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Histone Deacetylase Inhibitors Suppress Inflammatory Activation of Rheumatoid Arthritis Patient Synovial Macrophages and Tissue

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Macrophages contribute significantly to the pathology of many chronic inflammatory diseases, including rheumatoid arthritis (RA), asthma, and chronic obstructive pulmonary disease. Macrophage activation and survival are tightly regulated by reversible acetylation and deacetylation of histones, transcription factors, and structural proteins. Although histone deacetylase (HDAC) inhibitors (HDACis) demonstrate therapeutic effects in animal models of chronic inflammatory disease, depressed macrophage HDAC activity in patients with asthma, chronic obstructive pulmonary disease, or RA may contribute to inflammation in these diseases, potentially contraindicating the therapeutic administration of HDACis. In this study, we directly examined whether HDACis could influence the activation of macrophages derived from the inflamed joints of patients with RA. We found that inhibition of class I/II HDACs or class III sirtuin HDACs potently blocked the production of IL-6 and TNF-α by macrophages from healthy donors and patients with RA. Two HDACis, trichostatin A and nicotinamide, selectively induced macrophage apoptosis associated with specific downregulation of the antiapoptotic protein Bfl-1/A1, and inflammatory stimuli enhanced the sensitivity of macrophages to HDACi-induced apoptosis. Importantly, inflammatory and angiogenic cytokine production in intact RA synovial biopsy explants was also suppressed by HDACis. Our study identifies redundant, but essential, roles for class I/II and sirtuin HDACs in promoting inflammation, angiogenesis, and cell survival in RA.  The Journal of Immunology, 2010, 184: 2718–2728.

The improper recruitment, activation, and survival of macrophages contribute significantly to the pathology of many chronic inflammatory human diseases, including asthma, chronic obstructive pulmonary disease (COPD), atherosclerosis, and rheumatoid arthritis (RA). In RA, reciprocal activation of synovial macrophages, T lymphocytes, and stromal fibroblast-like synoviocytes by cytokines and cell–cell contacts promotes inflammation and joint destruction (1, 2). TNF-α is a primary activator of macrophages in RA synovial tissue, and evidence has emerged that engagement of TLR-2 and -4 also contributes to macrophage activation in RA (3–5). Clinical disease activity in RA correlates strongly with macrophage numbers in synovial tissue, as well as expression of macrophage-derived cytokines, such as TNF-α and IL-6 (6, 7). Macrophage survival under inflammatory conditions in the RA synovium is supported by the persistently high activation status of NF-κB, STAT3, and PI3K intracellular signaling pathways, which enhance macrophage resistance to apoptosis mediated by Fas–Fas ligand interactions and cytokine withdrawal (8). Because decreases in the number of synovial macrophages and the expression of inflammatory macrophage products correlate strongly with the clinical efficacy of therapeutic compounds (9, 10), the development and application of drugs modulating macrophage function and survival are likely to be of great clinical benefit in RA.

Inflammatory stimuli, such as TNF-α and the TLR-4 ligand LPS, induce the association of multiple transcription factors, including the NF-κB p65 subunit, AP-1, p53, and forkhead box O (FoxO) proteins, with transcriptional coactivators containing intrinsic histone acetyltransferase (HAT) activity. This promotes histone acetylation and the exposure of gene promoter regions for transcription (11). HAT enzymatic activity is counteracted by histone deacetylases (HDACs), four classes of which are expressed in mammals. Class I HDACs 1–3 and 8 are broadly expressed throughout mammalian tissues, whereas class II HDACs (4–7, 9, 10) display tissue-specific expression (11). Macrophage survival under inflammatory conditions in the RA synovium is supported by the persistently high activation status of NF-κB, STAT3, and PI3K intracellular signaling pathways, which enhance macrophage resistance to apoptosis mediated by Fas–Fas ligand interactions and cytokine withdrawal (8). Because decreases in the number of synovial macrophages and the expression of inflammatory macrophage products correlate strongly with the clinical efficacy of therapeutic compounds (9, 10), the development and application of drugs modulating macrophage function and survival are likely to be of great clinical benefit in RA.

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with COPD, decreases in HDAC2 and Sirt1 expression and activity are observed, correlating with disease severity (19, 20). Alveolar macrophages obtained from patients with asthma or COPD are sensitized to LPS-induced TNF-α, GM-CSF, and IL-8 production; restoration of HDAC activity normalizes patient macrophage cytokine responses (17, 21, 22). The potential pathological consequences of depressed HDAC activity were extended to RA, where, compared with healthy individuals and disease controls, the synovial tissue displays a marked reduction in total HDAC activity and HDAC1 and HDAC2 protein expression, particularly in synovial macrophages (23).

