Regulation of inflammation by histone deacetylases in rheumatoid arthritis: beyond epigenetics

Grabiec, A.M.

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CHAPTER 7

TRANSCRIPTIONAL PROFILING OF RHEUMATOID ARTHRITIS FIBROBLAST-LIKE SYNOVIOCYTES IDENTIFIES A SUBSET OF GENES WITH AU-RICH 3'-UTRs WHICH ARE SUPPRESSED BY HDAC INHIBITORS VIA REDUCTION OF mRNA STABILITY

Aleksander M. Grabiec[1,2], A. Marcel Willemsen[3], Antoine H. van Kampen[3], Paul P. Tak[2,4], Kris A. Reedquist[1,2]

[1]Department of Experimental immunology,
[2]Division of Clinical Immunology and Rheumatology,
[3]Bioinformatics Laboratory, Department of Clinical Epidemiology, Biostatistics and Bioinformatics, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

[4]GlaxoSmithKline, Stevenage, U.K.

ABSTRACT

Background and objectives: The production of inflammatory cytokines is tightly regulated by epigenetic and non-epigenetic mechanisms, among which acetylation and deacetylation of histones and non-histone proteins plays a prominent role. HDAC inhibitors (HDACi) suppress cytokine production by rheumatoid arthritis (RA) patient immune and stromal cells and are potent therapeutics in animal arthritis models. However, the molecular mechanism(s) by which HDACi mediate their anti-inflammatory effects is largely unknown. In our previous work we identified acceleration of mRNA degradation as a mechanism contributing to HDACi suppression of IL-6 production in RA fibroblast-like synoviocytes (FLS). This study was undertaken to characterize the role of reduced mRNA stability in HDACi-mediated regulation of RA FLS inflammatory activation.

Methods: RA FLS were treated with IL-1β in the presence or absence of ITF2357 and total RNA was extracted. Expression and mRNA stability of IL-1β-responsive genes was analyzed using quantitative PCR arrays. The frequencies of AU-rich elements (AREs) in the 3'-UTRs of the analyzed genes were determined using the Bioconductor package Biostrings. Production of IL-8, MMP1, MMP3 and TIMP1 was measured by ELISA.

Results: RA FLS exposure to ITF2357 reduced the mRNA levels of 85% of genes induced by IL-1β, including cytokines (IL-6, TNFα, IL-1α, IL-1β), chemokines (CCL2, IL-8, CXCL2, CXCL3, CXCL6, CXCL9, CXCL10), matrix-degrading enzymes (MMP1, MMP3, MMP13), and intracellular molecules regulating cellular inflammatory responses (COX-2, IRAK2, NFKB1, IRF1). In contrast, ITF2357 failed to modulate expression of genes non-responsive to IL-1β. Consistent with mRNA expression data, ITF2357 dose-dependently suppressed protein secretion of IL-8, MMP-1 and MMP-3, without affecting TIMP-1 production. Analyses of mRNA stability confirmed accelerated decay of IL-6 mRNA after FLS treatment with HDACi, and identified a number of other transcripts, including IL-8, COX-2, CXCL2, Bcl-XL and ADAMTS1, which are regulated by HDACi in a similar fashion. 3' UTRs of these transcripts were characterized by significantly higher frequencies of AREs than other HDACi-sensitive genes in RA FLS.

Conclusions: We demonstrate that HDACi prevent induction of the majority of genes induced by inflammatory stimulation of RA FLS and identify modulation of mRNA stability as an important mechanism underlying the anti-inflammatory effects of HDACi. However, kinetics analysis of other HDACi-sensitive genes also suggests the presence of additional molecular mechanisms by which HDACi regulate gene expression in RA FLS.

INTRODUCTION

Inflammatory cytokines and chemokines are central regulators of the immune system, and temporal and balanced regulation of their expression is necessary for an effective but self-limiting immune response. Excessive production and accumulation of cytokines leads to chronic inflammation and tissue damage typically associated with immune-mediated inflammatory diseases (IMIDs), including rheumatoid arthritis (RA).[1,2] In RA, cytokines such as tumor necrosis factor α (TNFα), interleukin (IL)-1β and IL-6 produced by leukocytes infiltrating the synovial tissue activate stromal fibroblast-like synoviocytes (FLS). FLS in turn invade surrounding tissues and secrete matrix-degrading enzymes, thereby contributing to irreversible destruction of bone and cartilage.[3,4] The importance of cytokines in RA pathobiology has been confirmed by successful introduction of anti-cytokine therapies to the treatment of RA,[5] and initial clinical efficacy of compounds targeting signaling pathways directly involved in cytokine production has recently been demonstrated.[6]

Expression of inflammatory cytokines is tightly regulated at multiple levels, among which modulation of mRNA stability plays an important role.[7,8] Cytokine mRNAs belong to the shortest-lived mRNAs, and the relative instability of these transcripts depends on the presence of conserved AU-rich elements (AREs) in their 3' untrasnlated regions (UTRs).[9] The importance of ARE-dependent regulation in cytokine responses has been demonstrated in an animal study using mice overexpressing human TNFα in which the 3' UTR sequence was substituted with the β-globin 3' UTR lacking the TNFα ARE motif. In this model, animals spontaneously developed chronic inflammatory arthritis associated with aberrant TNFα expression patterns, and this phenotype could be prevented by treatment with anti-TNFα antibodies.[10] Similarly, inflammatory arthritis was observed in mice lacking the endogenous ARE sequence within the 3' UTR of the TNFα gene.[11] Regulation of mRNA decay is mediated by ARE-binding proteins (ARE-BPs), such as AU-rich binding factor-1 (AUF1), tristetraprolin (TTP), KH-type splicing regulatory protein (KSRP) and HuR, which recruit ARE-containing mRNAs to the exosome where they undergo deadenylation and eventual degradation.[12] TTP-deficient mice develop autoimmune syndromes and arthritis,[13] and macrophages from mice lacking TTP are characterized by increased production and mRNA stability of TNFα.[14,15] ARE-mediated mRNA instability is not restricted to TNFα as ARE-BPs have been shown to affect mRNA decay of several other inflammatory mediators, including IL-2, IL-6, interferon γ, and both CC and CXC chemokine ligands.[16] Finally, microRNAs (miRNAs) contribute to the complex regulation of cytokine mRNA turnover via both ARE-dependent and -independent mRNA deadenylation and degradation.[17,18]

Activity of histone deacetylases (HDACs) is typically associated with epigenetic regulation of gene expression through the modulation of histone acetylation status resulting in changes of chromatin structure,[19,20] but in recent years it has became apparent that HDACs also target some 1700 non-histone proteins.[21] Many of these proteins are directly or indirectly involved in controlling gene expression: reversible protein acetylation affects activation of intracellular signaling pathways, transcription factor activity and retention, mRNA stability, protein translation and secretion.[22,23] HDAC inhibitors (HDACi) can suppress inflammatory cytokine production by murine and human immune cells,[24-27] and display therapeutic efficacy in animal models of chronic and acute inflammatory disorders, including colitis, lupus, multiple sclerosis, graft-versus-host disease and arthritis.[28,29] Although regulation of the components of mitogen-activated protein kinase (MAPK), signal transducer and activator of transcription (STAT), and nuclear factor-κB (NF-κB) pathways have been proposed to mediate anti-inflammatory effects of HDACi,[30-32] broader

analyses of molecular events underlying HDACi suppression of inflammatory cytokines failed to find a unifying mechanism related to HDACi modulation of signal transduction machinery.[33,34]

We recently reported that HDACi prevent IL-6 production in RA FLS and macrophages by promoting acclerated degration of IL-6 mRNA,[33] identifying a novel mechanism by which HDACi can suppress induction of inflammatory mediators. Other studies in cancer biology have also suggested that HDACi can modulate gene expression through effects on mRNA stability.[35-38] However, it is unknown how generally HDACi regulate inflammatory gene expression via effects on mRNA stability. This study was undertaken to analyze global effects of HDACi on expression profiles of genes relevant to RA pathology in RA FLS in the absence and presence of inflammatory activation, and to determine if modulation of mRNA stability was the sole mechanism by which HDACi regulate gene expression in these cells.

