Molecular mechanisms of histone deacetylases in rheumatoid arthritis fibroblast-like synoviocytes
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INFLAMMATORY CYTOKINES EPIGENETICALLY REGULATE RHEUMATOID ARTHRITIS FIBROBLAST-LIKE SYNOVIOTCYTE ACTIVATION BY SUPPRESSING HDAC5 EXPRESSION.

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ABSTRACT

Objectives: Epigenetic modifications play an important role in the regulation of gene transcription and cellular function. Here, we examined if pro-inflammatory factors present in the inflamed joint of patients with rheumatoid arthritis (RA) could regulate histone deacetylase (HDAC) expression and function in fibroblast-like synoviocytes (FLS).

Methods: Protein acetylation in synovial tissue was assessed by immunohistochemistry. The mRNA levels of HDAC family members and inflammatory mediators in the synovial tissue and the changes in HDAC expression in RA FLS were measured by quantitative (q)PCR. FLS were either transfected with HDAC5 siRNA or transduced with adenoviral vector encoding wild-type HDAC5 and the effects of HDAC5 manipulation were examined by qPCR arrays, ELISA and ELISA-based assays.

Results: Synovial class I HDAC expression was associated with local expression of tumor necrosis factor (TNF) and matrix metalloproteinase-1, while class IIa HDAC5 expression was inversely associated with parameters of disease activity (erythrocyte sedimentation rate, C-reactive protein, Disease Activity Score in 28 Joints). Interleukin (IL)-1β or TNF stimulation selectively suppressed HDAC5 expression in RA FLS, which was sufficient and required for optimal IFNB, CXCL9, CXCL10 and CXCL11 induction by IL-1β, associated with increased nuclear accumulation of the transcription factor, interferon regulatory factor 1 (IRF1).

Conclusions: Inflammatory cytokines suppress RA FLS HDAC5 expression, promoting nuclear localization of IRF1 and transcription of a subset of type I interferon response genes. Our results identify HDAC5 as a novel inflammatory mediator in RA, and suggest that strategies rescuing HDAC5 expression in vivo, or the development of HDAC inhibitors not affecting HDAC5 activity, may have therapeutic applications in RA treatment.
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INTRODUCTION

Genetic predisposition in rheumatoid arthritis (RA) contributes up to 50% of the risk of disease, with many of the identified genetic susceptibility loci associated with adaptive immune responses. An increasing effort is now being made on understanding how environmental factors, such as nutrition, infection, smoking or pollution, and the epigenetic regulatory mechanisms they influence, interact with genetic backgrounds to promote disease onset and progression in RA. These epigenetic regulatory mechanisms include the highly complex network of DNA methylation, histone modifications and expression of non-coding RNAs, which dynamically interact with each other to regulate cellular function in health and disease.

In RA, a rapidly increasing number of alterations in epigenetic regulatory processes contributing to disease development and progression has been described, particularly in relation to the imprinted pathogenic behavior of stromal fibroblast-like synoviocytes (FLS). A recent study identified more than 2000 loci showing differential DNA methylation patterns in RA and osteoarthritis (OA) FLS, mostly associated with genes relevant to the immune response. Moreover, aberrant miRNA expression in RA FLS contributes to both cellular activation and survival, and serum levels of circulating miRNA in patients with early arthritis may be associated with disease activity and progression. Histone modifications and histone-modifying enzymes comprise another distinct epigenetic regulatory mechanism. Histone acetyltransferases (HATs) modify N-terminal histone lysine residues and can confer to chromatin a hyperacetylated state associated with enhanced gene transcription. Histone deacetylases (HDACs) counteract HAT activity through the targeting of histones and non-histone signal transduction proteins and transcription factors.

The mammalian HDAC family is composed of the ubiquitously expressed class I HDACs (HDACs 1–3 and 8), tissue-enriched class II HDACs (HDACs 4–7, 9, 10), class III sirtuins (Sirt1–7) and class IV HDAC11. In chronic obstructive pulmonary disease (COPD), decreased HDAC activity is thought to play a central role in the development of the disease, and treatment of patients with COPD with theophylline restores HDAC activity and expression, improves lung function and decreases inflammatory cytokine levels. Early investigation of RA synovial tissue observed similarly depressed HDAC activity and expression, suggesting that a shift in HDAC/HAT activity favoring histone acetylation might constitute a general characteristic of chronic inflammatory diseases. However, others have reported that HDAC1 is elevated in RA, compared with OA, and positively correlates with tumor necrosis factor (TNF) expression. Moreover, suppression of HDAC activity with HDAC inhibitors (HDACi) decreases inflammatory cytokine production by immune and stromal cells derived from patients with RA, shows protection in animal arthritis models and has demonstrated clinical efficacy in the treatment of systemic-onset juvenile idiopathic arthritis. We undertook this current study to better understand the relationship between synovial HDAC expression and function, synovial protein acetylation, and clinical and local parameters of disease activity in RA.
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MATERIALS AND METHODS

