Molecular mechanisms of histone deacetylases in rheumatoid arthritis fibroblast-like synoviocytes
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Chapter 4

HISTONE DEACETYLASE 3 REGULATES THE INFLAMMATORY GENE EXPRESSION PROGRAMME OF RHEUMATOID ARTHRITIS FIBROBLAST-LIKE SYNOVIOCYTES.

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ABSTRACT

Objectives: Non-selective histone deacetylase (HDAC) inhibitors (HDACi) have demonstrated anti-inflammatory properties in both in vitro and in vivo models of rheumatoid arthritis (RA). Here, we investigated the potential contribution of specific class I and class IIb HDACs to inflammatory gene expression in RA fibroblast-like synoviocytes (FLS).

Methods: RA FLS were incubated with pan-HDACi (ITF2357, givinostat) or selective HDAC1/2i, HDAC3/6i, HDAC6i and HDAC8i. Alternatively, FLS were transfected with HDAC3, HDAC6 or interferon (IFN)-α/β receptor alpha chain (IFNAR1) siRNA. mRNA expression of interleukin (IL)-1β-inducible genes was measured by quantitative PCR (qPCR) array and signalling pathway activation by immunoblotting and DNA-binding assays.

Results: HDAC3/6i, but not HDAC1/2i and HDAC8i, significantly suppressed the majority of IL-1β-inducible genes targeted by pan-HDACi in RA FLS. Silencing of HDAC3 expression reproduced the effects of HDAC3/6i on gene regulation, contrary to HDAC6-specific inhibition and HDAC6 silencing. Screening of the candidate signal transducers and activators of transcription (STAT)1 transcription factor revealed that HDAC3/6i abrogated STAT1 Tyr701 phosphorylation and DNA binding, but did not affect STAT1 acetylation. HDAC3 activity was required for type I IFN production and subsequent STAT1 activation in FLS. Suppression of type I IFN release by HDAC3/6i resulted in reduced expression of a subset of IFN-dependent genes, including the chemokines CXCL9 and CXCL11.

Conclusions: Inhibition of HDAC3 in RA FLS largely recapitulates the effects of pan-HDACi in suppressing inflammatory gene expression, including type I IFN production in RA FLS. Our results identify HDAC3 as a potential therapeutic target in the treatment of RA and type I IFN-driven autoimmune diseases.
INTRODUCTION

Histone-modifying enzymes are epigenetic regulators implicated in the control of inflammatory processes, including immune and stromal cell activation, survival and proliferation. Histone acetyltransferases (HATs) acetylate lysine residues on histone tails, while histone deacetylases (HDACs) counterbalance HAT activity by deacetylating histone proteins. The delicate equilibrium between the acetylated state and the deacetylated state of chromatin orchestrates gene transcription. Furthermore, HATs and HDACs can also affect the acetylation status of non-histone proteins, thereby regulating signalling proteins and transcription factors to influence gene expression and cellular function. As a consequence, HDAC function could be essential to the development and perpetuation of chronic inflammatory diseases, such as rheumatoid arthritis (RA). In fact, HDAC activity and expression were shown to be altered in total peripheral blood mononuclear cells (PBMCs), synovial tissue and fibroblast-like synoviocytes (FLS) from patients with RA. Despite inflammatory mediators such as tumor necrosis factor (TNF) were found to positively associate with HDAC expression in synovial tissue and rapidly induce HDAC activity in FLS, single targeting of TNF may not be sufficient to restore the HDAC balance in immune cells of patients with RA. This suggests that multiple factors contribute to the altered HAT/HDAC balance and that inhibition of HDACs could have a therapeutic contribution to RA treatment.

We and others have shown that pan-HDAC inhibitors (pan-HDACi) reduce cytokine production in FLS and in immune cells from patients with RA, display antiarthritic properties in vivo and demonstrated primary clinical efficacy in the treatment of rheumatic diseases. Which HDAC or combination of HDACs is specifically involved in RA pathology, however, remains unknown. The HDAC family includes 18 members divided into class I HDACs (HDACs 1–3 and 8), class IIa HDACs (HDACs 4–5, 7 and 9), class IIb HDACs (HDACs 6 and 10), class III sirtuins (Sirt1–7) and class IV HDAC11. Accumulating evidence suggests that some of the class I and class IIb HDAC family members could contribute to RA pathology, as their synovial activity is elevated compared with disease controls and further increased by inflammatory stimuli, and inhibition of their activity is protective in animal models of arthritis. Class IIa HDAC9 deficiency was found to enhance regulatory T cell function and was protective in disease models of systemic lupus erythematosus and colitis, but there is little indication for a direct involvement of HDAC9 activity in regulating cytokine expression. Furthermore, our previous data indicated that synovial expression of class IIa HDACs does not positively correlate with RA disease parameters nor with mediators of inflammation, and that class IIa HDAC5 is a negative regulator of chemokine expression in RA FLS.