MATERIALS AND METHODS

Cell culture. FLS were isolated from synovial tissue specimens of patients with RA fulfilling the American College of Rheumatology revised criteria for RA,[39] cultured as previously described,[40] and used for experiments after overnight culture in Dulbecco's modified Eagle's medium (Gibco-BRL, Paisley, Scotland, UK) containing 1% fetal bovine serum (Invitrogen, Breda, The Netherlands). All studies were performed with FLS between passages 4 and 9.

RNA extraction and gene expression profiling. FLS were either left unstimulated or were treated with 100 nM ITF2357 (Italfarmaco, Cinisello Balsamo, Italy), 1 ng/ml IL-1β (R&D Systems, Minneapolis, MN), or the combination of both for 4 hours. Alternatively, cells were left untreated or were stimulated with 1 ng/ml IL-1β in the presence or absence of 250 nM ITF2357 for 1-8 hours. After FLS stimulation, total RNA was extracted using an RNeasy mini kit (Qiagen, Venlo, The Netherlands), including a DNAse step to remove genomic DNA. The concentration and purity of the RNA was determined with a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). cDNA was then synthesized from 1 µg of RNA using an RT^2 First Strand Kit (SABiosciences, Frederick, MD) and the expression of genes involved in the regulation of extracellular matrix, adhesion, inflammation, angiogenesis and cell survival was analyzed using RT^2 Profiler™ PCR Array sets (PAHS-013, PAHS-052, PAHS-072, SABiosciences) as described previously.[41] Briefly, cDNA was diluted, added to RT^2 SybrGreen Rox qPCR Master Mix (SABiosciences), and after vigorous shaking 25 µl of the experimental cocktail was added to each well of the PCR array plate. When PCR amplification was completed, threshold values were manually equalized for all analyzed samples and the threshold cycle (Ct) determined for each gene. StepOne Software v2.1 (Applied Biosystems) and Microsoft Excel spreadsheet software (Microsoft, Redmond, WA) were used to calculate expression of each analyzed gene relative to the mean expression of five housekeeping genes (B2M, HPRT1, RPL13A, GAPDH and ACTB).

Analysis of mRNA stability. FLS were left unstimulated or were treated with 250 nM ITF2357 for 30 min prior to stimulation with 1 ng/ml IL-1β. After 2 h of stimulation culture medium was discarded, cells were washed and fresh medium containing 10 µg/ml actinomycin D (ActD) (Sigma-Aldrich, St Louis, MO) was added. Cells were then harvested at 0, 2 and 5 hours following the addition of ActD, RNA was isolated, and the rates of mRNA degradation in the presence or absence of ITF2357 quantified using a customized RT^2 Profiler™ PCR Array set (SABiosciences) as described above.

Measurement of IL-8, MMP-1, MMP-3 and TIMP-1 production. RA FLS were either left unstimulated or were treated with increasing concentrations of trichostatin A (TSA) (Sigma-Aldrich) for 30 min prior to stimulation with 10 ng/ml IL-1β, and after 24 hours cell-free supernatants were harvested. IL-8 production was measured then using PeliKine Compact ELISA kit (Sanquin Reagents, Amsterdam, The Netherlands), and MMP-1, MMP-3 and TIMP-1 production was measured using DuoSet ELISA kit (R&D Systems) as per the manufacturer's instructions.

Statistical analyses. Data are presented as mean +/- SEM unless otherwise indicated. For analyzing dose responses to HDACi treatment, sets of data were analyzed by an overall Friedman test followed by post hoc Dunns' multiple comparison test. Differences in ARE frequencies in 3' UTRs between groups of genes were determined using the Fisher's exact test. p values < 0.05 were considered significant.

RESULTS

ITF2357 prevents the induction of IL-1β-responsive genes in RA FLS without affecting genes non-responsive to IL-1β. To gain more insight into mechanisms by which HDACi modulate inflammatory gene expression, we first analyzed how inhibition of HDAC activity affected RA FLS mRNA levels of a broad spectrum of genes relevant to disease pathology in RA. Cells were treated for 4 h with ITF2357, IL-1β, or the combination of both, and relative expression of genes regulating inflammatory activation, adhesion, angiogenesis and extracellular matrix homeostasis was determined using low density qPCR arrays. Stimulation with IL-1β induced expression of 25% of the 236 analyzed genes by more than 2-fold, and treatment with ITF2357 reduced expression levels of 85% of the genes responsive to IL-1β stimulation in RA FLS (Table 1). Notably, the degree of HDACi-mediated suppression of mRNA accumulation differed between genes: while transcriptional induction of MMPs, CXCL9, CXCL11, IFNβ and TLR4 was almost completely blocked in the presence of ITF2357, expression of other genes, such as IL-6, CCL2, CXCL2, CXCL3 and NFKB1, was suppressed by 20-50% (Fig. 1). Notably, expression of genes non-responsive to IL-1β stimulation was largely unaffected by ITF2357, with the exception of CCL2, CXCL10, NFKB1, ITGA2, BMP2 and 6 other genes (Table 1).

Next, to test whether HDACi-mediated reduction of mRNA accumulation of IL-1β-stimulated genes corresponded to changes at the protein level, we stimulated RA FLS with increasing doses of TSA and ITF2357 in the presence of IL-1β for 24 h and measured production of IL-8, MMP-1, MMP-3 and tissue inhibitor of metalloproteinases-1 (TIMP-1). Both HDACi dose-dependently inhibited IL-1β-mediated IL-8 production, reaching maximum inhibition of approximately 75% at 1 μM (Fig. 2A). All tested doses of TSA efficiently prevented MMP-1 and MMP-3 production induced by IL-1β regardless of the tested dose (Fig. 2B *left and middle panel*), indicating that HDACi-responsive genes segregate depending on concentrations of HDACi required for suppression: while 250 nM TSA completely blocked MMP-1 and MMP-3 production, 1 μM TSA was needed to achieve 70% reduction of IL-8 secretion. Consistent with mRNA data, TSA failed to affect production of TIMP-1 (Fig. 2B *right panel*).

ITF2357 suppresses IL-1β-induced gene expression in RA FLS independently of the kinetics of mRNA induction. We next narrowed our analyses to a set of genes identified in the above experiments as regulated by IL-1β in RA FLS, and genes relevant to cellular

