EXTENDED REPORT

Histone deacetylase inhibitors suppress rheumatoid arthritis fibroblast-like synoviocyte and macrophage IL-6 production by accelerating mRNA decay

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ABSTRACT

Background  Histone deacetylase inhibitors (HDACi) display potent therapeutic efficacy in animal models of arthritis and suppress inflammatory cytokine production in rheumatoid arthritis (RA) synovial macrophages and tissue.

Objectives  To determine the molecular mechanisms contributing to the suppressive effects of HDACi on RA synovial cell activation, using interleukin 6 (IL-6) regulation as a model.

Methods  RA fibroblast-like synoviocytes (FLS) and healthy donor macrophages were treated with IL-1β, tumour necrosis factor (TNF)α, lipopolysaccharide or polyinosinic-polycytidylic acid (poly(I:C)) in the absence or presence of the HDACi trichostatin A (TSA) or ITF2357 (givinostat). IL-6 production and mRNA expression were measured by ELISA and quantitative PCR (qPCR), respectively. Protein acetylation and the activation of intracellular signalling pathways were assessed by immunoblotting. The DNA-binding activity of nuclear factor κB (NFκB) and activator protein 1 (AP-1) components was measured by ELISA-based assays.

Results  HDACi (0.25–1.0 µM) suppressed RA FLS IL-6 production induced by IL-1β, TNFα and Toll-like receptor ligands. Phosphorylation of mitogen-activated protein kinases and inhibitor of NFκB (IkBα) following IL-1β stimulation were unaffected by HDACi, as were AP-1 composition and binding activity, and c-Jun induction. TSA induced a significant reduction in nuclear retention of NFκB in FLS 24 h after IL-1β stimulation, but this did not reduce NFκB transcriptional activity or correlate temporally with reductions in IL-6 mRNA accumulation. HDACi significantly reduced the stability of IL-6 mRNA in FLS and macrophages.

Conclusions  Our study identifies a novel, shared molecular mechanism by which HDACi can disrupt inflammatory cytokine production in RA synovial cells, namely the promotion of mRNA decay, and suggests that targeting HDAC activity may be clinically useful in suppressing inflammation in RA.

INTRODUCTION

Excessive production of inflammatory mediators pivots crucially to pathology in many chronic immune-mediated diseases (IMDs), including rheumatoid arthritis (RA). In RA, activated immune cells infiltrating the synovial tissue secrete large quantities of tumour necrosis factor (TNF)α, interleukin 1 (IL-1), IL-8 and IL-6, among other cytokines and chemokines. These secreted products, as well as cell–cell contacts, activate stromal fibroblast-like synoviocytes (FLS), which are potent effector cells in RA, generating enzymes that degrade cartilage and bone, and serving as a primary source of inflammatory cytokines in the synovium.3 2

Production of inflammatory cytokines is tightly regulated at multiple levels, including activation of signalling pathways, induced and epigenetic mechanisms regulating transcription factor access to gene promoters, post-transcriptional mRNA processing and protein secretion. Each of these processes can be regulated by reversible protein acetylation. Inflammatory stimuli activate transcriptional coactivators possessing intrinsic histone acetyltransferase (HAT) activity, leading to histone acetylation and increased accessibility of gene promoters for transcription.4 Histone deacetylases (HDACs), including the ubiquitously expressed class I HDACs (HDACs 1–3 and 8) and tissue-restricted class II HDACs (HDACs 4–7, 9, 10), counteract the activity of HATs to terminate ongoing transcriptional processes.5