In this study, we investigated the potential differential contribution of class I and class IIb HDAC family members to the inflammatory status in RA FLS using the combination of selective HDACi and genetic silencing of individual HDAC expression.
CHAPTER 4

MATERIALS AND METHODS

Patient material and FLS isolation
FLS were derived from synovial tissue specimens obtained from patients with RA by needle arthroscopy, as previously described, cultured in medium containing 10% fetal bovine serum (FBS, Invitrogen), and used between passages 4 and 10. All patients fulfilled the criteria for the classification of RA and had active disease, including clinical arthritis of the joint from which the synovial biopsies were obtained. Clinical characteristics of patients are shown in Table 1. Informed written consent was obtained from patients prior to inclusion in the study.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>RA (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>58 (31–71)</td>
</tr>
<tr>
<td>Male to female ratio (n)</td>
<td>5:13</td>
</tr>
<tr>
<td>Disease duration (months)</td>
<td>229 (1–43)</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>31 (2–101)</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>11.3 (1.0–128.0)</td>
</tr>
<tr>
<td>DAS28</td>
<td>4.06 (2.80–6.63)</td>
</tr>
<tr>
<td>ACPA+: n/total (%)</td>
<td>11/18 (61)</td>
</tr>
<tr>
<td>RF+: n/total (%)</td>
<td>10/18 (56)</td>
</tr>
<tr>
<td>Medications: n/total (%)</td>
<td></td>
</tr>
<tr>
<td>NSAIDs</td>
<td>8/18 (44)</td>
</tr>
<tr>
<td>Steroids</td>
<td>4/18 (22)</td>
</tr>
<tr>
<td>DMARDs</td>
<td>15/18 (83)</td>
</tr>
<tr>
<td>Biologicals</td>
<td>10/18 (56)</td>
</tr>
</tbody>
</table>

The values are expressed as median (range), unless otherwise indicated. ACPA, anticitrullinated protein antibody; CRP, C reactive protein; DAS28, disease activity score in 28 joints based on ESR; DMARDs, disease-modifying antirheumatic drugs; ESR, erythrocyte sedimentation rate; NSAIDs, non-steroidal anti-inflammatory drugs; RA, rheumatoid arthritis; RF=rheumatoid factor.

FLS treatment and stimulation
FLS were cultured overnight in medium containing 1% FBS prior to incubation with cytokines. FLS were stimulated with 1 ng/mL interleukin (IL)-1β (R&D Systems), 1000 U/mL interferon (IFN)-β (Peprotech) or IFN-α (Bio-Connect Life Sciences). The pan-HDACi ITF2357 and inhibitors specific for HDAC1/2, HDAC3/6, HDAC6 and HDAC8 (Italfarmaco) were used at concentrations ranging from 20 nM to 2 mM. Information about the specificity of the HDACi has been previously published.

Statistical analysis
Data are presented as mean±SEM, unless otherwise indicated. Friedman test followed by Dunn’s post hoc test and repeated measures analysis of variance (ANOVA) followed by Bonferroni correction were used for analyzing sets of data requiring multiple compari-
HDAC3 regulates the inflammatory gene expression programme of RA FLS

sons. The ratio t test was used for all other comparisons. Data were analyzed using GraphPad software with p values <0.05 considered statistically significant.

Detailed descriptions of immunoblotting, HDAC activity, thiazolyl blue tetrazolium bromide (MTT) assay, mRNA expression analysis, ELISA, invasion assay, siRNA transfection, signal transducers and activators of transcription (STAT)1 DNA binding and immunoprecipitation are provided in online supplementary materials and methods.

RESULTS

Selective class I HDACi differentially regulate global protein acetylation in RA FLS.
Pan-HDACi are broad-acting anti-inflammatory agents that are beneficial in several disease models. As primary evidence from in vitro and animal studies of arthritis pointed to class I HDACs as important contributors in the pathogenesis of RA, we attempted to dissect the potential roles of individual class I HDACs in mediating the inflammatory activation of RA FLS, using both pan-HDACi and inhibitors selective for HDAC1/2, HDAC3/6 and HDAC8. Treatment of RA FLS with each inhibitor resulted in distinct effects on global protein lysine acetylation. Both pan-HDACi and HDAC3/6i dose-dependently induced hyperacetylation of tubulin, a known HDAC6 substrate (figure 1A, top panel, 52 kDa band), as well as histone 3 (H3) and histone 4 (H4) (figure 1A, top panel, 18 and 14 kDa, respectively). In contrast, HDAC1/2i and HDAC8i displayed minimal

