Regulation of inflammation by histone deacetylases in rheumatoid arthritis: beyond epigenetics
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Citation for published version (APA):
Grabiec, A. M. (2012). Regulation of inflammation by histone deacetylases in rheumatoid arthritis: beyond epigenetics

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CHAPTER 5

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 disorder (COPD). Macrophage activation and survival is tightly regulated by reversible acetylation and deacetylation of histones, transcription factors and structural proteins. While histone deacetylase (HDAC) inhibitors (HDACi) demonstrate therapeutic effects in animal models of chronic inflammatory disease, depressed macrophage HDAC activity in asthma, COPD, and RA patients may contribute to inflammation in these diseases, potentially contraindicating therapeutic administration of HDACi. In this study, we directly examined if HDACi could influence activation of macrophages derived from the inflamed joints of RA patients. We found that inhibition of either class I/II HDACs or class III sirtuin HDACs potently blocked production of interleukin-6 and tumor necrosis factor-α by both healthy donor and RA patient synovial macrophages. Two HDACi, Trichostatin A and nicotinamide, selectively induced macrophage apoptosis associated with specific down-regulation of the anti-apoptotic protein Bfl-1/A1, and inflammatory stimuli enhanced sensitivity of macrophages to HDACi-induced apoptosis. Importantly, inflammatory and angiogenic cytokine production in intact RA synovial biopsy explants was also suppressed by HDACi. Our data identify redundant but essential roles for class I/II and sirtuin HDACs in promoting inflammation, angiogenesis, and cell survival in RA.
INTRODUCTION

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. Tumor necrosis factor-α (TNFα) is a primary activator of macrophages in RA synovial tissue, and evidence has emerged that engagement of Toll-like receptor (TLR)-2 and TLR-4 also contributes to macrophage activation in RA. Clinical disease activity in RA correlates strongly with macrophage numbers in synovial tissue, as well as expression of macrophage-derived cytokines such as TNFα, interleukin (IL)-6, and IL-8. Macrophage survival under inflammatory conditions in the RA synovium is supported by the persistently high activation status of nuclear factor-κB (NF-κB), STAT3, and phosphatidylinositol 3-kinase (PI3K) intracellular signaling pathways, which enhance macrophage resistance to apoptosis mediated by Fas-FasL interactions and cytokine withdrawal. Because decreases in the number of synovial macrophages and the expression of inflammatory macrophage products, correlate strongly with the clinical efficacy of therapeutic compounds, 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 TNFα and the TLR-4 ligand lipopolysaccharide (LPS), induce association of multiple transcription factors, including the NF-κB p65 subunit, AP-1, p53, and forking box O (FoxO) proteins, with transcriptional coactivators containing intrinsic histone acetyltransferase (HAT) activity. This promotes histone acetylation and exposure of gene promoter regions for transcription. 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, while class II HDACs (4-7, 9, and 10) display tissue-specific expression. Class I/II HDACs and the structurally unrelated nicotinamide adenosine dinucleotide (NAD)-dependent class III HDAC Sirt1 can also regulate gene transcription indirectly through deacetylation of intracellular signaling proteins, including p53, NF-κB p65, c-jun, FoxO transcription factors, and components of the JAK/STAT pathway.

Increased HAT activity and reduced HDAC activity are observed in bronchial biopsies and alveolar macrophages isolated from patients with asthma, localized to the site of inflammation. In patients with COPD, decreases in HDAC2 and Sirt1 expression and activity are observed, correlating with disease severity. Alveolar macrophages obtained from patients with asthma or COPD are sensitized to LPS-induced TNFα, GM-CSF, and IL-8 production; restoration of HDAC activity normalizes patient macrophage cytokine responses. The potential pathological consequences of depressed HDAC activity were extended to RA, where, compared to 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.

