 nM ITF2357. The data were calculated as mRNA in picograms per nanogram of 18S RNA. A value of 1.0 was assigned for the unstimulated control of each donor (indicated as A, B, and C), and the fold change was calculated for each donor.

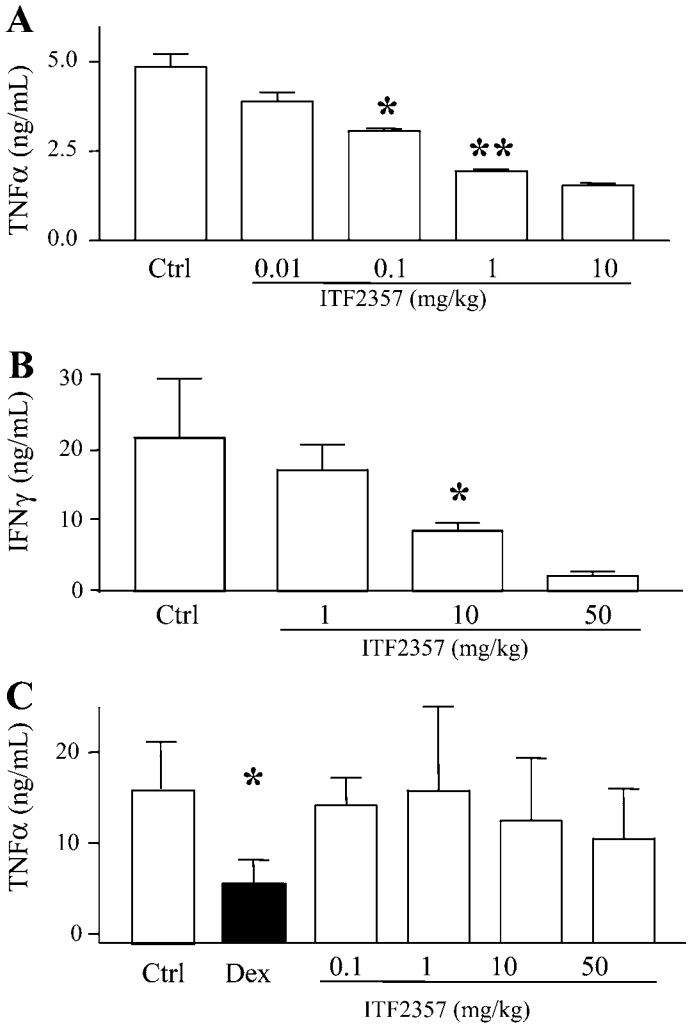

Figure 14. Effect of oral ITF2357 on LPS-induced circulating cytokines. (A) Mice received ITF2357 by gavage 1 h before intraperitoneal LPS (30 mg/kg). Dose-response of oral ITF2357 on serum TNFα (mean ± SEM) 90 min after LPS is shown. Control mice received an oral dose of water followed by LPS; 6 mice in each group. (B) Using another group of mice, an identical study was performed but blood was obtained 6 h after LPS. Mean ± SEM IFNγ is shown for 6 mice per group. (C) Similar to A and B, mice were pretreated with either dexamethasone (DEX at 10 mg/kg) or increasing doses of oral ITF2357 followed by an intravenous injection of anti-mouse CD3 (10 µg/kg). Serum was obtained 90 min after the anti-CD3 antibody and mean ± SEM TNFα is shown (5 mice per group). *P < 0.05; **P < 0.01 compared with water vehicle.

The mechanism for the reduction in IL-1β secretion is unclear because ITF2357 does not inhibit the enzymatic activity of caspase-1 in LPS-stimulated monocytes (unpublished observations).