Figure 1. Histone deacetylase inhibitors (HDACi) differentially affect global protein acetylation and HDAC activity.
(A) Rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS) were incubated with increasing concentrations (20, 100 and 500 nM) of either pan-HDAC1/2, pan-HDAC3/6 and pan-HDAC8 inhibitors or selective HDAC1/2, HDAC3/6 and HDAC8 inhibitors for 4 h. Total cell lysates were analyzed by western blotting with antibodies recognizing acetylated lysine (Ac Lysine), tubulin and histone 3 (H3). Proteins detected at 52, 18 and 14 kDa with antiacetylated lysine Ab correspond to acetylated tubulin, acetylated H3 and histone 4 (H4), respectively. The results are representative of four independent experiments. (B) RA FLS (n=4) were incubated with different HDACi for 4 h. Cell pellets were incubated with fluorogenic class-specific HDAC substrates and class I and class IIb HDAC activities were measured by fluorescent product release. The values represent arbitrary fluorescence values for cells untreated or treated with HDACi. *p<0.05, ****p<000.1, ratio t test.
to negligible effects on acetylation of these substrates. To confirm the pharmacological activity of the compounds, we measured the enzymatic activity of class I (figure 1B, upper panel), class Iib (figure 1B, lower panel and data not shown) and class Ila HDACs (data not shown) in lysates of FLS treated with the inhibitors. Pan-HDACi (p<0.0001), HDAC1/2i (p<0.05) and HDAC3/6i (p<0.05) significantly reduced class I HDAC activity, while a trend towards reduction in class I and class Iib HDAC activities was observed with HDAC8i and HDAC3/6i, respectively. Together, these data suggest that while each of the inhibitors displays pharmacological activity in RA FLS, HDAC3 and/or 6 are primarily responsible for mediating tubulin, H3 and H4 lysine acetylation.

Inhibition of HDAC3/6 displays similar effects to pan-HDACi in suppressing inflammatory gene expression in RA FLS.

To exclude the possible effects of compound toxicity in our analysis, we exposed FLS to increasing concentrations of HDACi and verified cell viability by MTT assay. Treatment for 24h had no discernable effect on overall FLS metabolic activity (figure 2A). To further assess the contributions of the different HDACi to the inflammatory activation of FLS, we analyzed the expression of a panel of 83 IL-1β-inducible genes in the presence or absence of the HDACi by quantitative PCR (qPCR) array (figure 2B and data not shown). Eighty per cent of the genes downregulated by more than twofold with pan-HDACi in each of the three RA FLS lines subjected to this analysis were similarly affected by HDAC3/6i. In contrast, only the lymphotoxin (LTA) gene was downregulated by HDAC8i and none by HDAC1/2i. To confirm the effects of HDAC3/6i on gene expression, we performed independent qPCRs on a selected subset of targets using mRNA from additional RA FLS lines treated with the inhibitor (figure 2C). In agreement with qPCR array data, HDAC3/6i significantly suppressed IL-1β-induced expression of interferon-β1 (IFNB1), CXCL9, CXCL10, CXCL11, CCL2, CCL3, IL6, IL8, matrix metalloproteinase (MMP)1 and MMP3. In contrast, HDAC1/2i and HDAC8i failed to inhibit IL6 and IL8 induction, even at concentrations as high as 2 mM (see online supplementary figure S1). Consistent with its effects on mRNA expression, HDAC3/6i significantly suppressed IL-6 and IL-8 protein production following IL-1β stimulation (figure 2D, left panel and right panel, respectively) and reduced RA FLS invasive capacities (figure 2E), an effect possibly associated with decreased levels of MMPs, and induced expression of TIMP1 (figure 2B, C).

**HDAC3, but not HDAC6, mediates IL-1β-induced gene expression in RA FLS.**

The HDAC3/6i used in these studies effectively targets both HDAC3 and HDAC6. HDAC3 has previously been identified as a key epigenetic modulator of inflammatory activation of murine macrophages and human PBMCs. Also, HDAC6 inhibition was shown to inhibit proinflammatory TNF-α and IL-6 cytokines in lipopolysaccharide (LPS)-stimulated THP-1 cells. To determine whether HDAC3 or HDAC6 might be responsible for the transcriptional changes observed with HDAC3/6i, we made use of an additional inhibitor specific for HDAC6. In initial experiments, we assessed the concentration of HDAC6i (1 mM) which induced a similar degree of tubulin acetylation as to HDAC3/6i (figure 3A) and had no effect on FLS viability (see online supplementary figure S2). Under these conditions, we observed no significant effect of HDAC6i on the expression of genes induced
HDAC3 regulates the inflammatory gene expression programme of RA FLS

Figure 2. Histone deacetylase (HDAC)3/6i recapitulates the effects of pan-HDAC inhibitors (HDACi) on inflammatory gene expression.