Paradoxically, diverse chemical classes of HDAC inhibitors (HDACis) demonstrated therapeutic potential in animal models of asthma (24), colitis (25), multiple sclerosis (26), graft-versus-host disease (27–29), endotoxic shock (30, 31), systemic sclerosis, and arthritis (32–35). Studies demonstrated that multiple class I/II HDACis prevent LPS-induced cytokine production in murine and human monocytes (25, 27, 30, 31, 36) and in human macrophages and dendritic cells, the transcription of chemokines regulating the recruitment of myeloid cells and T lymphocytes (37). Although these observations have generated interest in the application of these compounds to the treatment of RA (38, 39), reports that depressed HDAC activity may contribute to the inflammatory activation of synovial macrophages suggested that patients with RA might be resistant to therapeutic strategies targeting HDACs (23). However, the influence of HDACis on the inflammatory activation of RA synovial cells has not been examined. In this study, we directly investigated the effects of inhibitors of class I/II and class III sirtuin HDACs on the activation and survival of macrophages derived from healthy individuals and patients with RA and the global consequences of exposing intact synovial biopsy explants from patients with RA to HDACis.

Materials and Methods

Patients, monocye isolation, and cell culture

Patients with RA met the American College of Rheumatology revised criteria for RA (40). Patient characteristics are shown in Table I. All patients provided written informed consent, and these studies were approved by the Medical Ethics Committee of the Academic Medical Center, University of Amsterdam and the Institute of Rheumatology, Warsaw, Poland. Isolation of PBMCs from healthy volunteers and PBMCs and synovial fluid (SF) mononuclear cells from patients with RA was performed using Ficoll-Isopaque density gradient centrifugation (Nycomed, Pharma, Oslo, Norway). Peripheral blood (PB) and SF monocytes were purified from PBMCs and SF mononuclear cells by Standard Isotope Percoll gradient centrifugation (Amersham Biosciences, Piscataway, NJ). Monocytes were plated in 24-well plates (5 × 10⁵/ml) in IMDM (Invitrogen, Breda, The Netherlands) supplemented with 1% FBS (Invitrogen) for 1 h at 37°C, nonadherent cells were removed, and monocytes were cultured for 7–9 d in IMDM containing 10% FBS, 100 μg/ml gentamicin, and 5 ng/ml GM-CSF (BioSource International, Camarillo, CA) prior to use in experiments. Purity of monocytes and differentiation of monocytes into macrophages were confirmed by labeling of cells with APC-conjugated anti-CD14 Abs (BD Biosciences, San Jose, CA) and FITC-conjugated anti-CD68 Abs (Dako, Glostrup, Denmark), respectively, and FACS analysis (FACScalibur, BD Biosciences).

Measurement of cytokine production

Macrophages were left unstimulated or were stimulated for 24 h with TNF-α or LPS (both from Sigma-Aldrich, St. Louis, MO), in the absence or presence of compounds of suberoyl hydroxamic acid (SBHA), sodium phenylbutyrate (PheBut) (both from BIOMOL, Plymouth Meeting, PA), trichostatin A (TSA), or nicotinamide (NIC) (both from Sigma-Aldrich). Cell-free supernatants were harvested, and TNF-α, IL-6, -8, and -10 production were measured using PeliKine Compact ELISA kits (Sanquin Reagents, Amsterdam, The Netherlands), as per the manufacturer’s instructions. Alternatively, macrophages were left unstimulated or were stimulated for 6 h with LPS, in the absence or presence of HDACis, and 10 μg/ml brefeldin A was added for the final 4 h of stimulation. Cells were fixed, washed, permeabilized, and stained with PE-conjugated anti–IL-6 and APC-conjugated anti–TNF-α Abs (BD Biosciences) and subjected to FACS analysis using the FlowJo software package. Cytokine and chemokine production in RA synovial biopsy explant cultures was measured by ELISA, as above, or by multiplex immunoassays, as previously described (41).

Western blot analysis

Equivalent numbers of macrophages were lysed in 1× Laemmli’s buffer or in radioimmunoprecipitation lysis buffer (150 mM NaCl, 10 mM Tris, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 μg/ml leupeptin, 1 mM PMSF, 100 mM aprotinin, 200 μM benzamidine, and 2 μg/ml trypsin inhibitor [pH 7.6]). Lysates were resolved by electrophoresis on 4–12% gradient Bis-Tris SDS NuPAGE gels (Invitrogen), and proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) using a semidry transfer apparatus (Invitrogen). Membranes were washed in TBS (pH 8.0) containing 0.05% Tween-20 (Bio-Rad) (TBST), blocked in 2% milk (Bio-Rad)TBST, and incubated overnight at 4°C in primary Abs diluted in TBST. Primary Abs used included those specific for acetyl lysine, acetylated histone 3 (H3), acetylated histone 4 (H4) and H3 (Cell Signaling Technology, Beverly, MA), H4 (Upstate, Temecula, CA), survivin, actin (Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-2/1-A1 (provided by Dr. J. Borst, Netherlands Cancer Institute, Amsterdam, The Netherlands), tubulin, and acetylated tubulin (Sigma-Aldrich). Following washing, membranes were incubated in TBST containing HRP-conjugated anti-rabbit or anti-mouse Ig Abs (Bio-Rad) and developed using an ECL detection kit (Amersham, Little Chalfont, U.K.).