The reduction in secretion of LPS-induced IL-1β is not inconsequential from a clinical perspective. Indeed, severe systemic inflammatory diseases are due to greater release of active IL-1β from cultured PBMCs in vitro compared with PBMCs from healthy subjects. These diseases include systemic onset juvenile idiopathic arthritis (39), Muckle-Wells syndrome (26,40), familial cold-induced auto-inflammatory syndrome (41), neonatal-onset multisystem inflammatory disease (24,25,27), hyper IgD syndrome (42), and Schnitzler syndrome (43). The increased secretion of IL-1β in some of these diseases is due to a single point mutation in the NALP3 gene, which controls the activation of procaspase-1 (40,44). Alternatively, ITF2357 may affect the ability to secrete IL-1β via the specialized secretory lysosomal pathway and activation of phosphatidylcholine-specific phospholipase C (45). Another pathway in the secretion of IL-1β is the P2x7 receptor (46,47), which may also be affected by mechanisms of hyperacetylation. Nevertheless, when tested, ITF2357 did not inhibit the P2x7 receptor (unpublished observations). Therefore, the mechanism of IL-1β inhibition of secretion by ITF2357 remains unclear.

IFNγ is an important cytokine in several autoimmune diseases but is of particular importance in the pathogenesis of lupus erythematosus. The animal model for this disease is the *lpr/lpr* mouse, which develops a spontaneous proteinuria and lethal nephritis. Neutralization of IL-18 reduced the proteinuria and decreased the lethality (48). The early administration of TSA, a well-described inhibitor of HDAC, reversed the disease-associated cytokine imbalance (49). Daily injections of SAHA to *lpr* mice resulted in decreased hypersplenism and a decrease in the $CD4^-/CD8^-$ T cells (13), both considered pathological events in the development of the disease. Spontaneous autoantibodies deposited in the glomerulus were reduced in *lpr* mice treated with SAHA compared with vehicle-treated controls. Mesangial cells from these mice, when stimulated with LPS plus IFNγ, produce spontaneous nitric oxide, which was inhibited by SAHA. In our previous report, we also observed that mouse macrophages similarly treated with SAHA produce less nitric oxide (8). Therefore, the ability of ITF2357 to reduce IFNγ in vitro and in vivo as reported here is consistent with the efficacy of HDAC inhibition in the model of disease.

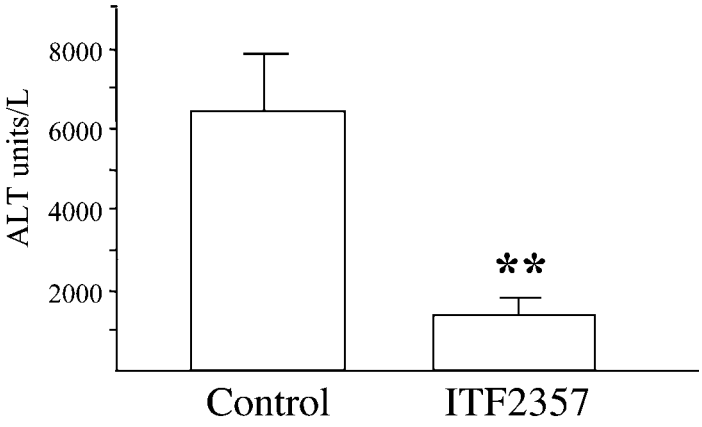

Figure 15. Effect of ITF2357 on ConA-induced hepatitis in BALB/C mice. BALB/C mice (10 per group) received either a single oral dose (100 µL) of 5 mg/kg of ITF2357 or an equivalent volume of water (Controls). After 30 min, both groups received an intravenous injection of ConA (200 µg per mouse). Mean ± SEM serum ALT are shown after 24 h. $^{**}P < 0.01$ versus ConA with water vehicle.