(A) Rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS) (n=5) were left untreated or preincubated with increasing concentrations (20, 100 and 500 nM) of pan-HDACi or selective HDACi for 30 min and further stimulated with interleukin (IL)-1β for 24 h. Changes in cell viability were analysed by MTT assay and presented as the mean absorbance±SEM at 590 nm. (B) FLS (n=3) were left untreated or preincubated with 250 nM of indicated HDACi for 30 min, prior to stimulation with IL-1β for 4 h. mRNA levels of 83 IL-1β-responsive genes were determined by quantitative PCR (qPCR) using a customized RT² Profiler PCR Array. Data are presented on heat map as row Z-scores computed from delta Ct values relative to a panel of five housekeeping genes. (C) RA FLS (n=6) were left untreated or incubated with 250 nM HDAC3/6i for 30 min and further stimulated with IL-1β for 4 h. Total RNA was extracted and changes in mRNA accumulation were analyzed by qPCR. *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001, ratio t test. (D) RA FLS (n=5) were left untreated or preincubated with increasing concentrations (20, 100 and 500 nM) of HDAC3/6i for 30 min and then stimulated with IL-1β for 24 h. IL-6 and IL-8 production was determined by ELISA. *p<0.05, **p<0.01, repeated measures ANOVA followed by Bonferroni correction for multiple comparison analysis. (E) RA FLS (n=4) were seeded into Boyden chambers, left untreated or preincubated with 250 nM HDAC3/6i for 30 min and then stimulated with IL-1β for 24 h. After 24 h of incubation, the number of invasive cells was determined. Graphs indicate the average number of cells per field. *p<0.05, repeated measures ANOVA followed by Bonferroni correction for multiple comparison analysis.
by IL-1β and suppressed by HDAC3/6i (figure 3B). While this suggested a primary role for HDAC3 in the effects of HDAC3/6i in suppressing FLS inflammatory activation, we sought to confirm this independently by knocking down HDAC3 and HDAC6 in FLS. Silencing efficiency at the level of mRNA and protein was confirmed for both HDAC3 (figure 3C, D, respectively) and HDAC6 (figure 3F, G). Silencing of HDAC3 significantly suppressed the IL-1β-mediated induction of genes targeted by HDAC3/6i (figure 3E and data not shown), contrary to HDAC6 silencing (figure 3H). Together, these results suggest that the HDAC3/6i prevents inflammatory gene expression primarily through its effects on HDAC3.

**HDAC3 regulates STAT1 phosphorylation independently of STAT1 acetylation.**

Macrophages deficient in HDAC3 display an impaired inflammatory gene expression programme upon LPS stimulation, partially dependent on altered transcriptional activation of STAT1. Sustained levels of STAT1 protein and its activated phosphorylated forms are elevated in RA FLS and synovium, likely contributing to the maintenance of the active inflammatory process. We therefore investigated whether HDAC3 might regulate gene expression in a STAT1-dependent manner in RA FLS. Treatment of FLS with HDAC3/6i had no effect on total STAT1 protein expression, but prevented STAT1 Tyr701 phosphorylation in response to IL-1β stimulation. In contrast, HDAC6i had no effect on STAT1 phosphorylation (figure 4A). Consistent with HDAC3/6i effects on STAT1 phosphorylation, which is required for its transcriptional activation, HDAC3/6i also completely blocked the induction of STAT1 DNA-binding activity in response to IL-1β (figure 4B). Silencing of HDAC3 expression similarly prevented IL-1β-induced STAT1 phosphorylation (figure 4C). It has been previously reported that STAT1 hyperacetylation is a prerequisite for STAT1 dephosphorylation and inactivation, so we examined if HDAC3/6i regulates the acetylation status of STAT1. We found that HDAC3/6i had no effect on STAT1 acetylation after 4 h of treatment (figure 4D) or at earlier time points (figure 4E). We conclude that while HDAC3 strongly regulates STAT1 activity in RA FLS, this does not occur via a direct acetylation event.

**HDAC3 controls the IL-1β-induced STAT1 phosphorylation via downregulation of IFN-β expression.**

STAT1 signalling is tightly regulated by the type I IFNs IFN-α and IFN-β and the type II IFN IFN-γ. In RA FLS, IFN-β is an essential mediator of TNF-dependent STAT1 activation and pharmacological inhibition of this pathway prevents T cell-attracting chemokine production in FLS. Here, we investigated whether regulation of IFN-β by HDAC3 might be responsible for the observed effects of HDAC3 inhibition on STAT1 activation and type I IFN gene responses in RA FLS. Exposure of FLS to IL-1β, IFN-α or IFN-β over time revealed differential kinetics of STAT1 phosphorylation (figure 5A). In particular, STAT1 phosphorylation in the presence of IL-1β was delayed compared with the responses induced by IFN-α or IFN-β. To verify whether late STAT1 activation by IL-1β would rely on type I IFN production, we silenced the expression of the IFN-α/β receptor alpha chain (IFNAR1) (figure 5B, C). IFNAR1 silencing potently blocked STAT1 phosphorylation in the presence of IL-1β or IFN-β (figure 5D), indicating that STAT1 activation by IL-1β is dependent on primary type
Figure 3. Histone deacetylase (HDAC)3 silencing suppresses the expression of HDAC3/6i target genes, contrarily to HDAC6 silencing or inhibition.