While some studies have indicated that decreased expression of HDACs in synovial tissue may contribute to pathology in RA,6 7 analyses of murine and human monocytes revealed that HDAC inhibitors (HDACi) are potent anti-inflammatory agents, which suppress lipopolysaccharide (LPS)-induced and TNFα-induced cytokine production.8–10 Also, HDACi uniformly ameliorate inflammation and prevent joint destruction in prophylactic and therapeutic protocols in animal arthritis models.11–16 These findings are relevant to RA as we have previously demonstrated that HDACi suppress IL-6 and TNFα production by RA synovial macrophages and synovial tissue explants.17 Moreover, RA FLS proliferation and survival in vitro is suppressed by HDACi.15 18 19 The exact mechanisms by which HDACi alleviate inflammation in acute and chronic inflammatory diseases remain unclear, but could be related to regulation of histone acetylation. Alternatively, HDACi may target some 1700 structural and signal transduction proteins, many of which are relevant to RA, including components of the mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription (STAT) pathways, transcription factors such as p53, nuclear factorκB (NFκB) p65 and c-Jun, as well as regulators of mRNA stability, protein degradation and secretion.20–22 Further understanding of
the molecular mechanism(s) contributing to anti-inflammatory effects of HDACi may facilitate hypothesis-driven decisions as to the suitability of HDACi in the treatment of RA, especially now that one HDACi, ITF2357 (givinostat; Italfarmaco, Cinisello Balsamo, Italy), has demonstrated initial clinical efficacy in the treatment of systemic onset juvenile idiopathic arthritis (SOJIA).13,24

Expression of IL-6 in RA synovial tissue strongly correlates with disease activity and inflammation severity in RA,25 and targeting of IL-6 signalling using tocilizumab, an anti-IL-6 receptor monoclonal antibody, demonstrates clinical efficacy in RA.26 Here we examined the mechanism by which HDACi might suppress IL-6 expression in RA FLS and macrophages, assessing effects on intracellular signalling pathways leading to IL-6 transcription and post-transcriptional regulatory events. We identify inhibition of IL-6 mRNA stability as a novel common mechanism by which HDACi regulate inflammatory gene expression in RA.

MATERIALS AND METHODS

Cell culture and stimulation

FLS were isolated from synovial biopsies of patients with RA (n=18) fulfilling the American College of Rheumatology revised criteria for RA,27 cultured as previously described,28 and used for experiments between passages 4 and 9, following overnight culture in medium containing 1% fetal bovine serum (FBS; Invitrogen, Breda, The Netherlands) (see supplementary table 1 for patient characteristics). Monocytes were isolated from buffy coats (Sanquin Reagents, Amsterdam, The Netherlands) of healthy donors (HDs) and differentiated into macrophages as described previously.17 Cells were treated with medium alone or medium containing trichostatin A (TSA) (Sigma-Aldrich, St Louis, Missouri, USA) or ITF2357 at the indicated concentrations for 30 min, followed by stimulation with IL-1β (0.2–10 ng/ml) (R&D Systems, Minneapolis, Minnesota, USA), TNFα (10 ng/ml) (Biosource International, Camarillo, CA, USA), LPS (1 μg/ml) or polyinosinic:polycytidylic acid (poly(I:C)) (10 μg/ml) (both from Sigma-Aldrich) for up to 24 h.

Measurement of cell viability

RA FLS viability was assessed 24 h post treatment by incubating cells with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) (1 mg/ml) for 2 h, followed by solubilisation of cells in acidic isopropanol solution containing 0.1% Igepal CA-630 (Sigma-Aldrich) and optical density measurement at 590 nm.

Quantitative measurement of IL-6 protein and mRNA expression

IL-6 protein production was measured in cell-free supernatants 24 h post stimulation using a PeliKine Compact ELISA kit (Sanquin Reagents). mRNA expression in FLS and macrophages was assessed by quantitative PCR (qPCR) as described in detail in the supplementary methods.

Protein extraction and immunoblotting

For determination of protein acetylation status and signalling pathway activation, FLS were left untreated or treated with TSA or ITF2357 for 30 min, prior to further incubation in medium alone or IL-1β for the time periods indicated in the figure legends. Protein extraction from whole cell lysates was as previously described, and nuclear fraction extraction and analysis by immunoblotting are described in detail in the supplementary methods.17

Measurement of transcription factor DNA-binding and transcriptional activity

DNA binding activities of NFκB components p65 and p50, and activator protein 1 (AP-1) components c-jun, JunB and JunD in FLS nuclear fractions were determined using a TransAM transcription factor ELISA (Active Motif, Carlsbad, California, USA) according to the manufacturer’s instructions. NFκB transcriptional activity in FLS was assessed using a recombinant adenoviral vector encoding a NFκB–luciferase reporter. Construction of the vector, generation of viral particles, transduction of FLS and measurement of luciferase activity are described in detail in the supplementary methods.