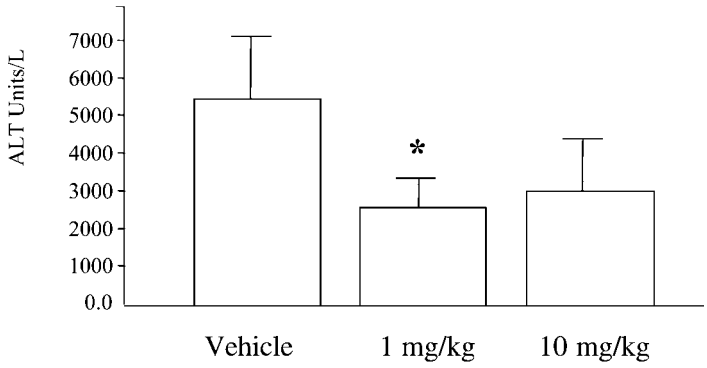

Figure 16. Effect of ITF2357 on ConA-induced hepatitis in C57Bl/6 mice. C57BL/6 mice (10 per group) received either a single oral dose of 1 or 10 mg/kg ITF2357 (100 µL), whereas control mice received water (Controls). After 30 min, all mice received an intravenous injection of ConA (200 µg per mouse). Mean ± SEM serum ALT are shown after 24 hours. $^{*}P < 0.05$ versus ConA with water vehicle.

The use of LPS as an inducer of cytokines is widely accepted as a model of systemic inflammation, particularly sepsis. However, with the exception of inflammatory bowel disease, nonmicrobial products such as autoantibodies or endogenous cytokines themselves trigger most cytokine-mediated autoimmune diseases. IL-12 and IL-18 are primarily macrophage products, which in turn stimulate T lymphocytes to produce IFNγ and IL-6. In the present study, we report that ITF2357 reduces LPS- as well as IL-12/IL-18–induced IFNγ and IL-6. In vivo, we also show that intravenous ConA-induced hepatitis was suppressed by ITF2357.

ITF2357 suppressed IFNγ induced by LPS in vivo and in freshly cultured PBMCs. IFNγ induced by LPS is a result of IL-12/IL-18 production from macrophages. In fact, mRNA levels for IFNγ in PBMCs stimulated with LPS was nearly completely absent 24 h after stimulation. However, either in vitro or in vivo, direct stimulation of T-cell receptor using agonistic anti-CD3–induced IFNγ was unaffected by ITF2357. It appears that IL-12/IL-18 induces IFNγ via a signaling pathway that is sensitive to inhibition by ITF2357, whereas the pathway for IFNγ via the T-cell receptor is not. The inhibition of IL-12/IL-18–induced IFNγ by ITF2357 might be at the level of IL-12 signaling because IL-18's effect on IFNγ production from T cells depends on IL-12–induced IL-18 receptor chains (50,51).

There is a growing body of evidence that HDAC inhibitors reduce severity in animal models of autoimmune diseases. For example, autoantibody-induced arthritis is a model for immune-complex–mediated disease (52) and the administration of the HDAC inhibitor FK228 to mice reduced arthritis scores of joint swelling and synovial inflammation as well as subsequent bone and cartilage destruction (12). In these mice, synovial tissue levels of TNFα and IL-1β were decreased (12). In cultured human synovial cells, FK228 inhibited proliferation and was associated with acetylation of the promoter region of the p21 gene (12). In a model of adjuvant arthritis, phenylbutyrate and TSA inhibited the expression of TNFα as well as reduced joint swelling, infiltration of mononuclear cells, and pannus formation (11). The expected loss in joint cartilage and bone destruction in this model was not observed in animals treated with these agents.

Relevant to the issue of HDAC inhibition of cytokines is the mouse model of GvHD. Human and mouse studies reveal a dysregulation of proinflammatory cytokines with the loss of gastrointestinal tract integrity contributing to GvHD. In mice with acute GvHD, the administration of SAHA reduced circulating levels of IFNγ, TNFα, IL-1β, and IL-6 (28). In addition, intestinal histopathology, clinical severity, and mortality were reduced compared with vehicle-treated animals (28). However, SAHA did not impair graft-versus-leukemia responses and significantly improved leukemia-free survival by using 2 different tumors (28). These findings are consistent with the ability of ITF2357 to suppress LPS-induced TNFα and IFNγ as well as IL-12/IL-18–induced IFNγ and IL-6. In addition, the data are also consistent with the failure of ITF2357 to reduce IFNγ stimulated by anti-CD3 agonistic antibodies as reported in the present studies.