(A) Rheumatoid arthritis (RA) fibroblast-like synovocytes (FLS) (n=3) were left untreated or incubated with either 250 nM HDAC3/6i or increasing concentrations (250, 500 and 1000 nM) of HDAC6i for 4 h. Protein lysates were analyzed by western blotting with antibodies recognizing acetylated tubulin and tubulin. (B) RA FLS (n=3) were left untreated or preincubated with 250 nM HDAC3/6i or HDAC6i for 30 min, prior to stimulation with interleukin (IL)-1β for 4 h. Changes in mRNA accumulation were analyzed by quantitative PCR (qPCR), and data were presented as expression relative to GAPDH. (C) RA FLS (n=4) were left not transfected or were transfected for 48 h with 20 nM control non-targeting siRNA (siScrb) or 20 nM HDAC3-specific siRNA (siHDAC3). HDAC3 knockdown efficiency was verified at the mRNA level by qPCR. (D) FLS were transfected as in (C) to confirm HDAC3 protein silencing by immunoblotting. Total protein lysates were analyzed with Abs recognizing HDAC3 or control H3. The signal intensity of five independent experiments was subsequently quantified by densitometry analysis. (E) FLS (n=6) were transfected as in (C) and further stimulated with 1 ng/mL IL-1β for 4 h. Changes in mRNA expression were analyzed as in (B). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ratio t test. (F) FLS (n=3) were transfected with either non-targeting siRNA (siScrb) or 20 nM HDAC6-specific siRNA (siHDAC6) using the same protocol as in (C) to confirm HDAC6 silencing on mRNA. (G) Confirmation of HDAC6 silencing on protein level was assessed as in (D), protein lysates were immunoblotted for HDAC6 or control actin, and densitometry analysis of three independent experiments is shown. (H) FLS (n=4) were transfected as in (C) and further stimulated with 1 ng/mL IL-1β for 4 h. Changes in mRNA expression were analyzed as in (B). *p<0.05, ratio t test.
I IFN signalling. Consistent with this, and in contrast to HDAC3/6i suppression of STAT1 phosphorylation in response to IL-1β, activation of STAT1 by exogenously added IFN-β was unaffected by HDAC3/6i (figure 5E). Silencing of IFNAR1 expression prevented the expression of a subset of genes regulated by HDAC3 (eg, CXCL9 and CXCL11), but left others (IL6 and IL8) unaffected (figure 5F). These data indicate that HDAC3 contributes to the activation of RA FLS in part through promoting type I IFN production and subsequent autocrine effects on STAT1-dependent gene expression and indicate that selective inhibition of HDAC3 could dampen the inflammatory activation of FLS.

Figure 4. Histone deacetylase (HDAC)3 regulates STAT1 phosphorylation, but not STAT1 acetylation.

(A) Rheumatoid arthritis fibroblast-like synoviocytes (FLS) (n=3) were left untreated or preincubated with 250 nM HDAC3/6i or HDAC6i for 30 min and further stimulated with interleukin (IL)-1β for 4 h. Protein lysates were analyzed by western blotting with antibodies recognizing STAT1 Tyr701 phosphorylation (p-STAT1 Tyr701), STAT1 or control H3. A representative of four independent experiments is shown. (B) Transcription factor DNA-binding assays were used to analyze DNA-binding activity of STAT1 in FLS nuclear extracts (n=6). The data are presented as the percentage of the absorbance values relative to unstimulated cells. **p<0.01, Friedman test followed by Dunn’s multiple comparison analysis. (C) FLS were transfected as in figure 3C and further stimulated with IL-1β for 4 h. Protein lysates were analyzed by western blotting with antibodies recognizing phospho-STAT1(Tyr701) and H3 and signal intensity of six independent experiments was subsequently quantified by densitometry analysis. *p<0.05, Wilcoxon matched pairs test. (D and E) FLS were preincubated with 250 nM HDAC3/6i for 30 min and stimulated with IL-1β for either 4 h (D) or 1 and 2 h (E). Cell lysates were precleared, immunoprecipitated with STAT1 Ab and immunoblotted with either STAT1 or acetylated lysine (Ac Lys) Abs. The input represents 10% of the whole cell lysate used for the immunoprecipitation.
Figure 5. Histone deacetylase (HDAC)3/6i effects on STAT1 regulation reflect the suppression of interferon (IFN)-β.