Measurement of macrophage cytokine mRNA expression

Macrophages were left untreated or were stimulated for 4 h with LPS in the presence or absence of 2 μM TSA or 20 mM NIC, harvested, washed with PBS, and total RNA was isolated using a GenElute RNA isolation kit (Sigma-Aldrich). RNA was reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen), and cDNA was amplified by PCR using primers specific for IL-6, TNF-α, IL-1β (Eurogentec, Philadelphia, PA), IL-8, and GADPH (Invitrogen). Following electrophoresis, PCR products were visualized using a Gene Flash imaging system (Westburg, Leusden, The Netherlands).

Measurement of cellular apoptosis

Macrophages were left untreated or were treated with increasing concentrations of HDACis in the presence or absence of TNF-α (10 ng/ml) or LPS (1 μg/ml) for 24–72 h, and apoptosis was evaluated by Annexin V-FITC (IQ Products, Groningen, The Netherlands) binding, propidium iodide (PI) exclusion, and FACS analysis. Data were expressed as the percentage of nonviable (Annexin V+ and/or PI+) cells. To assess disruption of macrophage mitochondrial membrane potential, cells were left untreated or were treated for 24 h with TSA or NIC and incubated with MitoTracker Red CMXRos (Molecular Probes, Eugene, OR) prior to FACS analysis.

Profiling of pro- and antiapoptotic gene expression

Expression of pro- and antiapoptotic genes was assessed by PCR-based multiplex ligation-dependent probe amplification (MLPA) analysis using the SALSA MLPA Kit R011 (MRD-Holland, Amsterdam, The Netherlands), as described previously (42). Data tables containing peak area values for each examined gene were exported and further analyzed with Microsoft Excel spreadsheet software. The sum of all peak data was set as 100% to correct for fluctuations in total signal between samples. Individual peaks were calculated relative to 100% value.

Synovial biopsy acquisition and culture

Synovial biopsies were obtained from patients with established RA by small-bore arthroscopy under local anesthesia, as previously described (43). Intact biopsies were cultured for 48 h in complete DMEM supplemented with 10% FCS and TNF-α (10 ng/ml) (BioSource International) in the absence or presence of increasing concentrations of HDACis. Cell-free tissue culture supernatants were harvested for cytokine analysis.

Statistical analyses

The data are presented as mean ± SEM (ELISA, multiplex immunoassay, and apoptosis measurements) or mean ± SD (MLPA). The distribution of all data sets was analyzed using the Shapiro–Wilks normality test. Because the majority of experimental data did not follow a Gaussian distribution, nonparametric analyses were used for all further comparisons. For comparisons within groups in which multiple comparisons were required, sets of data were analyzed using an overall Friedman test or the Kruskal–Wallis test where appropriate, followed by the post hoc Dunn multiple comparison test using cells not exposed to HDACis as reference controls.
Comparisons between groups were performed using the Wilcoxon signed-rank test. In experiments in which values for samples not treated with HDACi were normalized to 100%, remaining samples were expressed as the mean percentage of control values, and differences between samples were also analyzed using the Wilcoxon signed-rank test. $p$ values $< 0.05$ were considered significant.

**Results**

**TSA and NIC suppress macrophage production of IL-6 and TNF-$\alpha$ but not IL-8**

We initiated our studies by examining the influence of HDACi on human macrophage production of IL-6 and IL-8, two macrophage-derived cytokines important in RA. Healthy donor (HD) PB-derived macrophages were cultured in the absence or presence of increasing concentrations of TSA, an inhibitor of class I/II HDACs or, as a control, NIC, a general inhibitor of class III NAD-dependent sirtuin HDACs. Macrophages were then stimulated with medium alone, TNF-$\alpha$, or LPS. In the absence of additional stimulation, exposure of macrophages to TSA or NIC had no influence on basal IL-8 or -6 or TNF-$\alpha$ production (Fig. 1A). TSA and NIC also failed to influence TNF-$\alpha$–induced IL-8 production (Fig. 1B). However, TSA inhibited TNF-$\alpha$–induced IL-6 production in a dose-dependent manner, reaching maximum inhibition of $\sim 50\%$ at $1\mu M$ ($p < 0.05$). Surprisingly, although a proinflammatory role for Sirt1 has not been described, NIC also suppressed TNF-$\alpha$–induced IL-6 ($p < 0.001$). Both compounds also effectively blocked IL-6 and TNF-$\alpha$ production induced by LPS (Fig. 1C). TSA achieved 70% inhibition of IL-6 production at $1\mu M$ and >80% inhibition of IL-6 production at higher concentrations ($p < 0.001$) in LPS-stimulated macrophages. A similar 80% reduction in LPS-induced IL-6 production was observed when macrophages were treated with NIC ($p < 0.001$). TNF-$\alpha$ generated in response to LPS was also reduced by both tested compounds. Treatment with 250 nM TSA resulted in 60% reduction in TNF levels generated in response to LPS, and this effect was not increased at greater TSA concentrations. Strikingly, in the presence of NIC, LPS-induced TNF production was blocked by $> 95\%$ ($p < 0.001$). NIC had no significant effect on LPS-induced IL-8 production; a trend toward enhanced IL-8 production in response to LPS was noted in macrophages exposed to low concentrations of TSA (250 nM), which was reversed as TSA concentrations were increased.