Patients and synovial tissue samples
Synovial tissue specimens for immunohistochemical analyses were obtained from 12 patients with RA and 12 patients with OA (cohort III) undergoing surgical joint replacement at the Clinic of Orthopaedic Surgery, Schulthess Hospital Zurich, fixed in paraformaldehyde and embedded in paraffin. Synovial biopsies (six to eight per patient) for immunohistochemical analyses were also obtained from 18 patients with RA and 12 patients with psoriatic arthritis (PsA) (cohort IV) by arthroscopy, as were samples for immunohistochemical (cohort II) and mRNA (cohort I) expression studies from 20 and 19 patients with RA, respectively. Samples were embedded in TissueTek OCT (Miles Diagnostics), snap-frozen and stored in liquid nitrogen until further processing, as previously described.23 Patients with RA, PsA and OA fulfilled the 1987 revised criteria of the American College of Rheumatology (ACR), the CASPAR criteria for PsA and ACR criteria for knee OA, respectively.24–26 Informed written consent was provided by all patients prior to their inclusion in these studies, which were approved by the medical ethics committees of the Academic Medical Center, University of Amsterdam, The Netherlands, and the Schulthess Hospital, Zurich, Switzerland. Clinical characteristics of patients are shown in table 1.

Quantitative measurement of mRNA expression
For analysis of synovial tissue gene expression, total RNA was isolated from biopsies of 19 patients with RA using RNA STAT-60 (Invitrogen) according to the manufacturer’s instructions, and cleaned using RNEasy columns (Qiagen) and DNAse treatment. RNEasy Micro Kit (Qiagen) was used for RNA extraction from FLS. Quantity and purity of RNA was assessed using a Nanodrop spectrophotometer (Nanodrop Technologies). About 150–500 ng of 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 online supplementary table 1. For qPCR array analysis, 1000 ng of RNA was reverse transcribed using an RT² HT First Strand Kit (Qiagen), cDNA was mixed with SybrGreen qPCR Mastermix (Qiagen) and expression of 84 genes involved in FLS activation was analyzed using RT² Profiler customized qPCR arrays. qPCR reactions were performed on a StepOne Plus
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Real-Time PCR System (Applied Biosystems) and relative mRNA expression was calculated using StepOne Software V.2.1 (Applied Biosystems) as the ratio between the gene of interest and the expression of housekeeping gene(s) using the CT method.

Immunohistochemical analyses and digital image analysis
Serial sections from TissueTek-embedded biopsy samples were cut with a cryostat (5 μm), fixed with acetone and endogenous peroxidase activity blocked with 0.3% hydrogen peroxide in 0.1% sodium azide/phosphate-buffered saline. Sections were stained overnight at 4°C with rabbit antibodies against acetylated lysine (acLys) or acetylated histone 3 (Lys18) (acH3) (both from Cell Signaling Technology). Equivalent concentrations of control rabbit antibodies (anti-fluorescein isothiocyanate, Thermo Scientific) were applied for control sections. Sections were then washed and incubated with horseradish peroxidase (HRP)-conjugated swine anti-rabbit antibodies (Dako), followed by incubation with biotinylated tyramide and streptavidin-HRP, and development with aminoethylcarbazole (AEC, Vector Laboratories). Sections were subsequently counterstained with Gill’s haematoxylin (Klinipath) and mounted in Kaiser’s glycerol gelatine (Merck). For quantitative analysis of protein acetylation, stained sections were randomly coded by an independent observer, blinded to antibodies used and clinical diagnosis. Stained slides were analyzed by computer-assisted image analysis using the Qwin analysis system (Leica) as previously described. Values of integrated optical densities (IOD)/mm² were obtained and corrected for total number of nucleated cells per square millimeter.