(A) Rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS) were stimulated with either 1 ng/mL interleukin (IL)-1β or 1000 U/mL IFN-α and IFN-β at different time points, as shown in the figure. Protein lysates were analyzed by western blotting with antibodies recognizing phospho-STAT1(Tyr701), STAT1 and H3. (B and C) RA FLS (n=3) were left not transfected or were transfected for 48 h with 20 nM control non-targeting siRNA (siScrb) or 20 nM IFN-α/β receptor alpha chain (IFNAR1)-specific siRNA (siIFNAR1). IFNAR1 knockdown efficiency was verified at the mRNA level by quantitative PCR (qPCR) in (B) and at the protein level by western blot in (C). In (C), total protein lysates were analyzed with Abs recognizing IFNAR1 or control actin (representative of three independent experiments). (D) FLS (n=2) were transfected as in (B), stimulated with either 1 ng/mL IL-1β for 4 h or with 1000 U/mL IFN-β for 1 h and total protein lysates were processed as in (A). (E) FLS (n=2) were left untreated or incubated with 250 nM HDAC3/6i for 30 min, prior to stimulation with either 1 ng/mL IL-1β for 4 h or with 1000 U/mL IFN-β for 1 h. Protein lysates were analyzed as in (A). (F) FLS (n=7) were transfected as in (B) and stimulated with 1 ng/mL IL-1β for 4 h. Changes in mRNA accumulation were analyzed by qPCR, and data were presented as the mean±SEM mRNA expression relative to GAPDH. *p<0.05, ratio t test.
DISCUSSION

In RA, substantial lack of responsiveness to available therapies is leading to the growing necessity to identify novel therapeutic targets which could suppress inflammatory cytokine production. Further on, relapse phenomena after reducing or stopping conventional and disease-modifying antirheumatic drug treatment may indicate a pathological epigenetically imprinted status of the immune and stromal cells that contributes to the perpetuation of inflammatory activation. Indeed, distinct DNA methylome signatures and elevated HDAC expression have been observed in long-term cultured RA FLS compared with osteoarthritis (OA) FLS, and pharmacological inhibition of proteins reading or modifying epigenetic marks was shown to prevent the inflammatory activation of RA FLS.

Pan-HDACi were described as potent anti-inflammatory drugs in several immune-mediated diseases and in a variety of solid and hematologic tumours. Despite their safety and approved use for severe malignancies, including cutaneous T-cell lymphoma, evidence from cancer clinical trials has raised the potential of undesirable effects occurring upon HDACi treatment, such as thrombocytopenia, caused by defective megakaryocyte differentiation and platelet formation and possibly associated with tubulin hyperacetylation. Hence, selective HDAC inhibition may help to improve the therapeutic margin of safety. Emerging evidence indicates that specific class I HDAC family members (HDAC1–3, 8) could have a major role in the transcriptional regulation of inflammatory mediators, both in arthritis models and in other inflammatory diseases. Here, we compared the effects of ITF2357, a pan-HDACi shown to repress inflammation in vitro and in vivo models of arthritis, with HDAC1/2, HDAC3/6 and HDAC8 inhibitors on gene expression in RA FLS. We found that inhibition of HDAC3/6, but not of HDAC1/2 nor HDAC8, highly resembled the effects of pan-HDACi, as it led to suppression of genes associated with RA pathogenesis, including cytokines, MMPs, as well as IFNB1 and IFN-related genes. Importantly, both HDAC1/2 and HDAC8 inhibitors that were used in this study are remarkably selective and retain selectivity at high doses, indicating that the lack of effect on the genes that we screened is unlikely to be associated with ineffective HDAC enzymatic inhibition. In line with this possibility, transcriptome analysis of HDAC1-knockout FLS revealed that HDAC1 is predominantly implicated in the control of cell migration and proliferation, rather than cytokine transcription. Additionally, the acetylation signature of PCI34051, a selective HDAC8 inhibitor, was found to be restricted to a limited set of targets, particularly SMC3. Taken together, HDAC1, HDAC2 and HDAC8 are likely to play roles in responses to other stimuli, target other genes not screened in our study, regulate the inflammatory response at later time points or have cell-specific roles.

HDAC3 was previously shown to be an important epigenome modifier in the transcriptional regulation of inflammatory genes, as HDAC3 depletion prevents LPS-induced macrophage activation and its pharmacological disruption regulates atherogenic macrophage polarisation and cytokine production in RA patient PBMCs. On the other hand, HDAC6 plays a role in immunological tolerance in macrophages and its deficiency enhances regulatory T cells (T_{reg}) suppressive functions. Screening of a subset of genes affected by HDAC3/6i revealed that HDAC3 knockdown reproduced the effects
of HDAC3/6i, though to a more moderate extent that reflected partial knockdown efficiency. In contrast, both HDAC6 silencing and inhibition showed null or mild effects on transcriptional regulation, indicating that HDAC3 has a primary role in mediating the IL-1β-induced activation of FLS.