Two other inhibitors of class I/II HDACs (SBHA and PheBut) demonstrated similar effects on macrophage cytokine production. Both HDACi dose-dependently suppressed IL-6 production in response to LPS and TNF-$\alpha$ and TNF-$\alpha$ production in response to LPS (Fig. 2). As observed with TSA, SBHA and PheBut failed to affect TNF-induced IL-8 production, but lower concentrations of SBHA ($p < 0.01$) and PheBut enhanced LPS-induced IL-8 production. These agonistic effects disappeared as HDACi concentrations were increased (Fig. 2B).

**HDACi suppression of cytokine production does not correlate with global changes in histone acetylation**

Effects of HDACi on macrophage cytokine production did not strictly correlate with the effects of these compounds on global histone acetylation (Fig. 3). Incubation of macrophages with TSA and SBHA resulted in a time-dependent increase in the acetylation of H3 and H4, also observed to a lesser degree and with delayed kinetics in PheBut-treated cells. Sirt1 was reported to target H3 and H4 in vitro (44), but exposure of macrophages to NIC failed to induce detectable acetylation of H3 or H4. Acetylation of multiple nonhistone cellular proteins, ranging from 20–40 kDa in size, was...
observed with each HDACi. Together, these experiments verified the biological activity of the HDACi in human macrophages but failed to reveal a close association between HDACi-dependent histone acetylation and the ability of HDACIs to regulate macrophage cytokine production.

HDACis suppress cytokine gene transcription

In monocytes, suberoylanilide hydroxamic acid and ITF2357 HDACis prevent LPS-induced gene transcription of TNF-α and the secretion, but not the transcription, of IL-1β (30, 31, 36). To address which mechanism(s) might contribute to HDACi suppression of macrophage cytokine production, we examined treated macrophages by intracellular staining and FACS analysis. When macrophages were stimulated with LPS in the presence of brefeldin A, TNF-α accumulated in the cells (Fig. 4A, left panel). However, exposure of macrophages to TSA or NIC reduced TNF-α synthesis to background levels observed in unstimulated macrophages. In the absence of brefeldin A, although only a minor accumulation of TNF-α was observed in LPS-treated macrophages, no intracellular retention of TNF-α was induced by the presence of TSA or NIC (Fig. 4A, middle and right panels). Similar results were obtained with regard to LPS-induced IL-6 (Fig. 4B). Deregulated acetylation of tubulin has been identified as a mechanism by which HDACis can influence cytokine secretion (45). However, we found that TSA, but not NIC, could induce tubulin acetylation in LPS-treated macrophages (Fig. 4C), arguing against a general role for tubulin acetylation in the inhibition of cytokine production. Instead, TSA and NIC blocked LPS-induced accumulation of IL-6 and TNF-α mRNA (Fig. 4D), while having no effect on the accumulation of IL-8 and IL-1β mRNA, as previously observed in human monocytes (36). Together, these results indicate that HDACis suppress IL-6 and TNF-α production by blocking cytokine synthesis rather than secretion.

HDACis block cytokine production in synovial macrophages from patients with RA

We next addressed whether synovial macrophages from patients with RA (Table I) were also susceptible to the anti-inflammatory properties of HDACis observed in HD PB-derived macrophages. Monocytes were isolated from SF of five patients with RA and differentiated into macrophages. Basal levels of IL-6 production in RA SF macrophages were similar to those observed in HD macrophages, but basal IL-8 production was strongly elevated in RA SF macrophages (RA SF: 76.47 ± 33.93 ng/ml; HD: 17.93 ± 6.65 ng/ml). Additionally, the concentrations of IL-8 detected after TNF-α stimulation (RA SF: 107.6 ± 55.3 ng/ml; HD: 45.5 ± 15.9 ng/ml) were also elevated in RA SF-derived macrophages, indicating that proinflammatory properties of RA SF macrophages were maintained ex vivo. Because we noted wide variation in basal and inducible levels of cytokine

FIGURE 2. SBHA and PheBut suppress inflammatory cytokine production in human macrophages. Macrophages were stimulated with TNF-α (n = 7) (A) or LPS (n = 7) (B) for 24 h in the absence or presence of increasing concentrations of PheBut or SBHA. Tissue culture supernatant concentrations of IL-8 (left panels), IL-6 (middle panels), and TNF-α (right panels) were determined by ELISA. *p < 0.05; **p < 0.01; ***p < 0.001.