Paradoxically, diverse chemical classes of HDAC inhibitors (HDACi) demonstrated therapeutic potential in animal models of asthma, colitis, multiple sclerosis, graft versus host disease, endotoxic shock, systemic sclerosis, and arthritis. Studies demonstrated that multiple class I/II HDACi prevent LPS-induced cytokine production in murine and human monocytes, and in human macrophages and dendritic cells, the transcription of chemokines regulating the recruitment of myeloid cells and T lymphocytes. Although these
observations have generated interest in the application of these compounds to the treatment of RA.\textsuperscript{38,39} reports that depressed HDAC activity may contribute to the inflammatory activation of synovial macrophages have suggested that RA patients might be resistant to therapeutic strategies targeting HDACs.\textsuperscript{23} However, the influence of HDACi 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 HDACi.

**MATERIALS AND METHODS**

**Patients, monocyte isolation and cell culture.** Patients with RA met the American College of Rheumatology (ACR) revised criteria for RA.\textsuperscript{40} Patient characteristics are shown in Table 1. 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 peripheral blood (PB) mononuclear cells (PBMCs) from healthy volunteers and PBMC and synovial fluid mononuclear cells (SFMCs) from patients with RA was performed using Ficoll-Isopaque density gradient centrifugation (Nycomed, Pharma, Oslo, Norway). PB and SF monocytes were purified from PBMCs and SFMCs by Standard Isotone Percoll gradient centrifugation (Amersham Biosciences, Piscataway, NJ). Monocytes were plated in 24-well plates (5x10\textsuperscript{5}/ml) in Iscove modified Dulbecco medium (IMDM, Invitrogen, Breda, The Netherlands) supplemented with 1% fetal bovine serum (FBS, Invitrogen) for 1 hour at 37°C, non-adherent cells were removed, and monocytes cultured for 7-9 days in IMDM containing 10% FBS, 100 μg/ml gentamycin 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 antibodies (BD Biosciences, San Jose, USA) and FITC-conjugated anti-CD68 antibodies (Dako, Glostrup, Denmark), respectively, and FACS analysis (FACSCalibur, BD Biosciences).

**Table 1. Clinical features of patients with RA included in the study (n = 10).**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>56.5 (34-70)</td>
</tr>
<tr>
<td>Male: Female</td>
<td>3:7</td>
</tr>
<tr>
<td>Disease duration [years]</td>
<td>5 (1-19)</td>
</tr>
<tr>
<td>ESR [mm/hour]</td>
<td>46 (16-120)</td>
</tr>
<tr>
<td>CRP [mg/l]</td>
<td>47.5 (3-98)</td>
</tr>
<tr>
<td>RF [kU/l]</td>
<td>19 (7-358)</td>
</tr>
<tr>
<td>ACPA [kU/l]</td>
<td>199 (42-5043)</td>
</tr>
</tbody>
</table>

ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; RF, rheumatoid factor; ACPA, anti-ctitrullinated protein antibodies
**Measurement of cytokine production.** Macrophages were left unstimulated or were stimulated for 24 hours with TNFα or LPS (both from Sigma-Aldrich, St. Louis, MO), in the absence or presence of increasing concentrations of suberoyl bis-hydroxamic acid (SBHA), sodium phenylbutyrate (PheBut) (both from Biomol International LP, Plymouth Meeting, PA), trichostatin A (TSA) or nicotinamide (NIC) (both from Sigma-Aldrich). Cell-free supernatants were harvested, and TNFα, IL-6, IL-8, and IL-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 hours with LPS, in the absence or presence of HDACi, and 10 μg/ml brefeldin A was added for the final four hours of stimulation. Cells were fixed, washed, permeabilized, and then stained with PE-conjugated anti-IL-6 and APC-conjugated anti-TNFα antibodies (BD Biosciences), and subjected to FACS analysis using FlowJo software package. Cytokine and chemokine production in RA synovial biopsy explant cultures was measured either by ELISA as above, or by multiplex immunoassays, as previously described.41

**Western blot analysis.** Equivalent numbers of macrophages were lysed in 1x 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 nM aprotinin, 200 μM benazmidine 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 transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA) using a semidry transfer apparatus (Invitrogen). Membranes were washed in Tris-buffered saline (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 antibody diluted in TBS/T. Primary antibodies used included those specific for acetyl lysine, acetylated histone 3 (H3), acetylated histone 4 (H4) and H3 (Cell Signaling, Beverly, MA), H4 (Upstate, Temecula, CA), actin (Santa Cruz Biotechnology, Santa Cruz, CA), Bfl-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 immunoglobulin antibodies (Bio-Rad) and developed using an enhanced chemiluminescence (ECL) detection kit (Amersham, Little Chalfont, UK).