Statistical analyses

Data are presented as mean±SEM unless otherwise indicated. For analysing HDACi treatment dose responses, data sets were subjected to an overall Kruskal–Wallis test followed by post hoc Dunns’ multiple comparison test, using cells not exposed to HDACi as reference controls. In mRNA stability experiments, the rate of mRNA degradation over time was determined in each experimental condition and area under the curve (AUC) values were calculated and compared using the Mann–Whitney U test. The Mann–Whitney U test was used for all other comparisons. p values ≤0.05 were considered statistically significant.

RESULTS

HDACi suppress RA FLS IL-6 production

Multiple HDACi block IL-6 production by monocytes and macrophages derived from HD, as well as synovial macrophages and tissue from patients with RA.9,10,27 To determine if HDACi could also inhibit RA FLS IL-6 production, we treated RA FLS with increasing concentrations of TSA or ITF2357 prior to stimulation with agonists found in RA synovial tissue. Both HDACi dose-dependently suppressed IL-6 production induced by IL-1β. TSA achieved 50% inhibition of IL-6 production at 0.25 μM and more than 80% at 1 μM (p<0.001). A trend towards reduced IL-6 production was observed at clinically relevant concentrations of ITF2357 (0.25 μM), reaching 80% inhibition at 1 μM (p<0.01) (figure 1A). HDACi also suppressed IL-6 production in response to TNFα and the Toll-like receptor (TLR) ligands LPS and poly(I:C) by 50% to 80% (p<0.05) (figure 1B). HDACi suppression of IL-6 production could not be attributed to changes in FLS viability following exposure to HDACi, as measured by MTT reduction (figure 1C). As previously observed in RA macrophages, TSA suppressed the induction of IL-6 mRNA in FLS,17 1 μM TSA reduced IL-1β-induced IL-6 mRNA accumulation by 70% (p<0.01) (figure 2A), while 0.25 μM ITF2357 reached 45% inhibition (p<0.01) (figure 2B). ITF2357 blocked IL-6 mRNA accumulation in a concentration-dependent manner regardless of the dose of IL-1β used (figure 2C).

Suppression of IL-6 production by HDACi does not require epigenetic induction of transcriptional repressors

To determine if inhibition of IL-6 transcription by HDACi might result from induced expression of transcriptional corepressors or negative regulatory signalling components, we examined histone 3 (H3) and H4 acetylation following HDACi treatment. HDACi induced acetylation of H3 and H4, as well as a protein of 52 KD (identified as tubulin, data not shown) (figure 3A,B). No global changes in FLS protein acetylation status could be detected in response to IL-1β, TNFα or LPS alone (figure 3B). TSA and ITF2357 both induced H3, H4 and total cellular protein acetylation within 15 min of HDACi exposure (figure 3A), consistent
Basic and translational research

**Figure 1** Histone deacetylase inhibitors (HDACi) suppress interleukin 6 (IL-6) production by rheumatoid arthritis fibroblast-like synoviocytes (FLS) without affecting cell viability. A. FLS were stimulated with 10 ng/ml of IL-1β alone or in the presence of increasing concentrations of trichostatin A (TSA) or ITF2357 for 24 h (n=6–10). B. Alternatively, cells were left untreated or pre-exposed to 1 µM TSA followed by 24 h stimulation with IL-1β (10 ng/ml), tumour necrosis factor (TNF)α (10 ng/ml), lipopolysaccharide (1 µg/ml) or polyinosinic:polycytidylic acid (poly(I:C)) (10 µg/ml) (n=4). IL-6 levels in cell-free tissue culture supernatants were determined by ELISA. Results, presented as mean±SEM IL-6 concentration, were either subjected to the Kruskal–Wallis test followed by Dunn’s multiple comparison analysis with cells not treated with HDACi used as reference controls (A), or to the Mann–Whitney U test (B). *p<0.05; **p<0.01; ***p<0.001. C. Viability of cells after 24 h stimulation with IL-1β (10 ng/ml) in the presence or absence of increasing doses of TSA or ITF2357 was assessed by measurement of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction (n=5). Values representing changes in MTT processing are shown as mean±SEM optical density at 590 nm.