FIGURE 3. HDACis differentially induce acetylation of histone and nonhistone proteins in macrophages. Macrophages were left untreated in medium (med) or incubated for the indicated time (in minutes) in the presence of TSA (2 μM), SBHA (50 μM), PheBut (5 mM), or NIC (20 mM). Total cell lysates were prepared and analyzed by Western blotting with Abs recognizing acetyl lysine, acetylated (Ac)-H4, H4, Ac-H3, and H3. Results shown are representative of three independent experiments.
production by RA SF macrophages, the effects of HDACi on cytokine production in these cells were analyzed in terms of absolute concentrations of cytokines (Fig. 5A–C), as well as normalized to cytokine production by cells not treated with HDACis for each patient (Fig. 5D–F). Neither TSA nor NIC influenced basal RA SF macrophage IL-8 or -6 production (Fig. 5A, 5D). As observed in HD macrophages, TSA also failed to influence TNF-α– (Fig. 5B, 5E) or LPS-induced (Fig. 5C, 5F) IL-8 production in RA SF macrophages. In contrast, NIC blocked inducible IL-8 production by ∼30% (p < 0.01). Both HDACis blocked TNF-induced IL-6 production, with TSA demonstrating 50% inhibition at 1 and 2 μM (p < 0.05) and 70% inhibition by NIC at 20 μM (p < 0.01) (Fig. 5B, 5E). A clear dose-dependent reduction in IL-6 production mediated by LPS was also observed following HDACi treatment: TSA achieved 70% inhibition at 1 μM and >90% at 2 μM (p < 0.01); NIC achieved 90% inhibition at 20 mM (p < 0.01) (Fig. 5C, 5F). Collectively, these results suggest that the anti-inflammatory properties of HDACis are largely preserved in RA SF macrophages.

**HDACis induce apoptosis in human macrophages**

We next evaluated whether HDACi effects on macrophage cytokine production might be secondary to macrophage apoptosis. Macrophages were left untreated or were treated for 24 h with HDACis, and apoptosis induction was assessed by FACS analysis of cells stained with PI and Annexin V (Fig. 6A). Although lower doses of TSA (0.25 and 1 μM) and NIC (10 mM) that were capable of blocking macrophage cytokine production (Fig. 1) had no effect on macrophage survival, the highest concentrations of TSA (2 μM) and NIC (20 mM) resulted in a clear induction of apoptosis, as noted by positive staining with Annexin V and/or PI (Fig. 6A, 6B). TSA and NIC induced macrophage apoptosis in a time-dependent manner (Fig. 6C) associated with mitochondrial depolarization (Fig. 6D).

To evaluate the impact of HDACis on macrophage survival under inflammatory conditions, we incubated macrophages with increasing concentrations of TSA or NIC in the absence or presence of TNF-α (Fig. 7A) or LPS (Fig. 7B). Although TNF-α and LPS induced some degree of apoptosis in macrophage cultures, the cooperative effects of these stimuli with TSA or NIC were observed only at the highest HDACi concentrations tested (p < 0.05). Surprisingly, PheBut and SBHA failed to induce apoptosis in macrophages at any concentration tested and failed to enhance TNF-α– or LPS-induced apoptosis (Fig. 7C). Thus, these
experiments indicated that HDACis suppress macrophage cytokine production independently of apoptosis induction.

**HDACis induce apoptosis in RA synovial macrophages**

Because chronic activation of prosurvival NF-κB, PI3-K, and STAT signaling pathways protect RA SF-derived macrophages against many apoptotic stimuli (8), we examined whether these cells were also protected against HDACi-induced apoptosis. Direct comparison of PB- and SF-derived macrophages from patients with RA indicated no significant differences in the rate of apoptosis under basal conditions or following stimulation with TNF-α or LPS (Fig. 8, left panel). TSA alone induced 40% apoptosis in RA PB and SF macrophages, whereas NIC treatment led to a slight increase in apoptosis in both cell populations. The presence of proinflammatory stimuli, such as TNF-α (Fig. 8, middle panel) or LPS (Fig. 8, right panel), sensitized RA PB and SF macrophages to TSA-induced apoptosis. The percentage of apoptotic cells detected after NIC treatment was also slightly elevated in the presence of TNF-α or LPS. However, HDACis induced apoptosis in both cell populations to a similar degree. These data suggest that although there are no obvious differences in the apoptotic responses of RA PB and SF macrophages to HDACis, inflammatory stimuli, in general, sensitize macrophages to HDACi-induced apoptosis.