Statistical analyses
Data are presented as mean±SEM unless otherwise indicated. The Spearman’s correlation coefficient was used for correlation analyses. The Friedman test and Dunns’ post-hoc test were used for analyzing sets of data requiring multiple comparisons. The Mann–Whitney U test or the Wilcoxon matched pairs test were used for all other comparisons. The p values <0.05 were considered statistically significant.

Detailed descriptions of staining of paraffin-embedded sections, FLS culture and stimulation, HDAC activity and ELISA assays, immunoblotting, siRNA transfection and adenoviral transduction are provided in online supplementary materials and methods.

RESULTS

Synovial class I HDAC expression is associated with elevated expression of inflammatory mediators.

HDAC1 mRNA expression has been shown to positively correlate with TNF mRNA levels in RA synovial tissue. Here, we screened the synovial tissue from patients with RA to examine if this association is restricted to HDAC1 or can be extended to other HDACs (cohort I). TNF mRNA expression demonstrated a strong positive correlation with the mRNA expression of HDAC1 (figure 1A, top panel, R=0.651, p=0.003), and also to a lesser extent with other class I HDAC family members (figure 1A middle panel; HDAC2: R=0.523, p=0.022;
HDAC3: R=0.570, p=0.011). Synovial matrix metalloproteinase-1 (MMP-1) expression was also positively associated with the expression of HDAC1 (R=0.502, p=0.029) and HDAC2 (R=0.512, p=0.025), while a trend towards a positive correlation between MMP-1 and HDAC3 was also noted (figure 1B). No correlation was observed between TNF or MMP-1 expression and any of the class II HDACs, or the class I member HDAC8 (data not shown). In contrast, while expression of interleukin (IL)-6 was not associated with any of the class I HDACs (figure 1C top and middle panels and data not shown), we found a significant inverse relationship between expression of IL-6 and the class IIa member HDAC5 (figure 1C bottom panel, R=−0.477, p=0.039).

We also analyzed the relationship between HDAC mRNA levels and clinical parameters of disease activity in RA. Significant negative correlations were observed between

![Figure 1. Expression of class I histone deacetylases (HDACs) correlates positively with tumor necrosis factor (TNF) and matrix metalloproteinase (MMP)-1 mRNA levels in rheumatoid arthritis (RA) synovial tissue, while the class IIa HDAC5 expression is negatively associated with disease activity.](image)

The mRNA expression of HDAC1–10, TNF, MMP-1 and interleukin (IL)-6 was analyzed in the synovial tissue of 19 patients with RA (cohort I) by qPCR and calculated relative to the mean 18S rRNA and glyceraldehyde-3-phosphate dehydrogenase expression. Analyses of correlations between synovial expression of HDAC family members and (A) TNF, (B) MMP-1 and (C) IL-6 mRNA levels, and between (D) HDAC5 mRNA expression and the C-reactive protein (CRP) levels, erythrocyte sedimentation rate (ESR) and disease activity score in 28 joints (DAS28) were performed using Spearman’s correlation coefficient. Spearman R and p values are indicated in each graph, and circles indicate individual patient values.

Figure 1. Expression of class I histone deacetylases (HDACs) correlates positively with tumor necrosis factor (TNF) and matrix metalloproteinase (MMP)-1 mRNA levels in rheumatoid arthritis (RA) synovial tissue, while the class IIa HDAC5 expression is negatively associated with disease activity.
HDAC5 expression and the levels of serum C-reactive protein (CRP) (figure 1D top panel; R=−0.664, p=0.007), erythrocyte sedimentation rate (ESR) (figure 1D middle panel; R=−0.557, p=0.013) and patient disease activity score in 28 joints (DAS28) (figure 1D bottom panel; R=−0.567, p=0.011). Notably, there were no correlations between patient DAS28 score and mRNA levels of any other HDAC family member (data not shown). To validate these findings, we retrieved HDAC5 and IL-6 expression data from a previously published study analyzing the synovial tissue from a cohort of 62 patients with RA by microarrays. To validate these findings, we retrieved HDAC5 and IL-6 expression data from a previously published study analyzing the synovial tissue from a cohort of 62 patients with RA by microarrays. In this cohort we also observed significant negative correlations between

Figure 2. Protein acetylation levels are equivalent in rheumatoid arthritis (RA), osteoarthritis (OA) and psoriatic arthritis (PsA) synovial tissue.