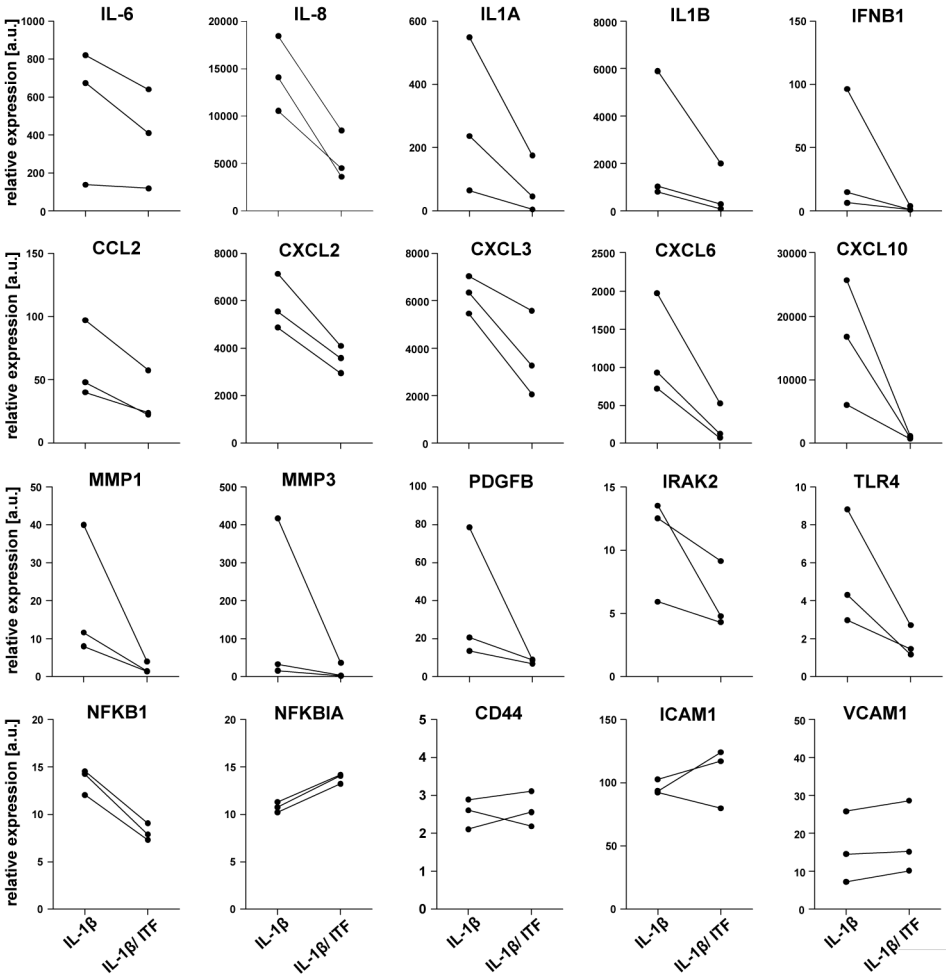

Figure 1. Selection of genes responsive and non-responsive to HDACi treatment in IL-1β-stimulated RA FLS. FLS (n = 3) were either left unstimulated or were stimulated with 1 ng/ml IL-1β with or without 100 nM ITF2357. Total RNA was then extracted, cDNA was synthesized and expression of 236 genes involved in adhesion, cellular inflammatory responses, angiogenesis and extracellular matrix regulation analyzed using qPCR arrays. Results are presented as relative mRNA expression in FLS treated with IL-1β alone and in the presence of ITF2357 and values for each analyzed cell line are depicted.

inflammatory activation and survival reported in other studies as responsive to HDACi treatment. To gain more insight into temporal changes in gene expression levels in the presence or absence of HDACi, we stimulated RA FLS with IL-1β for 1-8 hours with or without ITF2357 and analyzed kinetics of mRNA regulation of 89 selected genes using customized qPCR arrays (Table 2). We observed several different patterns of time-dependent gene induction by IL-1β: while expression of some genes was induced early after IL-1β treatment and gradually increased further over time (CCL2, VCAM1), expression of other genes

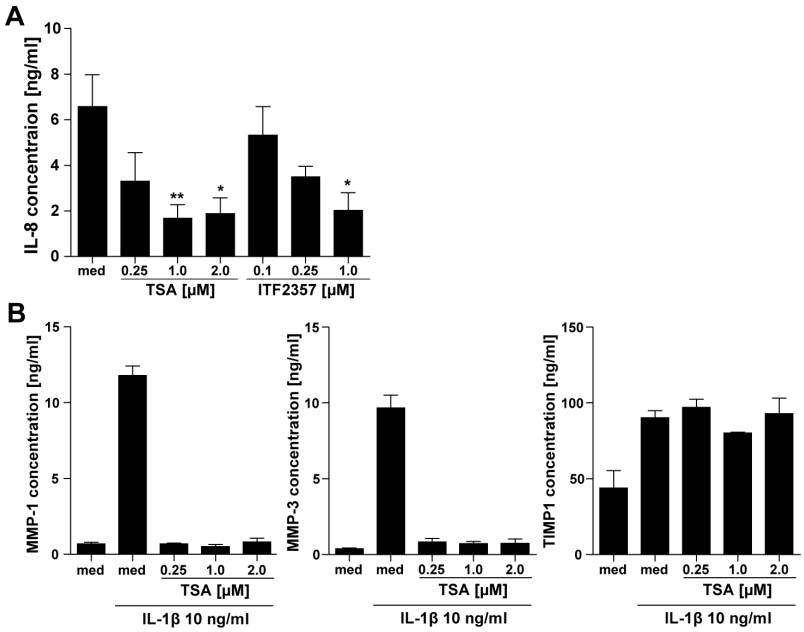

Figure 2. HDACi suppress IL-8, MMP-1 and MMP-3, but not TIMP-1 production by RA FLS. FLS were stimulated with 10 ng/ml IL-1β alone or in the presence of increasing concentrations of TSA or ITF2357. After 24 hours of incubation cell-free tissue culture supernatants were collected and levels of IL-8, MMP-1, MMP-3 and TIMP-1 were determined by ELISA. (*A*) Results, representing mean IL-8 concentration +/- SEM of 5 independent, were analyzed using the Friedman test followed by post hoc Dunns' multiple comparison test with cells not exposed to HDACi used as reference controls. $^{*}p < 0.05$; $^{**}p < 0.01$. (*B*) Data are presented as mean concentration of MMP-1 (*left panel*), MMP-3 (*middle panel*) and TIMP-1 (*right panel*) +/- SD and show a representative experiment.

was almost unaffected during the first 2 hours of stimulation and then rapidly increased 4 hours post-treatment (CXCL9, CXCL10, IL1B, PTGS2). We also identified a subset of genes characterized by a biphasic induction profile with apparent peaks at 1 and 4 hours after IL-1β stimulation (CXCL2, CXCL3, SELE). Finally, a class of genes was induced by IL-1β in a rapid but transient manner: their expression peaked 1 hour after treatment and then gradually decreased to basal levels 8 hours after exposure to IL-1β (TNF, NFKBIA, SOCS3) (Fig. 3 and data not shown). However, we failed to find any straightforward relationships between temporal changes in mRNA levels in response to IL-1β stimulation and the susceptibility of specific genes to regulation by HDACi. ITF2357 uniformly reduced expression of the vast majority of the analyzed IL-1β-inducible transcripts regardless of the kinetics of gene induction. Amongst the genes regulated by HDACi, suppression of mRNA accumulation was apparent throughout the time of gene induction, but different degrees of downregulation were observed (Fig. 3). Strikingly, out of 89 analyzed genes we identified only one gene upregulated by HDACi. While IL-1β had no effect on mRNA levels of the pro-apoptotic Bcl-2 family member BCL2L11 (also known as BIM), treatment with ITF2357 caused a rapid but transient induction of BCL2L11 expression.

Table 1. Regulation of gene expression by HDACi in RA FLS.