**TSA and NIC suppress macrophage expression of antiapoptotic Bfl-1 protein**

To gain more insight into the molecular mechanisms by which HDACis induce macrophage apoptosis, we left macrophages untreated or incubated them for 24 h in the presence of TSA or NIC and quantitatively measured mRNA expression of 33 genes representing essentially all known direct regulators of cellular apoptosis (Fig. 9A). Compared with untreated macrophages, macrophages exposed to TSA demonstrated no significant changes in the expression of the control housekeeping genes β2M and GUS (Fig. 9A, 9B). TSA treatment decreased the expression of the antiapoptotic Bcl-2-like protein Bfl-1 (also known as A1) by 70% (p < 0.05), whereas NIC reduced Bfl-1 expression by 30%; however, this trend did not reach statistical significance (Fig. 9B). Expression of two inhibitor of apoptosis protein (IAP)-like gene products was also affected by treatment with HDACis: livin expression was decreased 70% following TSA and NIC exposure, whereas the expression of survivin was selectively suppressed by TSA (p < 0.05). However, mRNA levels of these genes in untreated macrophages were low (representing <1% of total gene transcripts within the assay) (Fig. 9A). TSA and NIC ablated Bfl-1 protein expression as detected by Western blotting of macrophage lysates (Fig. 9C), and upregulation of Bfl-1 protein expression following TNF-α stimulation was also inhibited by TSA and NIC (Fig. 9D).

**HDACis block cytokine production in synovial tissue explants from patients with RA**

Studies in isolated macrophages derived from patients with RA cannot take into consideration adaptive changes of the cells in culture, fail to preserve cell–cell contacts and cytokine networks, and ignore the potential effects of HDACis on other synovial cells, such as lymphocytes and fibroblast-like synoviocytes (FLS). Each of these possibilities would impact upon the potential application

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**FIGURE 5.** TSA and NIC suppress cytokine production in SF macrophages from patients with RA. Macrophages were derived from SF and were left unstimulated (A, D) or were stimulated for 24 h with TNF-α (10 ng/ml) (B, E) or LPS (1 μg/ml) (C, F) in the absence or presence of increasing concentrations of TSA or NIC. Tissue culture supernatants were harvested and assessed for IL-8 (left panels) and IL-6 (right panels) by ELISA. Results are expressed as cytokine concentrations (A–C; each patient is represented by a different symbol [i.e., □, ○, ●, and ▲]) or concentrations were normalized to 100% in each experiment for samples not treated with HDACis and expressed as the percentage of control (D–F). Results represent the mean ± SEM of four or five independent experiments. *p < 0.05; **p < 0.01.
of HDACis in the clinical setting. Therefore, we examined the influence of increasing concentrations of HDACis on cytokine production in intact synovial biopsy explants from patients with clinically active RA obtained during arthroscopic surgery. All tested HDACis markedly inhibited synovial tissue production of IL-6 (Fig. 10A) and IL-8 (Fig. 10B). We extended our analyses to gain a more global insight into the effects of HDACis on the regulation of secreted products relevant to pathology in RA and/or previously identified as HDACi-regulated gene products in myeloid lineage cells (Table II) (1, 37). Because of the expected non-Gaussian distribution of data and the large number of conditions tested, nonparametric statistical analyses could not be performed on data presented in Fig. 10 and Table II, and data are presented descriptively. Among tested chemokines, CCL2 and CXCL-12 production was potently blocked by each of the HDACis, CCL5 was selectively suppressed by PheBut, and none of the HDACis significantly impacted upon CCL3 production. Levels of IL-15, which supports the survival of effector memory T cells, were largely unaffected, although NIC effected a modest suppression. Production of the Th17-polarizing cytokines IL-12 p40 and IL-23 was not influenced, except following NIC treatment, when a 40% decrease in IL-23 levels was observed. Additionally, IL-17 levels in explant culture supernatants were unaffected, although NIC effected a modest suppression. HDACi cytokine suppression was not limited to proinflammatory gene products, because we also observed that IL-10 content in explant cultures was uniformly suppressed by HDACis. Finally, proangiogenic vascular endothelial growth factor production was potently suppressed by TSA and PheBut and more modestly suppressed by NIC. Collectively, our data demonstrate that in RA synovial tissue, HDACis suppress the production of distinct, but overlapping, profiles of proinflammatory cytokines, chemokines, and growth factors.

FIGURE 6. TSA and NIC induce apoptosis in human macrophages. A, Representative plots of macrophages stained with PI and Annexin V-FITC and assessed by FACS following 24 h incubation in medium alone or medium containing TSA (2 μM) or NIC (20 mM). B, Analysis of macrophage apoptosis as assessed by staining with PI and Annexin V-FITC and FACS analysis following 24 h incubation in medium alone (med) or medium containing increasing concentrations of TSA (μM) or NIC (mM). Cells were considered nonviable if staining was positive for Annexin V or PI. Values represent the mean ± SEM of 10 independent experiments. C, Time-course analysis of apoptosis induction as measured in A of macrophages treated for 24, 48, or 72 h with medium alone (med), TSA (2 μM), or NIC (20 mM). Data represent the mean ± SEM for three independent experiments. D, Measurement of mitochondrial integrity by FACS analysis of macrophages labeled with MitoTracker following 24-h treatment with medium alone, TSA (2 μM), or NIC (20 mM). Graphs are representative of three independent experiments.