(A) Representative photomicrographs of RA tissue sections stained with control rabbit antibody (control Ig), or with antibodies recognizing acetylated lysine (aclLys) and acetylated histone 3 (Lys18) (acH3). Synovial tissue sections from (B) 12 patients with RA and 12 patients with OA (cohort III), and from (C) 18 patients with RA and 12 patients with PsA (cohort IV) were stained with antibodies recognizing aclLys and acetylated histone 3 (Lys 18) (acH3). Integrated optical density (IOD/mm²) values were calculated by computer-assisted digital image analysis and corrected for tissue cellularity. Differences between patient groups were analyzed using the Mann–Whitney U test. (D) Synovial biopsy sections from 20 patients with RA (cohort II) were stained with antibodies against aclLys and acH3 and the relationship between IOD/mm² and patient C-reactive protein (CRP) levels was calculated using Spearman’s correlation coefficient and presented as in figure 1.
synovial HDAC5 expression and serum CRP levels as well as expression of IL-6 (see online supplementary figure 1).

**RA and OA synovial tissue contain comparable levels of acetylated proteins.** Previous studies of HDACs in RA and other rheumatic diseases have analyzed total HDAC activity and expression in peripheral blood and synovial tissue, but it has not been assessed if reported differences result in altered acetylation of histones and non-histone proteins in the rheumatoid joint. To address the relationship between synovial HDAC expression and acetylation of histone and non-histone proteins, we performed immunohistochemical staining on synovial tissue, comparing RA and OA (cohort III), and RA and PsA (cohort IV) in two independent studies, using antibodies specific for acH3 (Lys18), a histone epigenetic marker associated with inflammation, and total acetylated lysine (acLys). While no specific staining was observed with control rabbit antibody, a strong nuclear staining was detected in the RA synovial tissue with anti-acH3 and anti-acLys antibodies. Protein acetylation was apparent both in the mono-nuclear cells infiltrating the synovial sublining and in the intimal lining layer (figure 2A left panel). Quantitative analysis of protein acetylation status on two sets of stainings from two independent cohorts revealed no significant differences in acLys and acH3 levels between RA and OA (cohort III, figure 2B), nor between RA and PsA (cohort IV, figure 2C), together indicating that protein acetylation levels are equivalent in both inflammatory and non-inflammatory forms of arthritis and are not associated with differential HDAC expression.

We next tested the hypothesis that protein acetylation status in synovial tissue might be related to patient disease activity. We performed immunohistochemical staining on synovial tissue from an additional independent group of 20 patients with RA (cohort II). No correlation was observed between acetylation markers and serum CRP levels (figure 2D), ESR or DAS28 (data not shown).

**Inflammatory stimuli suppress HDAC5 expression in RA FLS.** As we observed an inverse relationship between disease activity in RA and synovial expression of HDAC5, we next examined the potential effect of diverse inflammatory stimuli on the expression of class I and class II HDACs in RA FLS. mRNA expression of class I HDACs was not significantly affected by 24 h exposure to IL-1β, TNF or lipopolysaccharide (LPS) (figure 3A). In contrast, the class IIa HDAC5 was significantly down-regulated by 60% following TNF or IL-1β stimulation for 24 h. A decrease in mRNA expression was also observed at earlier time points after IL-1β (figure 3B). While the toll-like receptor (TLR)4 ligand LPS only moderately suppressed HDAC5 expression, the TLR3 ligand poly(I:C) also resulted in significant down-regulation of HDAC5 mRNA (see online supplementary figure S2). Inflammation-mediated down-regulation of HDAC5 mRNA by IL-1β and TNF was also reflected in reduced HDAC5 protein expression (figure 3C, D). We also examined HDAC activity in FLS using enzymatic substrates distinguishing between each class of HDACs. Class I and class IIb HDAC enzymatic activity was significantly enhanced after 24 h stimulation with IL-1β and TNF, respectively, while no significant changes occurred in class IIa HDAC activity (figure 3E). Collectively, these results indicate that class I, IIa and IIb HDAC family member expression and activity are differentially regulated in RA FLS by inflamma-
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Figure 3. Interleukin (IL)-1β down-regulates histone deacetylase 5 (HDAC5) but not class I HDACs in rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS).