Of the several genes coding for the different classes of HDAC, HDAC3, a class I deacetylase, has been studied for its effects on the transcription of TNFα. Overexpression of HDAC2, HDAC3, and HDAC5 reduced the ability p65-driven transcriptional activation of luciferase reporter gene (53). Others have reported that HDAC3 associates with a nonhistone substrate, acetylated RelA (p65), and deacetylates the molecule (54). In doing so, acetylation/deacetylation of p65 controls the transcriptional responses by affecting the binding to inhibitory κB (54). It is important however to distinguish between the effects of HDAC inhibition on cytokine production in transformed cell lines and those in primary cells or effects in vivo, which are the focus of the present study.

In another study (55), it was found that the interaction of IκBα is not affected by p65 acetylation and that p65 acetylation reduces the binding of NFκB to DNA. The implication is that acetylation of p65 is an essential step in turning off the transcriptional activity of NFκB. Therefore, inhibition of HDAC activity is consistent with inhibition of NFκB-driven genes such as TNF. Another study also shows that acetylation reduces the activity of transcription factor

HMG1 (56). However, in the case of ITF2357, inhibition of NFκB would not explain the lack of suppression of IL-8 production.

Inhibition of phosphorylation of mitogen-activated protein kinase (MAPK) 11 (also known as MAPK p38) prevents transcription of TNFα and other proinflammatory cytokines by blocking the binding of activating transcription factor 2. ITF2357 exerts no effect on the MAPK p38–regulated pathway (data not shown). Nonhistone interaction of HDAC3 has been described with MAPK 11 (57), in which the deacetylation of this kinase prevents participation in LPS-induced gene expression. It was demonstrated that overexpression of HDAC3 suppresses LPS induction of TNF gene expression (57). Not unexpectedly, inhibition by TSA overcomes the deacetylation by HDAC3 and increases the production of TNFα in LPS-stimulated macrophage cell lines (57).

Also in macrophage cell lines, PMA-stimulated TNFα production is increased upon inhibition of HDAC (58) due to acetylation of histones H3 and H4. However, in primary monocytes, only upon maturation during 7 days of culture does a similar acetylation takes place and increased expression of TNFα is observed (58). The inhibition of TNFα production by p50 homodimers binding to the TNFα promoters is well established. Nevertheless, this reduction in TNFα is reversed by HDAC inhibition and increased TNFα is observed (59). To resolve the discrepancies between increased or decreased TNFα production by inhibition of HDAC, one may conclude that in cell lines increased TNFα production is consistently observed, which may be due to a different acetylation state of nuclear histones or of other cellular proteins substrates of HDAC. On the other hand, inhibition of TNFα gene expression or synthesis by ITF2357 or SAHA is consistently observed in primary cells in in vitro and in vivo models of disease.

The treatment of lupus-prone mice with TSA reduced proteinuria, the infiltration of destructive inflammatory cells into the glomerulus, and spleen weight (9). The clinical benefit of TSA in these mice was associated with decreases in IL-12, IFNγ, and IL-6 and consistent with the data of the present report in primary human cells. TSA is nonspecific in inhibiting HDAC3 and other HDACs. SAHA has been reported to inhibit each of the 11 HDACs (60). Although the evidence that SAHA and ITF2357 reduce inflammation in animal models of disease (8-10,13) via suppression of TNFα, IL-1β, IFNγ, and IL-6 production, is consistent with hyperacetylation of a histone target in primary cells, we cannot rule out that nonhistone substrates have a role in the anti-inflammatory properties of ITF2357.

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