The anti-inflammatory properties of HDAC3 depletion in macrophages were described to be dependent on altered STAT expression and function. This observation is in line with previous works showing that bulk HDAC activity and the expression of class I HDACs are required to regulate the Janus kinase (JAK)/STAT signalling. In RA FLS, we found that IL-1β-induced STAT1 Tyr701 phosphorylation, an indicator of STAT1 activation, was abrogated by HDAC3/6i. Notably, drugs interfering with the JAK/STAT signalling are beneficial in the treatment of patients with RA failing to respond to methotrexate, pointing to a relevant targetable pathway for the disease. As STAT signalling is regulated by HDACs, we wondered whether direct acetylation of STAT1 protein could interfere with normal STAT1 function. Evidence in literature indicates, in fact, that STAT1 acetylation is necessary for its subsequent dephosphorylation. However, we could not detect STAT1 acetylation after incubation with HDAC3/6i. Previous papers reporting STAT1 acetylation at late time points might indicate that this phenomenon is rather secondary and cannot, anyhow, explain HDAC3/6i early effects on gene regulation. On the contrary, we report that HDAC3/6i indirectly regulates STAT1 activation by primary suppression of type I IFN production. Given the variety of genes regulated by HDAC3/6i, we investigated to what extent selective suppression of IFN signalling could affect global gene expression in FLS. As expected, only the expression of a subset of genes involved in classical type I IFN response, such as CXCL9 and CXCL11, but not IL6 and IL8, was suppressed by IFNAR1 silencing. Thus, inhibition of the IFN signature is a relevant, but not exclusive, mechanism for the regulation of inflammatory gene expression by HDAC3/6i. In line with this possibility, previous findings from our group and by others identified control of mRNA decay as a distinct mechanism by which HDACi control gene expression. Specifically, IL6 transcript stability was significantly reduced after pan-HDACi treatment in RA FLS, suggesting that a similar regulation could occur upon selective HDAC3 inhibition.

While the process underlying HDAC3-mediated regulation of type I IFN signalling in FLS needs to be further characterized, and the action range of HDAC3/6i has yet to be investigated, data from this study and others provide strong evidence that HDAC3 can act as a crucial epigenetic regulator of inflammation. Our results suggest that the development of selective HDAC3 inhibitors could be beneficial in the therapy of inflammatory disorders, such as RA and other rheumatic diseases characterized by type I IFN signature, while limiting possible side effects of pan-HDACi.

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REFERENCES

HDAC3 regulates the inflammatory gene expression programme of RA FLS


Supplementary Figure 1.
RA FLS (n=2) were either left untreated or were incubated with increasing concentrations (250, 500, 1000 and 2000 nM) HDAC1/2i or HDAC8i for 30 min, prior to stimulation with 1 ng/ml IL-1β for 4 h. Total RNA was extracted, and changes in mRNA accumulation of IL6 (A) and IL8 (B) were analyzed by quantitative (q)PCR. Data are presented as the mean ± SEM mRNA expression relative to GAPDH.

Supplementary Figure 2.
FLS (n=3) were either left untreated or were incubated with 2µM HDAC6i for 30 min, prior to stimulation with 1 ng/ml IL-1β for 4 h. Changes in cell viability were analyzed by MTT assay and presented as the mean absorbance ± SEM at 590 nm.
SUPPLEMENTARY MATERIALS AND METHODS

Protein extraction and immunoblotting
FLS were lysed in Laemmli’s buffer or FLS nuclear fractions were extracted using a Nuclear Extraction Kit (Active Motif) and protein content quantified with a BCA Protein Assay Kit (Pierce). Equivalent amounts of total protein lysate or nuclear extract were resolved by electrophoresis on 4-12% Bis-Tris SDS NuPAGE gels (Invitrogen). Proteins were then transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories), membranes were blocked in Tris buffered saline (pH 8.0) containing 0.05% Tween-20 (Bio-Rad) and 4% milk (Bio-Rad), washed and probed overnight at 4°C with antibodies recognizing HDAC3 (Abcam), HDAC6 (Santa Cruz), actin (Santa Cruz), H3 (Cell Signaling), acetyl lysine (Cell Signaling), Tubulin (Sigma), acetyl tubulin (Sigma), IFNAR1 (Abcam), STAT1 (Santa Cruz), p-STAT1 (Tyr701) (Invitrogen). After washing, membranes were incubated with HRP-conjugated swine anti-rabbit or goat anti-mouse immunoglobulin secondary antibody (Dako) and protein visualization and quantification was performed using an ImageQuant LAS4000 system. Densitometry analysis was done using ImageJ software. All lanes were plotted, upon which the areas of the graphs were measured. To analyse the relative expression, we calculated the ratio between the expression of HDAC3 and H3, HDAC6 and actin, or IFNAR1 and actin.