FIGURE 7. Inflammatory stimuli sensitize human macrophages to TSA- and NIC-induced apoptosis. Macrophages were left unstimulated in medium (med) or were stimulated for 24 h in the presence of TNF-α (10 ng/ml) (A) or LPS (1 μg/ml) (B) in the absence (control) or presence of increasing concentrations of TSA or NIC, and the percentage of nonviable cells was assessed as in Fig. 6. Data represent the mean ± SEM of at least five independent experiments. *p < 0.05; Wilcoxon signed-rank test. C, Alternatively, macrophages were incubated in medium alone (med), medium containing 2 μM TSA, or medium containing increasing concentrations of PheBut or SBHA alone or in combination with TNF-α or LPS. Data represent the mean ± SEM of at least five independent experiments. *p < 0.05; **p < 0.01. Kruskal–Wallis test followed by post hoc Dunn multiple comparison test using cells not exposed to HDACis as a reference control.

FIGURE 8. HDACis induce apoptosis in RA synovial macrophages. PB- and SF-derived macrophages from patients with RA were incubated in medium alone (left panel), TNF-α (10 ng/ml, middle panel), or LPS (1 μg/ml, right panel) for 24 h in the absence (control) or presence of TSA or NIC, and the percentage of nonviable cells was assessed. Results represent the mean ± SEM of at least four independent experiments.
FIGURE 9. TSA and NIC suppress macrophage expression of antiapoptotic Bfl-1 protein. A, Relative expression of 33 apoptosis regulatory genes and two control genes (β2-microglobulin/B2M and β-glucuronidase/GUS) measured by reverse transcription-MLPA in macrophages incubated for 24 h in medium (med) alone or medium containing TSA (2 μM) or NIC (20 mM). Data for each gene are expressed relative to the total signal in the sample as the mean ± SD of five independent experiments. B, Expression levels of select genes detected by MLPA in macrophages following incubation for 24 h in medium (med) alone or medium containing TSA or NIC. Data are presented as fold induction relative to untreated control ± SD (n = 5). *p < 0.05. C and D, Protein expression of Bfl-1 in macrophages. Macrophages were left unstimulated (C) or were stimulated with TNF-α (10 ng/ml) in the presence or absence of TSA (2 μM) or NIC (20 mM) for 24 h (D); protein extracts were examined by Western blotting for Bfl-1/A1, α-tubulin, or actin.

Discussion

A broad range of HDACis, representing all chemical classes of these compounds, showed protective effects in prophylactic and therapeutic models of RA. Uniformly, these compounds reduce disease severity, paw swelling, synovial infiltration and hyperplasia, inflammatory cytokine production, and joint destruction in rodents (32–35). However, the potential of extrapolating these protective effects of HDACis from animal models to human rodents (32–35). However, the potential of extrapolating these protective effects of HDACis from animal models to human chronic inflammatory disease has been strongly questioned by findings that HDAC activity is depressed at sites of inflammation in patients with asthma, COPD, or RA, particularly in macrophages (17–19, 23). Restoration of HDAC activity in macrophages from patients with asthma or COPD normalizes homeostatic GM-CSF, TNF-α, and IL-8 production and glucocorticoid sensitivity (17, 21, 22). By extension, depressed HDAC activity in RA synovial macrophages was suggested to contribute directly to pathology through epigenetic mechanisms promoting the transcription of inflammatory genes and/or could render patients refractory to HDACi treatment (23, 46). However, the influence of HDACis on macrophage cytokine production in these diseases has not been examined. In this study, we demonstrated that inhibition of class I/II HDACs or class III NAD-dependent sirtuin HDACs can suppress inflammatory activation of synovial macrophages from patients with RA. Two of these HDACis (TSA and NIC) promote macrophage apoptosis, especially in the presence of inflammatory stimuli.

Our findings appear to be incompatible with a model in which relative decreases in HDAC activity promote inflammatory gene transcription by histone-dependent chromatin remodeling. We find no evidence that HDACis enhance the production of IL-8 or other cytokines under homeostatic conditions or following stimulation by TNF-α in macrophages derived from HDs or patients with RA. At the lowest tested concentration of SBHA, we noted a significant increase in LPS-induced IL-8 production in HD macrophages. However, in the rest of our studies, macrophage IL-8 production was unresponsive to the presence of HDACis, a finding independently noted in human monocytes, macrophages, and DCs by other investigators (30, 31, 37). In contrast, HDACis suppressed the expression of most of the secreted cellular activation products examined in our studies. Although we did not directly address whether HDACis regulate macrophage cytokine production by epigenetic or nonepigenetic mechanisms, several lines of evidence indicate that HDACis may exert their anti-inflammatory and proapoptotic effects through mechanisms independent of histone acetylation. First, we found no clear correlation between the ability of class I/II HDACis to induce macrophage histone acetylation and the ability of these compounds to suppress cytokine production. Specifically, inhibition of sirtuin HDACs with NIC failed to induce histone acetylation but potently blocked cytokine production. Second, recent studies demonstrated that HDACs evolved prior to histones, and some 200 nonhistone protein targets of HDACs have been described (47). Third, many nonhistone targets known to regulate macrophage activation and survival are targeted by class I/II and sirtuin HDACs, including FoxO, c-jun, NF-κB p65, and STAT transcription factors (38). Although we cannot rule out the possibility that HDACis mediate their anti-inflammatory effects via non-HDAC targets, other candidates have yet to be identified.