**Measurement of macrophage cytokine mRNA expression.** Macrophages were left untreated or were stimulated for 4 hours with LPS in the presence or absence of 2 μM TSA or 20 mM NIC, harvested, washed with PBS and total RNA isolated using a GenElute RNA isolation kit (Sigma-Aldrich). RNA was reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen) and cDNA was amplified by polymerase chain reaction (PCR) using primers specific for IL-6, TNFα, 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 HDACi in the presence or absence of TNFα (10 ng/ml) or LPS (1 μg/ml) for 24-72 hours, and apoptosis was evaluated by Annexin V-FITC (IQ Products, Groningen, The Netherlands) binding and propidium iodide (PI) exclusion, and FACS analysis. Data was 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 treated for 24 hours with TSA or NIC and incubated with MitoTracker Red CMXRos (Molecular Probes, Eugene, OR) prior to FACS analysis.

Profiling of pro- and anti-apoptotic 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 (MRC-Holland, Amsterdam, The Netherlands) as described previously. Data tables containing peak area values for each examined gene were exported and further analyzed with Microsoft Excel spreadsheet software (Microsoft, Redman, WA). 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. Intact biopsies were cultured for 48 hours in complete DMEM supplemented with 10% FCS and TNFα (10 ng/ml) (Biosource International) in the absence or presence of increasing concentrations of HDACi. Cell-free tissue culture supernatants were harvested for cytokine analysis.

Statistical analyses. The data are presented as means ± SEM (ELISA, multiplex immunoassay, and apoptosis measurements) or means ± SD (MLPA). The distribution of all data sets was analyzed using the Shapiro-Wilk 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 post hoc Dunn multiple comparison test using cells not exposed to HDACi 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 TNFα 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, TNFα, or LPS. In the absence of additional stimulation, exposure of macrophages to TSA or NIC had no influence on basal IL-8, IL-6 or TNFα production (Fig. 1A). TSA and NIC also failed to influence TNFα-induced IL-8 production (Fig. 1B). However, TSA inhibited TNFα-induced IL-6 production in a dose-dependent manner, reaching maximum inhibition of ~50% at 1 μM (p < 0.05). Surprisingly, although a proinflammatory role for Sirt1 has not been described, NIC also suppressed TNFα-induced IL-6 (p < 0.001). Both compounds also effectively blocked IL-6 and TNFα
production induced by LPS (Fig. 1C). TSA achieved 70% inhibition of IL-6 production at 1μM 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). TNFα generated in response to LPS was also reduced by both tested compounds. Treatment with 250 nM TSA resulted in 60% reduction in TNFα levels generated in response to LPS, and this effect was not increased further at higher TSA concentrations. Strikingly, in the presence of NIC, LPS-induced TNFα production was blocked by greater than 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 TNFα and TNFα production in response to LPS (Fig. 2). As observed with TSA, both SBHA and PheBut failed to affect TNFα-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 further 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 has been 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 HDACi to regulate macrophage cytokine production.