(A) RA FLS (n=4) were left untreated or were stimulated with 1 ng/mL IL-1β, 10 ng/mL tumor necrosis factor (TNF) or 1 mg/mL lipopolysaccharide (LPS) for 24 h, and changes in mRNA accumulation of HDAC1–10 were analyzed by quantitative (q)PCR. Data are presented as the mean±SEM mRNA expression relative to glyceraldehyde-3-phosphate dehydrogenase.

(B) RA FLS were left untreated or were stimulated with 1 ng/mL IL-1β for 1–8 h and changes in HDAC5 mRNA expression analyzed by qPCR and presented as above.

(C) RA FLS were left untreated or were stimulated with either 1 ng/mL IL-1β (n=5) or 10 ng/mL TNF (n=4) for 24 h. Protein extracts were prepared and analyzed by immunoblotting with anti-HDAC5 and anti-actin antibodies, and signal intensity was subsequently quantified by densitometry analysis. Values for the 0 h time point were normalized to 100% and remaining values were shown as the mean±SEM percentage of HDAC activity compared with controls. Insets represent the mean±SEM fluorescence values for cells untreated or stimulated for 24 h with IL-1β or TNF. *p<0.05, **p<0.01, Friedman test followed by Dunn’s multiple comparison analysis with unstimulated cells used as reference controls.
tory agonists. HDAC5 regulates IL-1β-dependent cytokine and chemokine production in RA FLS. The relative activities of each member of class IIa HDACs in vivo are unknown, and class IIa HDACs have enzymatic-independent cellular functions. To better understand the role of HDAC5 in RA FLS and the consequences of its down-regulation by inflammatory stimuli, we silenced its expression with HDAC5-specific siRNA. HDAC5 siRNA efficiently silenced expression of both HDAC5 mRNA and protein (figure 4A, B, respectively), without affecting the mRNA expression of any of the other HDACs (see online supplementary figure 3) or FLS viability (figure 4C). We then analyzed the effects of HDAC5 knockdown on the expression of selected genes regulated by IL-1β and/or TNF in RA FLS. Interestingly, we found that the mRNA expression of a specific subset of chemokines and cytokines, namely, CXCL9, CXCL10, CXCL11 and IFNB, was potentiated by HDAC5 silencing in RA FLS exposed to IL-1β (figure 4D and online supplementary figure 4A). We observed a similar trend towards enhanced TNF and IL-1β expression, but no effect on other cytokines or MMPs, including IL-6, IL-8, MMP-1 and MMP-3 (see online supplementary figure 4A and data not shown). As observed at the level of mRNA expression, specific silencing of HDAC5 significantly enhanced CXCL-10 protein production following IL-1β stimulation (figure 4E), while IL-6 secretion was unaffected (figure 4F). Reciprocally, RA FLS were transduced with either adenovirus encoding control green fluorescent protein or wild-type HDAC5 (HDAC5WT). Over-expression of HDAC5 protein (figure 4G) had no significant effects on cell viability (figure 4H). HDAC5 over-expression specifically suppressed IL-1β induction of CXCL9, CXCL10, CXCL11 and IFNB mRNA expression (figure 4I and online supplementary figure 4B), while failing to influence expression of IL-6, IL-8, MMP-1 or MMP-3 (see online supplementary figure S4B). Interestingly, analysis of CXCL10 mRNA expression in RA synovial tissue (cohort I) revealed a significant positive correlation between CXCL10 transcript levels and the levels of serum CRP and a trend towards negative correlation between CXCL10 and HDAC5 mRNA expression (see online supplementary figure S5), confirming a functional link between HDAC5, CXCL-10 and RA disease activity.

**Silencing of HDAC5 in RA FLS induces the nuclear retention of the IRF1 transcription factor.**

To investigate the potential mechanism of action by which HDAC5 might regulate RA FLS activation, we examined the effects of HDAC5 silencing on the function of STAT1, nuclear factor (NF)-κB and interferon regulatory factor (IRF)1 transcription factors known to be important in the regulation of CXCL-10 expression. Silencing of HDAC5 failed to modulate IL-1β-induced STAT1 tyrosine phosphorylation (figure 5A) and DNA-binding activity (figure 5B), and had no effect on the NF-κB p50 and p65 subunit DNA-binding activity (figure 5C). However, nuclear accumulation of IRF1 (figure 5D, E), but not IRF3 (data not shown), induced by IL-1β in RA FLS was further enhanced following HDAC5 silencing. Together, these data suggest that HDAC5 might regulate cytokine and chemokine production in RA FLS via specific modulation of IRF1.
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Figure 4. Histone deacetylase 5 (HDAC5) regulates CXCL chemokine production in rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS).