	ITF 100 nM		IL-1β 1 ng/ml		IL-1β 1ng/ml + ITF 100 nM			ITF 100 nM		IL-1β 1 ng/ml		IL-1β 1ng/ml + ITF 100 nM	
Gene symbol	**Fold induction**	**SD**	**Fold induction**	**SD**	**Fold induction**	**SD**	**Gene symbol**	**Fold induction**	**SD**	**Fold induction**	**SD**	**Fold induction**	**SD**
ADAMTS1	0.57	0.06	0.75	0.27	0.30	0.08	CXCL6	1.03	0.48	1209.56	673.10	242.75	249.40
ADAMTS13	0.82	0.12	0.75	0.13	0.47	0.15	CXCL9	7.36	5.97	1035.83	1412.89	4.38	3.60
ADAMTS8	1.39	0.25	1.74	0.33	1.74	0.53	CXCR4	1.78	0.80	17.74	24.09	9.18	7.45
ADORA2A	1.08	0.25	257.46	57.23	64.86	23.97	CYBB	0.84	0.23	0.39	0.16	0.88	0.25
AGGF1	0.73	0.18	0.78	0.05	0.70	0.07	DEFB4	1.05	0.08	135.23	191.47	37.50	43.69
AMOT	2.40	1.34	0.80	0.42	1.77	1.49	DMBT1	0.74	0.11	0.54	0.34	1.08	0.18
ANG	0.89	0.02	0.83	0.04	0.96	0.04	ECM1	0.86	0.02	0.88	0.07	0.95	0.19
ANGPT1	0.94	0.15	0.78	0.11	0.83	0.04	EDIL3	0.90	0.04	0.99	0.07	0.86	0.17
ANGPT2	0.32	0.14	0.68	0.19	0.46	0.12	EREG	0.86	0.12	164.28	62.97	50.43	23.31
ANGPTL1	0.71	0.09	2.34	0.61	0.79	0.05	FGF1	0.77	0.03	0.72	0.12	0.66	0.02
BAI1	1.67	0.19	1.09	0.44	1.24	0.76	FGF13	0.90	0.07	1.64	0.44	1.08	0.06
BMP2	2.68	0.64	26.14	14.80	6.24	4.44	FGF2	0.64	0.11	3.32	1.17	2.18	1.45
BTG1	1.26	0.08	4.41	0.40	2.43	0.41	FGFBP1	0.92	0.32	0.95	0.12	1.10	0.76
C5	0.90	0.07	0.51	0.14	0.60	0.15	FIGF	0.58	0.10	0.60	0.26	0.49	0.29
C8A	0.75	0.34	0.53	0.18	1.02	0.18	FN1	0.96	0.02	0.98	0.10	1.08	0.07
CAMP	0.66	0.35	0.72	0.44	1.15	0.26	FOXO4	0.86	0.28	0.53	0.12	0.55	0.07
CASP1	0.75	0.07	1.61	0.04	1.12	0.12	FST	0.29	0.06	2.03	0.56	0.44	0.11
CASP4	0.75	0.10	2.31	0.31	1.08	0.06	GRN	0.88	0.02	0.89	0.07	0.88	0.16
CCL15	0.96	0.19	0.99	0.11	1.03	0.04	GRP	0.89	0.31	0.95	0.18	0.88	0.30
CCL2	0.43	0.01	59.62	28.64	37.05	19.36	HAS1	1.40	0.44	1.18	0.20	1.74	1.29
CCR3	0.57	0.22	0.70	0.25	0.83	0.07	HGF	1.00	0.04	2.36	1.11	1.11	0.23
CD14	0.98	0.11	0.78	0.10	1.02	0.03	HMOX1	1.10	0.34	1.02	0.25	0.95	0.50
CD1D	1.05	0.08	1.01	0.08	1.00	0.07	ICAM1	0.65	0.05	96.17	5.67	107.05	23.72
CD44	1.10	0.08	2.54	0.40	2.62	0.46	IFNA1	1.22	0.26	1.99	0.53	1.61	0.26
CD55	0.86	0.06	0.93	0.10	0.96	0.11	IFNB1	0.81	0.33	39.34	49.64	2.09	1.63
CD59	1.03	0.04	0.97	0.08	1.02	0.18	IFNG	1.01	0.46	0.31	0.09	0.61	0.45
CDH1	1.41	1.59	1.24	0.94	0.83	0.94	IFNGR1	1.39	0.08	2.76	0.29	2.38	0.51
CHGA	0.76	0.32	0.78	0.06	0.92	0.76	IFNGR2	0.96	0.05	5.31	0.93	2.09	0.56
CHUK	0.98	0.10	1.19	0.03	1.14	0.10	IKBKB	0.85	0.04	1.63	0.10	1.02	0.09
CLEC3B	0.83	0.07	0.79	0.10	0.71	0.17	IL10	1.04	0.35	8.09	10.28	1.18	0.25
CNTN1	1.20	0.38	1.29	0.59	1.38	0.52	IL12A	0.80	0.12	1.04	0.22	2.17	0.46

Table 1. Regulation of gene expression by HDACi in RA FLS. (Continued)

Gene symbol	ITF 100 nM		IL-1β 1 ng/ml		IL-1β 1ng/ml + ITF 100 nM		Gene symbol	ITF 100 nM		IL-1β 1 ng/ml		IL-1β 1ng/ml + ITF 100 nM	
	Fold induction	SD	Fold induction	SD	Fold induction	SD		Fold induction	SD	Fold induction	SD	Fold induction	SD
COL11A1	1.15	0.21	0.61	0.06	0.90	0.20	IL12B	0.69	0.12	1.11	0.33	0.91	0.97
COL12A1	0.83	0.05	0.93	0.12	0.94	0.21	IL12RB2	2.11	0.79	1.06	0.06	1.99	0.74
COL14A1	0.88	0.08	0.74	0.08	0.74	0.23	IL17F	0.89	0.24	0.96	0.02	0.91	0.47
COL15A1	0.91	0.09	0.92	0.11	0.91	0.16	IL1A	0.54	0.02	283.80	245.82	75.63	89.03
COL16A1	0.82	0.11	1.34	0.27	1.10	0.21	IL1B	1.09	0.22	2583.76	2870.73	793.38	1051.93
COL18A1	0.93	0.02	0.91	0.16	0.88	0.14	IL1F10	0.88	0.32	1.85	1.44	1.14	0.31
COL1A1	1.00	0.07	0.76	0.20	0.86	0.25	IL1F5	0.80	0.31	16.28	18.16	1.55	0.08
COL4A2	1.07	0.08	0.98	0.10	1.19	0.22	IL1F6	0.61	0.12	2.65	3.81	0.96	0.36
COL4A3	0.76	0.18	0.69	0.26	0.76	0.33	IL1F7	0.63	0.30	1.26	0.98	0.92	0.13
COL5A1	0.95	0.02	1.01	0.02	1.09	0.14	IL1F8	0.98	0.03	1.71	0.17	0.71	0.23
COL6A1	0.97	0.08	0.94	0.06	1.04	0.13	IL1F9	0.77	0.27	115.55	135.45	5.39	4.16
COL6A2	0.89	0.14	0.81	0.20	0.74	0.32	IL1R1	1.26	0.22	0.94	0.07	1.32	0.18
COL7A1	1.25	0.20	2.32	0.32	2.00	0.75	IL1R2	1.70	0.58	0.69	0.02	1.27	0.62
COL8A1	1.19	0.27	1.03	0.30	0.93	0.41	IL1RAP	1.86	0.14	1.01	0.11	1.68	0.28
COLEC12	1.21	0.19	0.79	0.06	1.05	0.14	IL1RAPL2	0.98	0.21	1.25	0.62	0.89	0.21
CRP	0.98	0.05	0.92	0.04	0.90	0.04	IL1RL2	1.16	0.28	1.13	0.59	1.33	0.15
CSF3	0.89	0.11	6328.79	6806.96	4295.00	4336.03	IL1RN	1.30	0.96	221.00	308.87	10.22	10.85
CTGF	1.31	0.20	0.11	0.04	0.36	0.29	IL6	0.63	0.18	545.04	359.46	390.73	261.19
CTNNA1	0.84	0.10	0.92	0.04	0.92	0.00	IL8	0.90	0.37	14362.94	3950.44	5543.41	2587.95
CTNNB1	1.02	0.04	1.13	0.05	1.15	0.17	IRAK1	0.90	0.18	0.91	0.23	0.85	0.24
CTNND1	1.04	0.01	1.02	0.07	1.07	0.23	IRAK2	0.94	0.28	10.66	4.13	6.09	2.68
CTNND2	1.00	0.18	0.96	0.12	0.99	0.13	IRF1	1.24	0.19	12.07	1.19	16.24	4.70
CXCL10	0.45	0.18	16228.56	9874.66	929.50	231.90	ITGA1	1.05	0.14	1.30	0.51	1.28	0.48
CXCL11	1.18	0.92	1428.09	1634.30	38.30	28.75	ITGA2	2.44	0.88	0.76	0.11	1.91	1.45
CXCL12	0.94	0.07	1.04	0.11	0.90	0.12	ITGA3	1.06	0.02	0.78	0.10	0.87	0.26
CXCL13	0.87	0.29	2.12	2.39	0.63	0.34	ITGA4	0.95	0.13	0.79	0.08	0.87	0.23
CXCL14	1.01	0.08	1.04	0.10	1.11	0.07	ITGA5	1.20	0.04	0.95	0.09	1.41	0.44
CXCL2	1.09	0.31	5856.84	1164.99	3549.35	572.17	ITGA6	0.94	0.12	0.74	0.07	0.91	0.26
CXCL3	1.28	0.27	6283.79	788.24	3641.56	1785.63	ITGA7	1.37	0.34	0.92	0.21	0.98	0.37
CXCL5	1.45	1.08	605.44	300.05	282.10	232.17	ITGA8	1.04	0.07	1.33	0.41	1.21	0.22
ITGAL	1.06	0.41	1.41	1.00	1.30	0.90	PGLYRP3	0.92	0.40	0.71	0.19	1.21	0.06