with the possibility that histone hyperacetylation might lead to transcriptional induction of repressors of IL-6 transcription. However, in the absence or presence of the protein translation inhibitor cycloheximide (CHX), TSA suppressed IL-1β-induced IL-6 mRNA accumulation in FLS (p<0.05) (figure 3C). The failure of CHX to rescue IL-6 mRNA accumulation suggested that de novo synthesis of a repressor protein is not required for HDACi suppression of IL-6 in RA FLS.

**HDACi fail to affect MAPK signalling and AP-1 activation in RA FLS**

Activation of MAPK and NFκB signalling pathways, leading to transcriptional activation of NFκB and AP-1, are required for IL-1β-induced IL-6 production, and can be modulated by reversible acetylation.20 29 We analysed the effects of TSA on the phosphorylation status of p38 and extracellular signal-regulated kinase (ERK) MAPK in RA FLS by immunoblotting. IL-1β induced rapid phosphorylation of p38 and ERK, which peaked 30 min post stimulation. TSA treatment affected neither phosphorylation levels, nor temporal regulation of p38 and ERK activation (figure 4A), indicating that HDACi blockade of RA FLS IL-6 production is not mediated by suppression of MAPK signalling.

Phosphorylation of c-Jun N-terminal kinase (JNK), activation of the c-Jun component of the AP-1 transcription factor and subsequent rapid induction of c-Jun expression are essential pre-requisites for optimal IL-6 gene transcription, and are sensitive to acetylation.20 31 However, similarly to p38 and ERK, IL-1β-stimulated JNK phosphorylation in FLS was unaffected by TSA (figure 4B). Also, IL-1β induced a transient fivefold increase of c-Jun mRNA levels which was unaffected by TSA (figure 4C). Stimulation with IL-1β caused a 2.5-fold increase in p-c-Jun DNA binding compared to unstimulated FLS (figure 4D). However, FLS exposure to TSA did not suppress the induction of c-Jun binding activity (figure 4D) and had no influence on DNA binding of Jun family members JunB and JunD (data not shown). Collectively, these results suggest that HDACi-dependent IL-6 suppression in RA FLS is not mediated by direct regulation of MAPK and AP-1 signalling pathways.

**HDACi modulate NFκB signalling in RA FLS**

NFκB signalling makes pivotal contributions to the induction of IL-6 expression, and the activity as well as nuclear retention of NFκB subunits is tightly regulated by reversible acetylation.32 In initial experiments, we found that TSA treatment had no effect on the magnitude or kinetics of inhibitor of NFκBα (IkBα) phosphorylation or degradation in RA FLS (figure 5A). To examine the influence of HDACi on the temporal regulation of NFκB nuclear retention, FLS were cultured in the absence or presence of TSA and then stimulated with medium alone or IL-1β, and

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nuclear accumulation of p50 and p65 NFκB subunits assessed by immunoblotting. TSA had little effect on nuclear p50 and p65 in the absence of inflammatory stimulation, but slightly increased the amounts of both proteins 1 h after IL-1β stimulation. However, nuclear retention of these subunits 24 h post stimulation was strongly reduced (figure 5B). In DNA-binding assays we observed a trend towards elevated p50 and p65 binding 1 h after IL-1β stimulation in the presence of TSA, while a significant reduction of DNA binding was observed only after 24 h (figure 5C). To determine if p65 retained transcriptional activity in the presence of TSA, we cotransduced RA FLS with adenoviral vectors encoding 4×NFκB-minimal promoter (MLP)-luciferase reporter, and evaluated the effects of TSA on luciferase enzymatic activity. TSA did not inhibit, and clearly increased, the transcriptional activity of NFκB early after stimulation with IL-1β (figure 5D). Together, these findings indicate that HDACi-
induced suppression of IL-6 mRNA, detectable 4 h after inflammatory stimulation, precedes inhibitory effects of HDACi on p50 and p65 DNA binding and nuclear retention.