Importantly, we observed that HDACis selectively suppress the production of a broad range of proinflammatory growth factors, chemokines, and cytokines in intact RA synovial tissue explants. We noted strong suppression of IL-8 expression in RA synovial explants following HDACi treatment, likely indicating targeting of other cell types in the synovial tissue, such as FLS. Previous
gene-array studies in LPS-treated human macrophages and DCs indicated that HDACis selectively suppress macrophage expression of chemokines and cytokines regulating Th1 function and the recruitment of monocytes and macrophages (37). In RA synovial explants, we observed suppression of the chemokines CCL2, CCL5, and CXCL-12, whereas CCL3 levels were unaffected. Although HDACis were shown to suppress murine DC production of cytokines needed for in vitro and in vivo differentiation and expansion of Th17 cells (48), we observed the suppression of IL-6 and -23, but not IL-12 p40, in RA synovial explants and no impact of these compounds on the levels of IL-17 secreted by the synovial tissue. However, IL-17 levels in untreated explants were minimally above the lower detection limit of our assay, consistent with recent findings that little IL-17 is detectable in SF of patients with established RA (49). Anti-inflammatory IL-10 production was also reduced in synovial explants treated with HDACis, highlighting that HDACis do not target only proinflammatory gene products. Because the genetic silencing of HDAC11 enhances IL-10 production by myeloid cells (50), our finding also underscores the difficulty of extrapolating the effects of inhibiting specific HDACs to predicting the effects of pharmacological HDAC inhibition in vivo.

Our finding that inhibition of class III sirtuin HDACs with NIC mirrored the ability of inhibitors of class I/II HDACs to suppress macrophage and synovial explant cytokine production, as well as induce macrophage apoptosis, is surprising. Like HDAC2, Sirt1 expression and activity are decreased in lung tissue of patients with COPD, and cigarette smoke suppresses Sirt1 expression by transcriptional and posttranslational mechanisms (20). The contribution of depressed Sirt1 activity to pathology in COPD is suggested by the observation that genetic silencing of Sirt1 expression in human monocytic cell lines enhances NF-κB transcriptional activity and homeostatic IL-8 production (20). Additionally, mice genetically deficient for Sirt1 develop autoimmune disease marked by Ab deposition in liver and kidneys and symptoms of diabetes (51). Together, these observations would predict that pharmacological inhibition of sirtuin promotes inflammation.

We also identified the antiapoptotic Bcl-2 family member Bfl-1 as a target of HDACis in macrophages. HDACis were demonstrated to induce apoptosis in cancer cell lines through modulation of extrinsic death-receptor pathways (through modulation of Fas, FLIP, and TRAIL) and intrinsic mitochondrial pathways (via regulation of expression of Bcl-2 family members, including Bcl-2, Bcl-7A, survivin, XIAP, Bid, Bim, Bmf, Noxa, and Puma) (52). In macrophages, sustained activation of STAT, PI3K, and NF-κB signaling pathways promotes cell survival by upregulating the expression of FLIP, Bfl-1, and McI-1 (53–59). We found that TSA and NIC selectively suppressed homeostatic macrophage expression of Bfl-1 and prevented the upregulation of this antiapoptotic protein following macrophage exposure to TNF-α. Bfl-1 protects cells against apoptosis by neutralizing proapoptotic Bid and Bak proteins (60), and an essential role for Bfl-1 in maintaining human macrophage survival was established in gene-silencing experiments (59). Curiously, although PheBut and SBHA inhibited macrophage cytokine production as effectively as TSA, they failed to induce apoptosis of macrophages. This may indicate that although TSA, PheBut, and SBHA fail to discriminate between classes I and II HDAC isoforms in vitro (61), these compounds...
may selectively inhibit distinct HDACs in vivo. Further research is
needed to determine whether diverse HDACs, including Sirt1,
regulate macrophage activation and survival through common or
convergent signaling pathways.

Importantly, the induction of macrophage apoptosis by HDACis
was significantly enhanced in the presence of TNFR and TLR
ligands, inflammatory stimuli readily found in RA synovial tissue.
Similarly, HDACis induce apoptosis of RA stromal FLS only in
the presence of TRAIL (34, 62). Selective effects of HDACis on cells
in inflammatory tissue were also observed in vivo; topical appli-
cation of TSA and PheBut induced cell cycle inhibitor expression
in the tissue of arthritic, but not nonarthritic, rats (32). Thus,
therapeutic administration of HDACis might selectively induce
apoptosis at sites of inflammation in RA. Our results suggest that
although HDAC activity may be reduced in RA synovial tissue
(23), residual HDAC activity plays an essential role in maintaining
cellular activation and survival and presents a potential opportu-
nity for therapeutic application of HDACis in RA.

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Disclosures
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