Table 1. Regulation of gene expression by HDACi in RA FLS. (Continued)

	ITF 100 nM		IL-1β 1 ng/ml		IL-1β 1ng/ml + ITF 100 nM			ITF 100 nM		IL-1β 1 ng/ml		IL-1β 1ng/ml + ITF 100 nM	
Gene symbol	Fold induction	SD	Fold induction	SD	Fold induction	SD	Gene symbol	Fold induction	SD	Fold induction	SD	Fold induction	SD
ITGAM	0.67	0.46	1.26	0.72	0.74	0.19	PLG	0.96	0.19	0.99	0.11	1.03	0.04
ITGAV	0.91	0.03	0.92	0.07	1.03	0.19	PPBP	1.04	0.13	1.16	0.41	1.07	0.24
ITGB1	0.91	0.01	0.92	0.06	0.97	0.18	PRL	0.69	0.15	0.84	0.33	1.10	0.70
ITGB2	0.81	0.08	0.89	0.04	0.89	0.13	PROC	3.28	1.48	0.66	0.41	1.55	0.45
ITGB3	1.12	0.33	1.39	0.72	1.29	0.67	PROK1	0.99	0.51	1.11	0.24	1.29	1.15
ITGB4	1.24	0.30	1.29	0.35	1.10	0.14	PTAFR	0.64	0.34	0.79	0.56	1.20	0.26
ITGB5	0.87	0.04	0.79	0.03	0.83	0.11	PTN	0.94	0.16	0.95	0.02	1.05	0.12
KAL1	1.11	0.07	0.76	0.04	0.95	0.35	RHOB	1.87	0.36	0.22	0.05	0.65	0.24
KITLG	1.27	0.19	1.88	0.06	1.60	0.21	RNH1	0.85	0.02	0.84	0.06	0.89	0.06
KLK3	1.59	0.83	1.67	1.00	3.68	4.94	RUNX1	1.29	0.07	1.22	0.14	1.72	0.67
LALBA	0.72	0.43	0.58	0.09	0.94	0.76	S100A12	1.05	0.08	0.99	0.07	0.96	0.11
LAMA1	0.84	0.04	0.66	0.08	0.78	0.18	SELE	1.45	0.89	2672.32	2110.70	390.69	419.33
LAMA2	0.81	0.05	0.81	0.02	0.83	0.11	SELL	1.04	0.39	0.95	0.14	0.94	0.09
LAMA3	0.93	0.14	0.88	0.15	0.79	0.09	SELP	1.96	0.29	2.10	0.99	2.15	0.98
LAMB1	0.91	0.06	0.77	0.09	0.86	0.25	SERPINA1	0.90	0.20	11.74	11.09	1.65	0.85
LAMB3	1.26	0.28	12.19	5.34	2.48	1.06	SERPINC1	0.94	0.18	0.61	0.14	0.96	0.21
LAMC1	0.79	0.09	0.86	0.03	0.85	0.07	SERPINE1	1.52	0.27	1.00	0.21	1.64	0.45
LBP	0.97	0.22	1.29	0.27	1.31	0.25	SERPINF1	1.07	0.11	1.09	0.11	1.36	0.22
LEP	0.58	0.18	1.02	0.38	0.56	0.20	SFTPD	0.87	0.28	0.65	0.16	0.95	0.20
LTF	0.74	0.28	0.66	0.25	0.73	0.18	SGCE	0.95	0.03	0.94	0.05	1.04	0.06
LY96	0.92	0.04	0.88	0.09	0.87	0.13	SPARC	0.87	0.02	0.83	0.04	0.85	0.17
LYZ	1.51	0.88	1.32	1.23	0.73	0.33	SPG7	1.00	0.16	0.81	0.19	0.70	0.19
MAPK14	1.07	0.09	0.76	0.17	1.16	0.06	SPINK5	0.76	0.37	0.53	0.48	0.25	0.14
MAPK8	1.14	0.14	1.16	0.12	1.60	0.05	SPP1	0.99	0.11	0.87	0.13	0.99	0.15
MDK	1.02	0.03	0.99	0.01	1.01	0.10	STAB1	0.96	0.27	1.21	0.62	1.21	1.20
MIF	0.96	0.05	1.03	0.05	0.88	0.13	TGFA	1.45	0.50	8.24	5.23	4.97	4.37
MMP1	0.94	0.04	19.90	17.53	2.30	1.50	TGFB1	1.36	0.15	0.97	0.12	1.25	0.26
MMP10	1.02	0.18	9.06	11.69	1.81	1.27	TGFBI	0.89	0.04	0.93	0.02	1.00	0.15
MMP11	0.98	0.17	0.94	0.09	1.02	0.20	THBS1	0.84	0.13	0.74	0.15	0.94	0.31
MMP12	0.79	0.23	98.11	73.70	7.08	3.44	THBS2	0.84	0.05	0.74	0.02	0.95	0.14
MMP13	0.79	0.24	52.89	40.32	3.66	1.73	THBS3	0.86	0.14	0.75	0.24	0.69	0.28

Table 1. Regulation of gene expression by HDACi in RA FLS. (Continued)

Gene symbol	ITF 100 nM		IL-1β 1 ng/ml		IL-1β 1ng/ml + ITF 100 nM		Gene symbol	ITF 100 nM		IL-1β 1 ng/ml		IL-1β 1ng/ml + ITF 100 nM	
	Fold induction	SD	Fold induction	SD	Fold induction	SD		Fold induction	SD	Fold induction	SD	Fold induction	SD
MMP14	0.89	0.04	0.95	0.19	0.79	0.26	TIE1	1.21	0.36	2.12	0.38	1.44	0.42
MMP15	1.88	0.75	1.47	0.89	1.45	0.67	TIMP1	1.00	0.03	1.27	0.04	1.53	0.11
MMP16	1.62	0.12	0.97	0.42	1.16	0.30	TIMP2	0.94	0.05	0.84	0.04	0.78	0.13
MMP2	0.88	0.08	0.87	0.07	0.79	0.20	TIMP3	1.94	0.77	0.43	0.19	1.14	0.32
MMP3	0.91	0.02	155.37	226.85	14.30	19.60	TLR1	0.46	0.09	1.69	0.38	0.79	0.19
MMP7	0.95	0.35	2.04	0.39	1.20	0.11	TLR10	0.80	0.20	0.75	0.16	0.85	0.18
MMP8	1.38	0.67	17.05	17.05	1.84	1.54	TLR2	0.86	0.16	11.93	4.43	9.83	3.54
MMP9	1.06	0.16	1.47	0.19	1.02	0.32	TLR3	0.89	0.12	1.43	0.08	1.18	0.20
MYD88	0.86	0.11	1.70	0.44	1.07	0.24	TLR4	0.69	0.09	5.37	3.06	1.79	0.81
NCAM1	1.89	0.62	0.64	0.37	2.41	1.47	TLR6	0.86	0.10	0.87	0.21	0.79	0.28
NCF4	0.76	0.38	1.02	0.23	1.34	0.36	TLR8	2.23	2.13	0.77	0.57	1.56	1.59
NFKB1	0.48	0.04	13.62	1.37	8.09	0.90	TLR9	1.54	0.86	1.30	1.41	1.31	1.26
NFKB2	0.80	0.14	1.04	0.03	1.23	0.11	TNC	1.06	0.03	4.00	1.04	4.67	1.37
NFKBIA	0.72	0.05	10.76	0.55	13.82	0.53	TNF	1.21	0.61	291.46	256.72	134.03	26.58
NLRC4	0.76	0.03	0.79	0.20	0.79	0.05	TNFRSF1A	0.96	0.09	1.03	0.14	1.06	0.09
NOS2	0.93	0.26	4.96	6.18	0.93	0.20	TNNI2	0.73	0.42	1.02	0.04	1.12	0.63
NPPB	0.88	0.27	1.06	0.07	1.69	1.70	TNNI3	1.21	0.08	1.15	0.19	1.83	1.86
NPR1	0.88	0.28	0.92	0.07	1.22	1.01	TOLLIP	1.07	0.06	0.98	0.07	1.09	0.18
PDGFB	2.40	1.95	37.66	35.70	8.23	1.15	TRAF6	0.98	0.04	1.27	0.06	1.04	0.08
PDGFD	0.90	0.02	0.76	0.05	0.78	0.02	TREM1	0.66	0.26	0.68	0.08	0.90	0.26
PECAM1	1.17	0.13	1.17	0.13	1.07	0.05	TYMP	0.92	0.07	2.26	0.63	0.94	0.56
PF4	0.98	0.42	2.96	1.34	1.96	0.97	VCAM1	0.73	0.04	15.88	9.39	17.98	9.54
PGF	1.51	0.48	0.79	0.44	1.33	0.51	VCAN	0.89	0.08	0.81	0.15	0.95	0.24
PGLYRP1	0.98	0.58	0.77	0.17	1.40	0.59	VEGFA	0.97	0.17	1.60	0.50	1.31	0.67
PGLYRP2	1.01	0.28	1.07	0.45	1.32	0.18	VTN	0.78	0.22	0.85	0.09	0.92	0.15