HDAC activity assays
HDAC substrates for the different HDAC classes, based on ε-N-acylated lysine, were derivatized on the carboxyl group with the fluorophore 7-amino-4-methylcoumarin (AMC) and analysed by fluorescence emission as previously described.1

MTT assay
Cell viability was assessed 24 h post-treatment with HDAC inhibitors. FLS were incubated 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 acidified isopropanol solution containing 0.1% Igepal CA-630 (Sigma-Aldrich) and absorbance measurement at 590 nm.

mRNA expression analysis
RNeasy Micro Kit (Qiagen) was used for RNA extraction. Quantity and purity of RNA was assessed using a Nanodrop spectrophotometer (Nanodrop Technologies). RNA was reverse-transcribed using a First-Strand cDNA synthesis kit (Thermo Scientific) and quantitative (q)PCR reaction was performed using Fast SybrGreen PCR Master Mix (Applied Biosystems). Sequences of the primers used are listed in supplementary table1. For qPCR array analysis, RNA was reverse-transcribed using an RT² HT First Strand Kit (Qiagen), cDNA was mixed with SybrGreen qPCR Mastermix (Qiagen) and expression of 83 genes involved in FLS activation was analysed using RT² Profiler customised qPCR arrays. qPCR reactions were performed on a StepOne Plus Real-Time PCR System (Applied Biosystems) and relative mRNA expression was calculated using StepOne Software V.2.1 (Applied Biosystems). The ratio between the gene of interest and the expression of GAPDH housekeeping gene, or the expression of five housekeeping genes (B2M, HPRT1, RPL13A,
HDAC3 regulates the inflammatory gene expression programme of RA FLS

GAPDH and ACTB) was calculated for qPCR and qPCR arrays, respectively, using the CT method.

**ELISA**

IL-6 and IL-8 were measured using the human IL-6 ELISA Set, and the human IL-8 ELISA Set (BD Biosciences) in cell culture supernatants according to the manufacturer’s instructions.

**Invasion assay**

24-well Boyden chambers provided with 8 µm transparent PET membranes (Corning) were coated with Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (3 mg/ml, Corning). To measure cell invasion, RA FLS were suspended in medium containing 1% FCS and either left untreated or stimulated with IL-1β with or without pretreatment with 250nM HDAC3/6i and seeded into the chambers. Medium supplemented with 10 % FBS was used as an attractant in the lower chamber. After 24h, the invaded cells on the lower side of the membrane were fixed with methanol and stained with crystal violet 0.5 %. The number of invading cells was averaged from three 10 field-of-view images.

**siRNA transfection**

RA FLS were transfected using DharmaFECT1 (Thermo Scientific). The day before transfection cells were incubated with DMEM containing 10% FBS which was then replaced with OPTI-MEM serum-reduced medium. HDAC3, HDAC6 or IFNAR1 specific siRNA (20nM) and control non-targeting siRNA (20nM), (Thermo Scientific) were mixed with DharmaFECT1 and incubated for 20 minutes at room temperature prior to transfection. 24 h after transfection, medium was replaced with DMEM containing 1% FBS for another 24 h.

**STAT1 DNA-binding**

STAT1 DNA-binding activity in FLS nuclear fractions was determined using a TransAM transcription factor ELISA (Active Motif) according to the manufacturer’s instructions. 20 µg of purified nuclear extracts were used for each experiment.

**Immunoprecipitation**

RA FLS were lysed in IP buffer (1% Triton x100, 10% glycerol, 20mM Tris HCl pH 7.4, 135 mM NaCl, 1.5 mM MgCl$_2$) with the addition of protease inhibitors (1 tablet in 10mL lysis buffer, Roche). The homogenate was left on ice for 20 min and centrifuged at 13000 rpm for 10 min at 4°C. Whole cell lysate was pre-cleared with G-sepharose (1µl for 50µl of cell lysate) and incubated with rotation at 4 °C for 1 h. Pre-cleared lysates were centrifuged at 1500 rpm for 1 min, supernatant collected and transferred to clean eppendorfs. Approximately 2 µg of protein of the pre-cleared lysate were stored and used as input for immunoblotting, while 20 µg proteins were further incubated with anti-STAT1 Ab (Santa Cruz, 1:100 dilution) overnight with rotation at 4 °C. The next day, lysate was incubated with G-sepharose (1µl for 50µl of cell lysate) for 3 h with rotation at 4 °C, centrifuged at 1500 rpm for 1 min and washed 3 times with IP lysis buffer. At the last step, superna-
tant was carefully removed and resuspended in Laemmli’s buffer for immunoblotting, as described in detail above. Anti-acetyl lysine Ab (Cell Signaling, 1:1000 dilution) was used for final Western-blot.

**Supplementary Table 1. Sequences of primers used for qPCR analysis.**

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<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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**REFERENCE LIST**