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**Figure 2.** SBHA and PheBut suppress inflammatory cytokine production in human macrophages. Macrophages were stimulated with TNFα (n = 7) (A) or LPS (n = 7) (B) for 24 hours 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 TNFα (right panels) were determined by ELISA. *p < 0.05, **p < 0.01, ***p < 0.001.
HDACi suppress cytokine gene transcription. In monocytes, suberoylanilide hydroxamic acid (SAHA) and ITF2357 HDACi prevent LPS-induced gene transcription of TNFα, and the secretion but not the transcription of IL-1β. 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, TNFα accumulated in the cells (Fig. 4A, left panel). However, exposure of macrophages to TSA or NIC reduced TNFα synthesis to background levels observed in unstimulated macrophages. In the absence of brefeldin A, while only a minor accumulation of TNFα was observed in LPS-treated macrophages, no intracellular retention of TNFα was induced by the presence of TSA or NIC (Fig. 4A, middle and right panels). Similar results were obtained in regard to LPS-induced IL-6 (Fig. 4B). Deregulated acetylation of tubulin has been identified as a mechanism by which HDACi can influence cytokine secretion. However, we found that only 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, both TSA and NIC blocked LPS-induced accumulation of IL-6 and TNFα mRNA (Fig. 4D), while having no effect on the accumulation of IL-8 and IL-1β mRNA, as previously observed in human monocytes. Together, these results indicate that HDACi suppress IL-6 and TNFα production by blocking cytokine synthesis rather than secretion.

HDACi block cytokine production in synovial macrophages from patients with RA. We next addressed whether synovial macrophages from patients with RA (Table 1) were also susceptible to the anti-inflammatory properties of HDACi observed in HD PB-derived macrophages.
Figure 4. TSA and NIC suppress macrophage IL-6 and TNFα transcription rather than secretion. (A), (B) Macrophages were left unstimulated in medium (med) or stimulated for 6 hours with LPS (1 μg/ml) in the absence or presence of TSA (2 μM) or NIC (20 mM). Brefeldin A (10 μg/ml) was included (+bref A, left panels) or omitted (-bref A, middle panels) for the last 4 hours of stimulation. Cells were subjected to intracellular staining for TNFα (A) and IL-6 (B) and FACS analysis. Data shown are the results of one of four independent representative experiments. Right panels represent mean ± SEM of cytokine staining from four independent experiments. (C) TSA, but not NIC, promotes acetylation of tubulin. Macrophages were left unstimulated in medium (med) or stimulated for 4 hours with LPS (1 μg/ml) in the absence or presence of TSA (2 μM) or NIC (20 mM). Cellular lysates were prepared and examined by Western blotting for acetylated (Ac-) and total tubulin (Tub) content. (D) TSA and NIC inhibit LPS-induced expression of IL-6 and TNFα, but not IL-8 and IL-1β mRNA. Macrophages were left unstimulated in medium (med) or stimulated for 4 hours with LPS (1 μg/ml) in the absence or presence of TSA (2 μM) or NIC (20 mM), and expression levels of IL-6, TNFα, IL-8, IL-1β, and GAPDH mRNA were analyzed by PCR.
Monocytes were isolated from SF of five RA patients 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 TNFα 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 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 HDACi for each patient (Fig. 5D-F). Neither TSA nor NIC influenced basal RA SF macrophage IL-8 or IL-6 production (Fig. 5A and D). As observed in HD macrophages, TSA also failed to influence TNFα (Fig. 5B and E), or LPS –induced (Fig. 5C and F) IL-8 production in RA SF macrophages. In contrast, NIC blocked inducible IL-8 production by ~30% (p < 0.01) (Fig. 5B and E). Both HDACi blocked TNFα-induced IL-6 production with TSA demonstrating 50% inhibition at 1 μM and 2 μM (p < 0.05), and 70% suppression of IL-6 production by NIC.

**Figure 5.** TSA and NIC suppress cytokine production in SF macrophages from patients with RA. Macrophages were derived from SF and left unstimulated (A, D) or were stimulated for 24 hours with TNFα (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 HDACi and expressed as the percentage of control (D-F). Results indicate the mean ± SEM of four or five independent experiments. *p < 0.05, **p < 0.01.
inhibition by NIC at 20 mM ($p < 0.01$) (Fig. 5B and E). A clear dose-dependent reduction of IL-6 production mediated by LPS was also observed following HDACi treatment: TSA achieved 70% inhibition at 1 μM and >90% at 2 μM ($p < 0.01$), and NIC achieved 90% inhibition at 20 mM ($p < 0.01$) (Fig. 5C and F). Collectively, these results suggest that the anti-inflammatory properties of HDACi are largely preserved in RA SF macrophages.