(A-F) RA FLS were left not transfected or were transfected for 48 h with 20 nM control non-targeting siRNA (scr) or 20 nM HDAC5-specific siRNA (siHDAC5). HDAC5 knockdown efficiency was verified at the (A) mRNA and (B) protein level by quantitative (q)PCR and immunoblotting, respectively. Results in (A) are presented as the mean±SEM HDAC5 mRNA expression of three independent experiments and in (B) a representative of four independent experiments is shown. (C) Changes in RA FLS viability after HDAC5 knockdown (n=4) were analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay and presented as the mean±SEM absorbance at 590 nm. (D) Transfected RA FLS (n=8) were stimulated with 1 ng/mL interleukin (IL)-1β for 4 h and mRNA expression of CXCL9, CXCL10, CXCL11 and IFNB relative to glyceraldehyde-3-phosphate dehydrogenase was determined by qPCR. Data are shown as mean±SEM fold induction compared to scrb. *p<0.05, **p<0.01, Wilcoxon matched pairs test. (E) CXCL-10 and (F) IL-6 production by control and transfected RA FLS stimulated with 1 ng/mL IL-1β for 24 h was determined by ELISA. Results are presented as the mean±SEM concentration from seven and five independent experiments for CXCL-10 and IL-6, respectively. *p<0.05, **p<0.01, Friedman test followed by Dunns’ multiple comparison analysis. (G-I) RA FLS were transduced with control GFP-encoding or wild-type HDAC5-GFP (HDAC5WT) encoding adenoviral vector for 24 h and serum-starved for another 24 h prior to further processing. (G) Overexpression of HDAC5 protein was confirmed by immunoblotting with anti-HDAC5 and anti-actin antibodies. The effects of HDAC5 overexpression on (H) cell viability and (I) mRNA expression of CXCL9, CXCL10, CXCL11 and IFNB after 4 h stimulation with 1 ng/mL IL-1β were determined as described above. Data in (H) are shown as the mean±SEM absorbance at 590 nm of four independent experiments, and in (I) as the mean±SEM relative mRNA expression of eight independent experiments. *p<0.05, **p<0.01, Wilcoxon matched pairs test.
DISCUSSION

Previous studies which reported reduced HDAC activity in RA synovium compared with disease controls,\(^\text{15}\) or, alternatively, increased expression of HDAC1 and HDAC activity in RA synovial tissue,\(^\text{16,17}\) have failed to reach a consensus on whether HDAC expression and activity might be altered, and how such alterations might contribute to pathology. Here, we observe a strong positive correlation of HDAC1, HDAC2 and HDAC3 mRNA levels with TNF expression in RA synovial tissue, and similar trends were observed for MMP-1. These positive correlations, together with our observation that inflammatory cytokines can stimulate class I HDAC activity in RA FLS, suggest that elevated class I HDAC might be important in promoting inflammation and tissue damage in RA. In line with this possibility, the class I-selective HDACi MS-275 is more effective in suppressing inflammation and bone destruction in collagen-induced arthritis in rodents than the pan-HDACi suberoylanilide hydroxamic acid.\(^\text{37}\) While specific contributions of HDAC1, HDAC2 and HDAC3...
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enzymatic activity to pathology in RA remain to be determined, combined siRNA-mediated silencing of HDAC1 and HDAC2 in RA FLS cooperatively slows cellular proliferation in response to platelet-derived growth factor. Genetic silencing of the HDAC3 expression in macrophages prevents their inflammatory activation in vivo, but recent studies have indicated that HDAC3 might also regulate gene expression independently of its enzymatic activity. We observed a significant inverse relationship between RA disease activity and synovial mRNA expression of HDAC5. Consistent with evidence that HDAC expression is dynamically regulated by inflammatory stimuli in macrophages, we observed that in RA FLS stimulation with either TNF or IL-1β leads to a rapid downregulation of HDAC5 mRNA and protein expression. Suppressed HDAC5 expression has also been reported in the bronchia of patients with COPD. We find that HDAC5 regulates the expression of a subset of genes involved in the type I interferon response in RA FLS, including CXCL9, CXCL10, CXCL11 and IFNB. CXCL-10, as well as other CXCL chemokines, has a major role in leukocyte homing to inflamed tissues and FLS invasiveness, emphasized by the initial clinical efficacy of an anti-CXCL-10 monoclonal antibody in patients with RA. Interestingly, the promoters of the genes, which we observed to be regulated by HDAC5, were not enriched for potential binding sites for the two known HDAC5-binding partners, MEF2A and YY1. While HDAC5 silencing failed to affect the phosphorylation, nuclear localization and/or DNA-binding activity of three transcription factors known to contribute to the type I interferon response (STAT1, NF-κB or IRF3), HDAC5 silencing promoted nuclear accumulation of IRF1. Although IRF3 is the main IRF family member involved in the regulation of CXCL-10 in response to TLR ligand signaling in RA FLS, elevated synovial lining IRF1 expression is observed in patients with RA compared with patients with OA, indicating involvement of both IRF1 and IRF3 in RA FLS activation. In macrophages, TNF predominantly activates IRF1, and IL1-β was recently shown to induce CXCL-10 expression via recruitment of IRF1 in and in vivo. The mechanism by which HDAC5 regulates IRF1 in RA FLS is independent of effects on IRF1 mRNA expression (data not shown), and may reflect direct or indirect effects on IRF1 nuclear retention. In this regard, it is noteworthy that class IIA HDACs have minimal enzymatic activity compared with other HDACs, and are regulated primarily by phosphorylation events that control their own subcellular localisation. While further studies are needed to characterize the role of HDAC5 in RA, and the mechanism by which it regulates IRF1, our results identify a specific role for HDAC5 in the activation of RA FLS, indicating that further development of the HDACi targeting specific HDACs will allow differential modulation of gene expression programmes contributing to pathology in RA and other immune-mediated inflammatory diseases.