FLS were left unstimulated. treated for 4 h with 100 nM ITF2357. 1 ng/ml IL-1β. or were pretreated with 100 nM ITF2357 for 30 min prior to stimulation with IL-1β. After 4 h of stimulation total RNA was extracted. reverse transcribed and changes in gene expression analyzed by low density qPCR arrays. Data were normalized to the expression of 5 housekeeping genes (B2M. HPRT1. RPL13A. GAPDH and ACTB) and are presented as mean fold induction compared to unstimulated cells +/- standard deviation (SD) of 3 independent experiments.

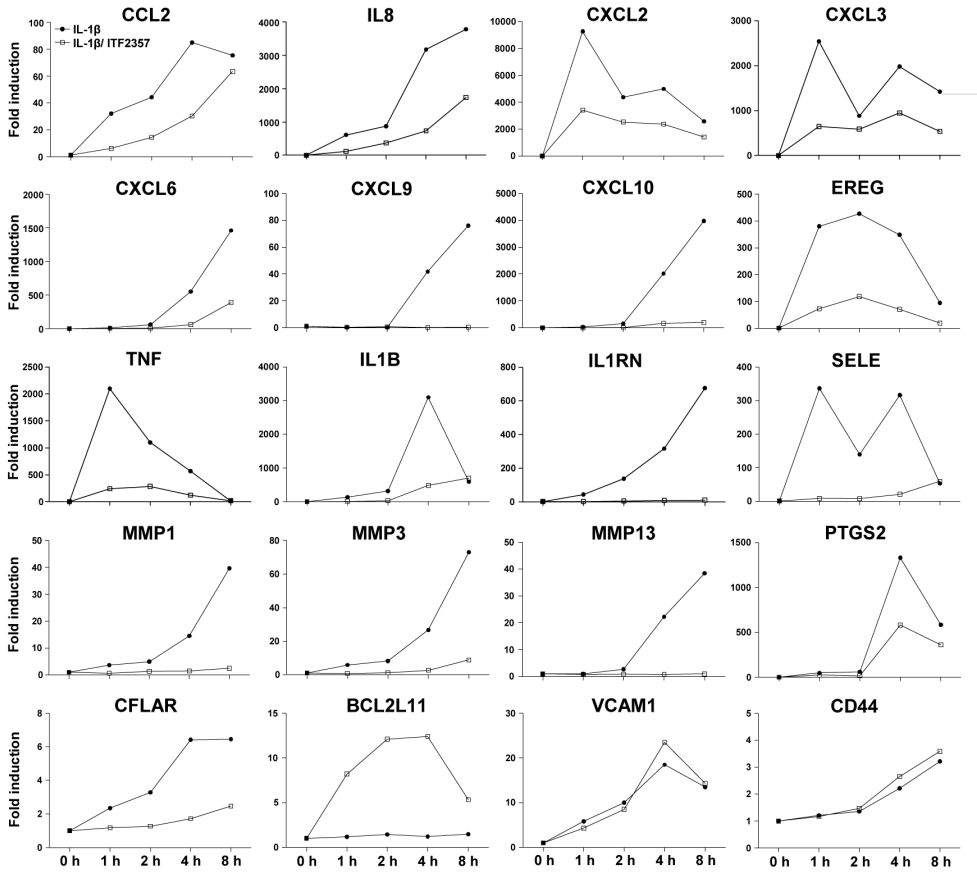

Figure 3. HDACi suppress expression of IL-1β-responsive genes regardless of kinetics of gene induction. RA FLS were left untreated (med) or were treated with IL-1β (1 ng/ml) with or without 250 nM ITF2357 for the indicated time (in hours). Total RNA was extracted, reverse transcribed, and temporal changes in mRNA accumulation of IL-1β-inducible genes were monitored by a customized low density qPCR array system. Data are presented as fold induction of mRNA levels compared to unstimulated cells in the presence or absence of ITF2357. Results of one experiment representative of two independent experiments are shown.

HDACi suppression of a subset of IL-1β-inducible genes is associated with reduced mRNA stability. In our previous study we demonstrated that HDACi accelerate mRNA decay of IL-6 in RA FLS and healthy donor macrophages.[33] To test whether this observation could be extended to other inflammatory mediators suppressed by HDACi in FLS, we analyzed effects of ITF2357 on the stability of transcripts previously selected for analyses of expression profiles by customized qPCR arrays (Table 2). We confirmed our previous observation that HDACi reduce IL-6 mRNA stability and identified 5 other transcripts, stability of which was substantially reduced in the presence of 250 nM ITF2357: IL-8, CXCL2, PTGS2, BCL2L1 and ADAMTS1 (Fig. 4A). The stability of other mRNAs remained unaffected by ITF2357, even though their expression was reproducibly suppressed by HDACi (Fig. 4B and data not shown).

Table 2. Genes selected for quantitative analysis of HDACi effects on RA FLS gene expression.

Gene Symbol	Refseq #	Gene Symbol	Refseq #	Gene Symbol	Refseq #
ADAMTS1	NM_006988	FOXO4	NM_005938	MMP12	NM_002426
ADAMTS13	NM_139025	GADD45A	NM_001924	MMP13	NM_002427
ADORA2A	NM_000675	HDAC1	NM_004964	MMP3	NM_002422
ANGPT2	NM_001147	HDAC10	NM_032019	MMP7	NM_002423
BCL2A1	NM_004049	HDAC11	NM_024827	MMP8	NM_002424
BCL2L1	NM_138578	HDAC2	NM_001527	PTGES	NM_004878
BCL2L11	NM_006538	HDAC3	NM_003883	MYD88	NM_002468
BIRC2	NM_001166	HDAC4	NM_006037	NCAM1	NM_000615
BIRC5	NM_001168	HDAC5	NM_005474	NFKB1	NM_003998
BMP2	NM_001200	HDAC6	NM_006044	NFKBIA	NM_020529
CCL2	NM_002982	HDAC7	NM_016596	NOS2	NM_000625
CD44	NM_000610	HDAC8	NM_018486	PDGFB	NM_002608
CDKN1A	NM_000389	HDAC9	NM_178425	PTGS2	NM_000963
CDKN1B	NM_004064	ICAM1	NM_000201	RHOB	NM_004040
CFLAR	NM_003879	IFNB1	NM_002176	SELE	NM_000450
CSF3	NM_000759	IFNGR1	NM_000416	SERPINA1	NM_000295
CXCL10	NM_001565	IFNGR2	NM_005534	SOCS3	NM_003955
CXCL11	NM_005409	IL1A	NM_000575	SOD2	NM_000636
CXCL2	NM_002089	IL1B	NM_000576	TIMP3	NM_000362
CXCL3	NM_002090	IL1F5	NM_012275	TLR1	NM_003263
CXCL5	NM_002994	IL1F9	NM_019618	TLR2	NM_003264
CXCL6	NM_002993	IL1RN	NM_000577	TLR4	NM_138554
CXCL9	NM_002416	IL6	NM_000600	TNF	NM_000594
CXCR4	NM_003467	IL8	NM_000584	VCAM1	NM_001078
DNMT3B	NM_006892	IRAK2	NM_001570	B2M	NM_004048
EREG	NM_001432	IRF1	NM_002198	HPRT1	NM_000194
FASLG	NM_000639	ITGA2	NM_002203	RPL13A	NM_012423
FGF2	NM_002006	LAMB3	NM_000228	GAPDH	NM_002046
FOXO1	NM_002015	MMP1	NM_002421	ACTB	NM_001101
FOXO3	NM_001455	MMP10	NM_002425		