**HDACi induce apoptosis in human macrophages.** We next evaluated if HDACi effects on macrophage cytokine production might be secondary to macrophage apoptosis. Macrophages were left untreated or were treated for 24 hours with HDACi, and apoptosis induction 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 and 6B). Both TSA and NIC induced macrophage apoptosis in a time-dependent manner (Fig. 6C) associated with mitochondrial depolarization (Fig. 6D).

To evaluate the impact of HDACi on macrophage survival under inflammatory conditions, we incubated macrophages with increasing concentrations of TSA or NIC in the absence or presence of TNFα (Fig. 7A) or LPS (Fig. 7B). Although TNFα and LPS induced some degree of apoptosis in macrophage cultures, 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 TNFα- or LPS- induced apoptosis (Fig. 7C). Thus, these experiments indicated that HDACi suppress macrophage cytokine production independently of apoptosis induction.

**HDACi induce apoptosis in RA synovial macrophages.** As chronic activation of pro-survival NF-κB, PI3K, and STAT signaling pathways protect RA SF-derived macrophages against many apoptotic stimuli, we examined if these cells were also protected against HDACi-induced apoptosis. Direct comparison of PB-derived and SF macrophages from patients with RA indicated no significant differences in the rate of apoptosis under basal conditions or following stimulation with TNFα or LPS (Fig. 8, left panel). TSA alone induced 40% apoptosis in both 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 TNFα (Fig. 8, middle panel) or LPS (Fig. 8, right panel), sensitized both 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 TNFα or LPS. However, HDACi 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 HDACi, 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 HDACi induced macrophage apoptosis, we left macrophages untreated or incubated them for 24 hours 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 expression of the control housekeeping genes β2-microglobulin (B2M) and β-glucuronidase (GUS) (Fig. 9A and 9B). TSA
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 hour 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 hour 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. *p < 0.05, ***p < 0.001. (C) Time-course analysis of apoptosis induction as measured in A of macrophages treated for 24, 48, and 72 hours (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-hour treatment with medium alone, TSA (2 μM), or NIC (20 mM). Graphs are representative of three independent experiments.

treatment decreased expression of the anti-apoptotic Bcl-2-like protein Bfl-1 (also known as A1) by 70% (p < 0.05), while 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 HDACi: livin expression was decreased by >70% following TSA and NIC exposure, while 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 TNFα stimulation was also inhibited by TSA and NIC (Fig. 9D).
Figure 7. Inflammatory stimuli sensitize human macrophages to TSA- and NIC-induced apoptosis. Macrophages were left unstimulated in medium (med) or stimulated for 24 hours in the presence of TNFα (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 assessed as in Fig 6. Data represent the means ± SEM of six or seven independent experiments. *p < 0.05; Wilcoxon signed-rank test. (C) Alternatively, macrophages were incubated in medium alone (med) or medium containing 2 μM TSA, or increasing concentrations of PheBut or SBHA alone or in combination with TNFα 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 HDACi as a reference control.

HDACi 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 potential effects of HDACi on other synovial cells such as lymphocytes and fibroblast-like synoviocytes (FLS). Each of these possibilities would impact upon potential application of HDACi in the clinical setting. Therefore, we examined the influence of increasing concentrations of HDACi on cytokine production by intact synovial biopsy explants from patients with clinically active RA obtained during arthroscopic surgery. All tested HDACi 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 HDACi 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 2). Because of the expected non-Gaussian distribution of data and the
Figure 8. HDACi induce apoptosis in RA synovial macrophages. PB+ and SF− derived macrophages from patients with RA were incubated in medium alone (left panel), TNFα (10 ng/ml, middle panel) or LPS (1 μg/ml, right panel) for 24 hours in the absence (control) or presence of TSA or NIC, and the percentage of nonviable cells assessed. Results represent the mean ± SEM of at least four independent experiments.