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REFERENCES


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Supplementary Figure 1. HDAC5 expression in RA synovial tissue negatively correlates with disease activity and IL-6 expression.

RNA was isolated from synovial tissue specimens from 62 RA patients and expression levels of HDAC5 and IL-6 were retrieved from the results of gene array analysis. Relationships between HDAC5 expression and IL-6 expression or C-reactive protein (CRP) levels were calculated using Spearman’s correlation coefficient and presented as in Figure 1.

Supplementary Figure 2. TLR ligands differentially regulate HDAC5 expression.

RA FLS were incubated with LPS (1 μg/ml), poly(I:C) (10 μg/ml) or LTA (10 μg/ml) for 24 h. mRNA expression of HDAC5 was analysed by quantitative PCR and data are presented as the mean±SEM HDAC5 expression relative to GAPDH of four independent experiments.

Supplementary Figure 3. HDAC5 siRNA specifically downregulates expression of HDAC5, but not other HDAC family members.

RA FLS were transfected with either control non-targeting siRNA (scrb) or HDAC5-specific siRNA (HDAC5si) as described in Figure 4 to verify the effect of HDAC5 silencing on other HDAC family members. RNA was extracted, reversed transcribed and mRNA expression of HDAC1-10 was analysed by quantitative PCR. Data are presented as mean±SEM mRNA expression relative to GAPDH (n=2).
Supplementary Figure 4. HDAC5 siRNA and overexpression regulate a subset of IL-1β-inducible inflammatory mediators.

(A) RA FLS (n=3) were transfected with control non-targeting siRNA (scrb) or HDAC5-specific siRNA (HDAC5si) as described in Figure 4. Cells were either left unstimulated or were stimulated with 1 ng/ml IL-1β for 4 h. mRNA expression of genes known to be important in FLS biology was determined by quantitative PCR using a customized RT2 Profiler™ PCR Array. Data are shown as mean±SEM mRNA expression relative to the average expression of five housekeeping genes (B2M, HPRT1, RPL13A, GAPDH and ACTB). (B) RA FLS (n=3) were transduced with control GFP-encoding or wild-type HDAC5-GFP (HDAC5WT) encoding adenoviral vector as described in Figure 4. Cells were then left untreated or were stimulated with 1 ng/ml IL-1β for 4 h and mRNA expression was analysed as in (A).

Supplementary Figure 5. Synovial CXCL10 expression positively correlates with patient CRP levels.