HDACi modulate stability of transcripts characterized by increased frequencies of ARE motifs in 3' UTRs. mRNA stability is tightly regulated by interactions between ARE sequences located within the 3' UTRs of the transcript and ARE-BPs,[9] and early studies in cancer cells have indicated that HDACi modulate activity of some ARE-BPs resulting in accelerated mRNA degradation.[35,36] Therefore, we analyzed potential differences in the frequencies of ARE sequences in the 3' UTR regions of transcripts for which mRNA stability was or was not responsive to HDACi.

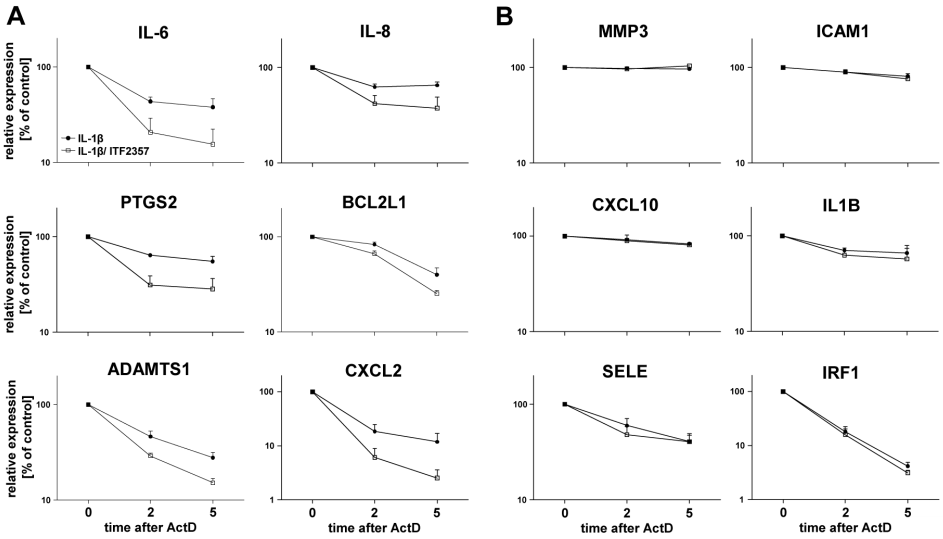

Figure 4. HDACi accelerate mRNA decay of a subset of IL-1β-responsive genes in RA FLS. FLS (n = 3) were stimulated with IL-1β (1 ng/ml) in the presence or absence of 250 nM ITF2357. After 2 h of stimulation transcription was blocked with 10 μg/ml of actinomycin D (ActD) and RNA was extracted at the indicated time points (h) from the start of ActD treatment. cDNA was then synthesized and the rates of mRNA degradation in the presence or absence of ITF2357 were determined using customized qPCR arrays. mRNA expression values for 0 h time point were normalized to 100%, and all remaining values were calculated as the mean percentage ± SEM of mRNA levels compared with controls. Graphs show (*A*) mRNAs degradation of which was accelerated in the presence of ITF2357, and (*B*) examples of genes which were not regulated by ITF2357 at the level of transcript stability.

We used the BiomaRt online interface to retrieve 3' UTRs for all known transcripts of the 89 analyzed genes from the UCSC database, and analyzed them for the presence of the following AREs: AUUUA pentamers, UUAUUUAUU and UUAUUUA(A/U)(A/U) nonamers, UAUUUAU heptamers, and UA[U]2-5AU variations. These motives were then counted based on exact pattern matching of IUPAC symbols using the Bioconductor package Biostrings (http://www.bioconductor.org/packages/2.2/bioc/html/Biostrings.html).[42] Subsequently we compared frequencies of the analyzed ARE sequences in the group of 6 mRNAs for which we observed reduced stability in the presence of ITF2357 to ARE frequencies in the remaining set of 83 transcripts not regulated by HDACi at the level of transcript stability.[43] We found an enrichment of most of the analyzed ARE motifs in the set of transcripts characterized by accelerated decay in the presence of ITF2357 compared to the other gene transcripts (Table 3). At least one AUUUA pentamer was present in 87.5% of the transcripts for which HDACi increased mRNA decay, compared to 27.3% of the transcripts of the other gene set ($p = 1.3 \times 10^{-6}$). Significant differences were also noted in the frequencies of UUAUUUAUU and UA[U]2-5AU variations ($p = 0.002$ and $p = 1.2 \times 10^{-6}$, respectively). The UUAUUUA(A/U)(A/U) nonamer was enriched in the regulated transcripts (18.8% compared to 6.5%) but this trend failed to reach statistical significance (Table 3). These data demonstrate that HDACi-induced acceleration of mRNA decay is selectively observed in gene transcripts enrichmed with ARE sequences in their 3' UTRs, and may suggest that HDACi mediate their effects on RA FLS mRNA stability at least in part through modulation of activity of ARE-BPs.

Table 3. Statistical analysis ARE sequence frequencies in 3' UTR regions of the analyzed transcripts. Genes were divided into two groups and the percentage (%) of transcripts containing at least one of the analyzed ARE motifs was calculated for mRNAs stability of which was regulated (6 genes) and not regulated (83 genes) by HDACi. The differences in ARE sequence frequencies between the analyzed sets of genes were determined using the Fisher's exact test.

	AUUUA		UUAUUUAUU		UUAUUUA (A/U)(A/U)		UAUUUAU		UA[U]2-5AU	
Gene set	% of mRNAs	*p* value	% of mRNAs	*p* value	% of mRNAs	*p* value	% of mRNAs	*p* value	% of mRNAs	*p* value
Regulated	87.5	1.3×10^{-6}	18.8	0.002	18.8	0.086	75	1.5×10^{-8}	75	1.2×10^{-6}
Not regulated	27.3		1.3		6.4		11.6		17.5	

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DISCUSSION

Stromal FLS are potent effector cells in RA, which are characterized by a semi-transformed and invasive phenotype similar to tumor cells, and contribute to joint destruction by secreting enzymes that degrade bone and cartilage. HDACi suppress RA FLS proliferation, induce cell cycle arrest and sensitize them to certain pro-apoptotic stimuli.[44] Little is known, however, about HDACi effects on FLS inflammatory activation. In our previous study we demonstrated that HDACi block the production of IL-6, a key inflammatory cytokine contributing to the pathobiology of RA, and showed that accelerated IL-6 mRNA degradation is at least partly responsible for this effect.[33] Here, we report that HDACi prevent the induction of the majority of IL-1β-regulated cytokines, chemokines, matrix-degrading enzymes and other mediators of inflammation, and identify a subset of genes regulated by HDACi through modulation of mRNA stability.