Figure 9. TSA and NIC suppress macrophage expression of anti-apoptotic 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 hours 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 hours 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), (D) Protein expression of Bfl-1 in macrophages. Macrophages were left unstimulated (C) or stimulated with TNFα (10 ng/ml) (D) in the presence or absence of TSA (2 μM) or NIC (20 mM) for 24 hours; protein extracts were examined by Western blotting for Bfl-1/A1, α-tubulin, or actin.
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 HDACi, CCL5 was selectively suppressed by PheBut, and none of the HDACi significantly impacted upon CCL3 production. Levels of IL-15, which supports 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, where a 40% decrease in IL-23 levels was observed. Additionally, IL-17 levels in explant culture supernatants were also unaffected by HDACi treatment. HDACi cytokine suppression was not limited to pro-inflammatory gene products, as we also observed that IL-10 content in explant cultures was uniformly suppressed by HDACi. Finally, proangiogenic vascular endothelial growth factor production was potently suppressed by TSA and PheBut, and more modestly suppressed by NIC.

Figure 10. HDACi suppress cytokine production in synovial biopsy explants from patients with RA.
Intact synovial biopsies from three patients with RA were stimulated for 48 hours with TNFα (10 ng/ml) in the presence or absence of increasing concentrations of TSA, SBHA, PheBut (PB) and NIC. Cell-free tissue culture supernatants were harvested and analyzed for IL-6 (A) and IL-8 (B) by ELISA. Data were expressed as cytokine concentrations (left panels; each patient is represented by a different symbol [i.e., ●, ■, ▲, ]). Bars indicate the means of the three independent experiments. Alternatively, values for samples not treated with HDACi were normalized to 100%, and remaining values expressed as the mean percentage ± SEM of cytokine production compared with controls (right panels).
Collectively, our data demonstrate that in RA synovial tissue, HDACi suppress the production of distinct but overlapping profiles of proinflammatory cytokines, chemokines, and growth factors.

**DISCUSSION**

A broad range of HDACi, representing all chemical classes of these compounds, showed protective effects in both 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. However, the potential of extrapolating these protective effects of HDACi 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. Restoration of HDAC activity in macrophages from patients with asthma and COPD normalizes homeostatic GM-CSF, TNFα, and IL-8 production and glucocorticoid sensitivity. 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. However, the influence of HDACi on macrophage cytokine production in these diseases has not previously 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 HDACi (TSA and NIC) promote macrophage apoptosis, especially in the presence of inflammatory stimuli.

### Table 2. Effects of HDACi treatment on intact synovial biopsy cytokine production.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Control, pg/ml</th>
<th>Production, % of control ± SEM</th>
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<tr>
<td>VEGF</td>
<td>132.3 ± 93.58</td>
<td>5.74 ± 2.49</td>
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<td></td>
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<td>19.26 ± 4.70</td>
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<td></td>
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<td>38.05 ± 12.48</td>
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<tr>
<td>CCL2</td>
<td>4930 ± 1995</td>
<td>7.27 ± 5.61</td>
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<td></td>
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<td>42.51 ± 11.13</td>
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<tr>
<td></td>
<td></td>
<td>24.01 ± 9.18</td>
</tr>
<tr>
<td>CCL5</td>
<td>1267 ± 444.2</td>
<td>51.98 ± 23.57</td>
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<td>27.27 ± 14.89</td>
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<td></td>
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<td>55.74 ± 26.65</td>
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<tr>
<td>CCL3</td>
<td>1596 ± 1261</td>
<td>184.5 ± 118.7</td>
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<td>68.87 ± 20.09</td>
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<td></td>
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<tr>
<td>CXCL-12</td>
<td>444.3 ± 55.34</td>
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<td>18.54 ± 9.27</td>
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<td>IL-10</td>
<td>4.623 ± 1.96</td>
<td>26.29 ± 19.78</td>
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<td>24.01 ± 11.05</td>
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<td>IL-21</td>
<td>237.8 ± 17.20</td>
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<td>35.03 ± 9.09</td>
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<td>IL-12 p70</td>
<td>3790 ± 2.18</td>
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<td>99.23 ± 0.77</td>
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<td>93.60 ± 5.27</td>
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<tr>
<td>IL-15</td>
<td>0.90 ± 0.12</td>
<td>96.40 ± 8.24</td>
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<td>88.21 ± 6.67</td>
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<td>IL-23</td>
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<td>87.02 ± 3.90</td>
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<td>57.23 ± 2.20</td>
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<td>IL-17</td>
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<td>104.4 ± 11.19</td>
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<td>97.41 ± 6.91</td>
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</table>