CXCL10 mRNA expression in the synovial tissue of 19 RA patients (cohort I) was analysed by qPCR and calculated relative to the mean 18S rRNA and GAPDH expression. Correlations between CXCL10 expression and C-reactive protein (CRP) levels or HDAC5 mRNA expression were calculated using Spearman’s correlation coefficient and presented as in Figure 1.
**SUPPLEMENTARY MATERIALS AND METHODS**

**Immunohistochemical analyses and digital image analysis**
Paraffin-embedded sections were deparaffinized, pretreated at 80°C for 30 minutes in 10 mM citrate buffer (pH 6.0) for antigen retrieval, and endogenous peroxidase activity was blocked with 1% hydrogen peroxide. Sections were then blocked with in Tris buffered saline (TBS) pH 7.4 containing 5% horse serum solution and 1% bovine serum albumin, and incubated overnight at 4°C with primary antibodies. After extensive washing sections were incubated with biotinylated goat anti-rabbit antibody (Jackson, Suffolk, UK) followed by incubation with HRP-conjugated streptavidin complex (Vector Laboratories). Bound antibodies were then visualized using AEC and sections counterstained as described in Materials and Methods.

**FLS culture and stimulation**
RA FLS were isolated from synovial biopsies of patients (n=19) fulfilling the American College of Rheumatology revised criteria for RA, and used for experiments between passages 4 and 9, following overnight culture in medium containing 1% fetal bovine serum (FBS; Invitrogen, Breda, The Netherlands). FLS were stimulated with IL-1β (1 ng/ml) (R&D Systems, Minneapolis, Minnestoa, USA), TNFα (10 ng/ml) (Biosource International, Camarillo, CA, USA), LPS (1 μg/ml), poly(I:C) (10 μg/ml) or LTA (10 μg/ml) for 1-24 h.

**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.\(^3\)

**Protein extraction and immunoblotting**
FLS were lysed in 1x Laemmli’s buffer or FLS nuclear fractions were extracted using a Nuclear Extraction Kit (Active Motif, Carlsbad, CA, USA) and protein content quantified with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Equivalent amounts of total protein lysate, cytoplasmic 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, Hercules, CA, USA), membranes were blocked in Tris-buffered saline (pH 8.0) containing 0.05% Tween-20 (Bio-Rad) and 2% milk (Bio-Rad), washed and probed overnight at 4°C with antibodies recognizing HDAC5 (Cell Signaling), actin (Santa Cruz, Heidelberg, Germany), H3 (Cell Signaling), IRF-1 (Santa Cruz), α-Tubulin (Sigma) and 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. ImageJ software was used for densitometry analysis and the ratio between the expression of HDAC5 and actin was calculated.

**siRNA transfection and adenoviral transduction**
RA FLS were transfected using DharmaFECT1 (Thermo Scientific, Schwerte, Germany).
CHAPTER 3

The day before transfection cells were seeded in 6 well plates in 2 ml DMEM containing 10% FBS which was then replaced with OPTI-MEM serum-reduced medium. 10-100 nM HDAC5-specific siRNA or control non-targeting siRNA (Thermo Scientific) were mixed with DharmaFECT1 and incubated for 20 minutes at RT prior to transfection. 24 h after transfection, medium was replaced with DMEM containing 1% FBS for an additional 24 h. Control adenoviruses encoding GFP (provided by Dr SW Tas, our institute) and adenoviruses encoding GFP-tagged HDAC5 (HDAC5WT) were amplified and titrated in HEK-293 cells. FLS were transduced with HDAC5WT or GFP adenoviruses (2.5×10^8 IU/ml), and 24 h after transduction were serum-starved for another 24 h in DMEM containing 1% FBS. Cell viability was assessed 48 h post-transfection or post-transduction by incubating cells with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, Haverill, United Kingdom) (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.

ELISA
RA FLS supernatants were analysed for CXCL-10 chemokine secretion by ELISA (R&D Systems) according to the manufacturer’s instructions. RA FLS nuclear extracts were used for IRF1 and IRF3 ELISA (Bio-Connect Diagnostics) according to the manufacturer’s instructions. DNA-binding activities of NF-kB components p65 and p50, and STAT1 in FLS nuclear fractions were determined using a TransAM transcription factor ELISA (Active Motif) according to the manufacturer’s instructions.

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

<table>
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<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>HDAC1</td>
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<td>TGGAGGCAAGAATTTAATG</td>
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<td>HDAC2</td>
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<td>HDAC3</td>
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<td>HDAC4</td>
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<td>GTGAGTCAGAGGGCTGTTT</td>
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