**HDACi modulate IL-6 mRNA stability in RA FLS and HD macrophages**

To gain more insight into the kinetics of IL-6 suppression by HDACi in RA FLS, we treated the cells with IL-1β for 1–8 h with or without TSA, and measured changes in IL-6 mRNA levels over time. IL-1β induced a 20-fold increase in IL-6 mRNA levels within 1 h, which remained stable until 4 h before further increasing. TSA failed to affect IL-6 mRNA accumulation 1 h after stimulation, but a strong reduction was observed at 4 h and became more pronounced at 8 h (p<0.05) (figure 6A). As we had previously ruled out a need for de novo repressor synthesis in HDACi-mediated IL-6 suppression, we examined if differences might be attributable to effects on mRNA stability. FLS were stimulated with IL-1β for 4 h in the presence or absence of TSA, and after inhibiting transcription with actinomycin D (ActD), we analysed time-dependent IL-6 mRNA decay. Accelerated degradation of IL-6 mRNA in TSA-treated FLS was detectable within 1 h of ActD treatment, becoming more pronounced at later time points (p<0.05 for AUC) (figure 6B). Similar effects were observed in the presence of 0.25 μM ITF2357 (figure 6C). The presence of TSA also significantly accelerated IL-6 mRNA decay in LPS-stimulated macrophages (figure 6D). Together, these results indicate that modulation of mRNA stability is a common mechanism by which IL-6 production is suppressed by HDACi in macrophages and RA FLS.

**DISCUSSION**

IL-6 acts pleiotropically to induce acute-phase protein synthesis, stimulate B cell antibody production, modulate T helper 17 (Th17) and cytotoxic T cell differentiation, activate endothelial cells and induce osteoclast differentiation. Under physiological conditions, proinflammatory and anti-inflammatory effects of IL-6 contribute to an effective but self-limiting acute immune response, but in RA and other IMIDs, excessive IL-6 production leads to deregulation of the immune system and pathology. Despite the dual role of IL-6 in regulating inflammation, targeting of the IL-6 receptor has shown strong clinical benefits in RA.26 However, since a substantial fraction of patients with RA remain non-responsive to currently available anticytokine therapies, there is growing interest in identifying novel therapeutic targets which could suppress inflammatory cytokine production, such as HDACi. Pharmacological inhibition of HDAC activity has shown anti-inflammatory effects in animal arthritis models,11–16 and synovial macrophages and tissue explants from patients with RA.17 Here, we show that HDACi reduce RA FLS IL-6 production induced by cytokines and TLR ligands, and identify modulation of IL-6 mRNA stability as a primary molecular mechanism contributing to this effect in FLS and macrophages.

RA FLS treatment with HDACi, as previously observed with RA synovial macrophages,17 rapidly induced acetylation of histone and non-histone proteins, raising the possibility that HDACi-induced expression of transcription corepressors might explain the suppressive effects of HDACi on IL-6 production. However, treatment of cells with CHX, to prevent translation of putative repressors, failed to rescue IL-6 production in the presence of HDACi. Consistent with this, even though HDACi induce H4 hyperacetylation in the IL-6 promoter region in murine bone marrow-derived macrophages, IL-6 production is downregulated,
β transduction events downstream of IL-1 reveals evidence supporting a role for HDACi in modulating acute signal of c-Jun induction and activation, or reduction of NF gene expression via blockade of MAPK signalling. However, we observed no effect of TSA on TLR signalling. However, we observed no effect of TSA on IL-1β-induced activation of p38, ERK and JNK MAPKs, c-Jun gene induction, or DNA binding activity of Jun family members. Our results highlight the difficulties of extrapolating effects of HDACi in various cell types to primary cells from RA synovial tissue in the absence of formal studies.