Cytokine concentrations were determined by Luminex analysis of tissue culture media of intact synovial explants (n = 3 patients with RA) treated for 48 hours with HDACi (1 μM TSA, 2mM PheBut, or 20 mM NIC) in the presence of TNF. Results indicate mean cytokine concentrations for control samples not treated with HDACi and mean % relative to control ± SEM for samples treated with HDACi.
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 HDACi enhance production of IL-8 or other cytokines under homeostatic conditions or following stimulation by TNFα 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 is unresponsive to the presence of HDACi, a finding independently noted in human monocytes, macrophages, and DCs by other investigators.\textsuperscript{30,31,37} In contrast, HDACi suppressed the expression of most of the secreted cellular activation products examined in our studies. Although we did not directly address in these studies whether HDACi regulate macrophage cytokine production by epigenetic or nonepigenetic mechanisms, several lines of evidence indicate that HDACi 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 HDACi 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.\textsuperscript{47} Third, many nonhistone targets known to regulate macrophage activation and survival are targeted by both class I/II and sirtuin HDACs, including FoxO, c-jun, NF-κB p65, and STAT transcription factors.\textsuperscript{38} Although we cannot rule out the possibility that HDACi mediate their anti-inflammatory effects via non-HDAC targets, other candidates have yet to be identified.

Importantly, we observed that HDACi selectively suppress 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 have indicated that HDACi selectively suppress macrophage expression of chemokines and cytokines regulating Th1 function and recruitment of monocytes and macrophages.\textsuperscript{37} In RA synovial explants, we observed suppression of the chemokines CCL2, CCL5, and CXCL-12, whereas CCL3 levels were unaffected. Although HDACi were shown to suppress murine DC production of cytokines needed for in vitro and in vivo differentiation and expansion of Th17 cells,\textsuperscript{48} we observed only suppression of IL-6 and IL-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.\textsuperscript{49} Anti-inflammatory IL-10 production was also reduced in synovial explants treated with HDACi, highlighting that HDACi do not target only proinflammatory gene products. Because the genetic silencing of HDAC11 enhances IL-10 production by myeloid cells,\textsuperscript{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 both transcriptional and posttranslational mechanisms.\textsuperscript{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. Additionally, mice genetically deficient for Sirt1 develop autoimmune disease marked by antibody deposition in liver and kidneys and symptoms of diabetes. Together, these observations would predict that pharmacological inhibition of sirtuin promotes inflammation. However, we found that in primary human macrophages and RA synovial tissue, NIC performs similarly to, if not more potently than, class I/II HDACi with regard to suppression of cytokine production.

We also identified the antiapoptotic Bcl-2 family member Bfl-1 as a target of HDACi in macrophages. HDACi were demonstrated to induce apoptosis in cancer cell lines through modulation of both 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-3, survivin, XIAP, Bid, Bim, Bmf, Noxa and Puma). In macrophages, sustained activation of STAT, PI3K, and NF-κB signaling pathways promotes cell survival by upregulating expression of FLIP, Bfl-1, and Mcl-1. 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 TNFα. Bfl-1 protects cells against apoptosis by neutralizing proapoptotic Bid and Bak proteins, and an essential role for Bfl-1 in maintaining human macrophage survival was in gene-silencing experiments. 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, these compounds may selectively inhibit distinct HDACs in vivo. Further research will be needed to determine whether diverse HDACs, including Sirt1, regulate macrophage activation and survival through common or convergent signaling pathways.

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

ACKNOWLEDGEMENTS

We thank Drs. C.A. Ambarus, M.J.H. Boumans, D.M. Gerlag, M.J.H. de Hair, and M.G.H. van de Sande for performing synovial arthroscopies, Dr. N. van Geloven for help with statistical analyses, and Dr. R. Lutter and Prof. Dr. P.J. Coffer for critical reading of the manuscript.
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