NFκB signalling is complexly regulated by reversible acetylation, as p65 can be acetylated on at least 5 unique lysine residues, each having a distinct effect on p65 function. Acetylation at Lys221 promotes p65 dissociation from IkBα and nuclear import, while acetylation at Ser310 enhances p65 transcriptional activity. Alternatively, p65 acetylation at Lys122 and Lys123 decreases DNA binding affinity, enhances IkBα association and promotes nuclear export. We observe a similarly complex regulation in RA FLS, as p65 nuclear import, DNA binding activity and transcriptional capacity are all enhanced by HDACi in the initial hours following IL-1β stimulation. However, HDACi promote dissociation from DNA and nuclear export at later time points, consistent with recent observations in an SV40 Tag-transformed RA FLS cell line. The changes we observe in NFκB activity are consistent with temporal acetylation of NFκB at distinct regulatory sites, but direct studies are needed to address this possibility. However, the kinetics of alterations in NFκB activity cannot explain the acute suppression of IL-1β-induced IL-6 production by HDACi, but may confer a protective effect in the face of chronic inflammatory stimulation.

While many of the HDACi effects on cellular activation have been attributed to epigenetic regulation or influences on signal transduction, studies in cancer biology have provided initial evidence that these compounds can modulate gene expression via regulation of mRNA stability. We observe a similar and shared mechanism of action in the suppression of IL-6 production in macrophages and RA FLS. mRNA stability is regulated by hundreds of gene products, some of which have been identified as functionally associated with HDACs. Selectivity is also observed in miRNAs targeted by these proteins as, for example, Zc3h12 RNAse specifically regulates IL-6 mRNA stability. Alternatively, as observed in cancer biology studies, HDACi might differentially modulate microRNA (miR) expression. IL-6-specific miRs have yet to be identified, but the expression of several miRs associated with inflammatory responses is altered in RA FLS and synovial tissue. Future studies will be needed to elucidate how HDACi enhance IL-6 mRNA degradation, and test how globally this mechanism regulates gene expression in RA synovial tissue.

![Figure 5](image-url)
endometrial cells, raising the possibility that HDAC-dependent mRNA destabilisation might regulate inflammatory gene expression through crossstalk with epigenetic DNA methylation pathways. The results presented here provide the first mechanistic evidence by which HDACi might suppress inflammatory cytokine production in RA, and, together with the initial proof of principle that has recently been obtained in SOJIA, sup-45

Figure 6 Histone deacetylase inhibitors (HDACi) accelerate interleukin 6 (IL-6) mRNA degradation in rheumatoid arthritis fibroblast-like synoviocytes (FLS) and healthy donor (HD) macrophages. A. FLS were left untreated (med) or were treated with IL-1β (10 ng/ml) with or without 1 µM trichostatin A (TSA) for the indicated time (h), total RNA was extracted, cDNA was synthesised and temporal changes in IL-6 mRNA accumulation were monitored by quantitative PCR (qPCR). Data represent the mean±SEM of five or six independent experiments. *p<0.05, Mann–Whitney U test. B. FLS were stimulated with IL-1β (10 ng/ml) in the presence or absence of 1 µM TSA (n=5) or (C) with IL-1β (1 ng/ml) in the presence or absence of 250 nM ITF2357 (n=3). Alternatively, (D) monocyte-derived HD macrophages (n=4) were stimulated with lipopolysaccharide (1 µg/ml). After 4 h of stimulation cells were washed and fresh medium containing 10 µg/ml of actinomycin D (ActD) was added. RNA was extracted at the indicated time points (h) from the beginning of ActD treatment and the rates of IL-6 mRNA degradation in the presence or absence of HDACi were examined by qPCR. Values for the 0 h time point were normalised to 100%, and remaining values were expressed as the mean±SEM percentage of IL-6 mRNA levels compared with controls. For (B) and (D) the areas under the curves (AUCs) obtained for cells treated with an inflammatory stimulus alone or in the presence of TSA were calculated and differences between AUC values were analysed by the Mann–Whitney U test (insets, mean AUC±SEM). *p<0.05.

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