HDACi suppress production of a broad range of inflammatory mediators by monocytes, macrophages, dendritic cells and other immune cells.[29,45] However, studies in cancer cells have demonstrated that HDACi modulate expression only of a small proportion of basally transcribed genes.[46,47] Also, TSA treatment affects the mRNA levels of only 4% of the genes expressed in cord tissues of experimental autoimmune encephalomyelitis mice.[48] Our findings confirm these observations: RA FLS 4 h exposure to ITF2357 in the absence of IL-1β stimulation resulted in more than 2-fold changes in the expression of 4.7% of the 236 analyzed genes, only 55% of which were upregulated (Table 1). Since we have previously demonstrated that FLS treatment with HDACi causes rapid increases in the histone acetylation status,[33] this observation indicates that global histone hyperacetylation is not sufficient to induce gene transcription. In line with this interpretation, even though treatment of murine macrophages with TSA causes rapid hyperacetylation of histone 4 at the *IL-6* promoter, IL-6 mRNA expression is suppressed,[34] suggesting complex relationships between histone modifications and mRNA induction. However, since local alterations in histone acetylation status in gene promoter regions are associated with the rates of transcription,[49,50] it remains to be determined if specific patterns of changes in histone acetylation can be detected locally at the promoters of the genes induced by ITF2357 in FLS.

Our analysis of 236 genes involved in several aspects of RA pathology demonstrates that ITF2357 potently blocks mRNA induction of many IL-1β-responsive genes encoding secreted products, including cytokines (TNFα, IL-1β, IL-6), chemokines (IL-8, CCL2, CXCL2-3, CXCL6, CXCL9-11), matrix-degrading enzymes (MMP-1, MMP-3) and growth factors (FGF2, PDGFB). We

also confirmed that the observed suppression of IL-8, MMP-1 and MMP-3 mRNA expression by HDACi directly corresponds to changes in protein production. These findings are relevant in the context of potential clinical application of HDACi in the treatment of IMIDs, as ITF2357 reduced expression of inflammatory genes in RA FLS at concentrations achievable in humans.[51,52] Curiously, ITF2357 suppressed IL-1β-mediated mRNA induction of IL-8 and IL-1β in FLS, both of which have previously been reported to be unaffected by HDACi at the gene expression level in monocytes and macrophages stimulated with LPS or TNFα.[25-27,53] These observations suggest that HDACi effects on IL-8 and IL-1β mRNA induction have a cell type- and/or stimulus-specific character, which may be attributable to differences in the expression of signaling or regulatory molecules utilized by myeloid and stromal cells upon inflammatory activation by different stimuli. ITF2357 also reduced the mRNA expression of receptors and components of intracellular signaling pathways responsible for cellular inflammatory activation, including TLR4, IRAK2, MYD88, and NFKB1. Although this effect cannot explain immediate suppression of mRNA induction of most analyzed genes, it suggests that anti-inflammatory effects of HDACi might also occur at later time points due to altered expression of transcription factors and signaling molecules. In line with this possibility, we and others have previously reported suppressed nuclear retention of NF-κB p50 and p65 subunits 24 h after exposure of RA FLS and FLS-like cell lines to HDACi.[33,54] Strikingly, HDACi completely blocked transcriptional induction of essentially all MMP genes responsive to IL-1β. HDACi regulation of MMPs has a cell type-independent character as inhibition of HDAC activity also suppresses MMP production by chondrocytes and prevents cytokine-induced cartilage destruction.[55] However, ITF2357 fails to affect MMP mRNA stability, and we have previously ruled out HDACi regulation of early signaling events known to regulate MMP transcription.[33] Further research is therefore needed to characterize mechanisms underlying MMP suppression by HDACi.

We extended our previous observation that HDACi accelerate IL-6 mRNA decay in RA FLS, and identifed an additional set of genes, including IL-8, CXCL2, cyclooxygenase-2 (PTGS2) and BCL2L1 (Bcl-XL), which are regulated in a similar fashion. Analyses for the presence of ARE motifs typically recognized by ARE-BPs in the 3' UTRs of the monitored genes revealed increased frequency of most of these sequences in transcripts regulated by ITF2357 through reduction of mRNA stability. Although it is not possible to determine which ARE-BPs might be responsible for HDACi-induced acceleration of mRNA decay because most of these proteins bind several classes of ARE motifs,[56] results of this *in silico* analysis raise the possibility that reversible acetylation may modulate the expression, subcellular localization, or activity of ARE-BPs. Indeed, an early study in breast cancer cells identified reductions in the cytoplasmic levels of HuR, an ARE-BP that increases transcript stability, as a mechanism underlying accelerated mRNA decay in the presence of HDACi.[36] Furthermore, butyrate, a short-chain fatty acid with HDACi activity, suppresses TNFα production by macrophage-like synovial cells and this effect is associated with induction of the TTP family member BRF-1 (TIS11B).[57] Alternatively, since accelerated mRNA degradation of the regulated transcripts is observed as early as 2 h following stimulation, it is possible that HDACi modulate ARE-BP protein turnover through acetylation-mediated protein stabilization: acetylation promotes the stability and function of p53 and FoxP3 by preventing their ubiquitylation and subsequent proteasomal degradation.[58,59] It is unknown, however, whether this mechanism is also responsible for regulation of protein stability of ARE-BPs.

Regulation of mRNA stability is not restricted to ARE-BPs – downregulation of gene expression by miRNAs is mediated, among other mechanisms, by acceleration of mRNA turnover. Depending on the degree of complementarity, miRNAs either promote endonucleolytic cleavage of the

target mRNA, or reduce the stability of the transcript by recruiting protein complexes catalyzing mRNA deadenylation followed by 5' decapping, which exposes the message to degradation by 5' exonucleases.[18,60] miRNA expression is regulated, in part, by epigenetic mechanisms, including changes in histone acetylation,[61] and initial studies in cancer cells have demonstrated that treatment with HDACi rapidly modulates miRNA expression profiles.[62,63] Notably, HDACi restore expression of miRNA-16 in chronic lymphocytic leukemia cells.[64] miRNA-16 is a tumor suppressor miRNA containing a UAAAUAUU sequence complementary to the ARE motif, which interacts with TTP to modulate the stability of a subset of mRNAs, including TNFα and PTGS2.[65] It is therefore possible that induction of miRNA-16 or other ARE-targeting miRNAs by HDACi is one of the mechanisms underlying the acceleration of mRNA decay observed in HDACi-treated FLS. Finally, miRNAs can modulate mRNA stability indirectly by modulating expression of ARE-BPs: application of predictive algorithms has identified a number of ARE-BPs, including TTP, AUF1 and HuR, as potential miRNA targets,[66] and a recent report has confirmed miRNA-29a as a direct suppressor of TTP expression.[67] Additional experiments are therefore needed to verify the potential involvement of miRNA induction in HDACi-mediated mRNA degradation, and to test the relationship between regulation of miRNAs and ARE-BPs by HDACi.

Our bioinformatic analyses have unexpectedly revealed striking differences in the frequencies of ARE motifs between splice variants of certain mRNAs. For example, comparison of two protein-coding IL-6 transcripts of similar length revealed that one of them (ENST00000404625) contains six AUUUA pentamers, while only one AUUUA motif can be found the other transcript (ENST00000420258). Alternative splicing within the 3' UTR region of the transcript is a well-characterized phenomenon,[68] and differences in mRNA stability and, consequently, expression of proteins encoded by splice variants differing in the presence of ARE-rich motifs in their 3' UTRs are associated with pathology in systemic lupus erythematosus.[69-71] Although it remains to be determined which mRNA variants of the analyzed genes are expressed in FLS, this observation raises the possibility that the reported discrepancies in HDACi regulation of certain genes under different experimental conditions might be attributed to differential expression of 3' UTR splice variants. In this regard, a recent study has reported that HDACi augment IL-6 production by murine bone marrow-derived macrophages,[72] while we and others have observed reduction of IL-6 expression by HDACi in RA FLS,[33] as well as human monocytes and macrophages.[25-27] Likewise, differential regulation of IL-8 transcript stability by different 3'UTR splice variants might explain the differences in HDACi effects on IL-8 production in IL-1β-stimulated FLS and macrophages stimulated with LPS or TNFα.[25-27]

Collectively, the data presented here demonstrate that HDACi prevent induction of the majority of genes induced in cytokine-stimulated RA FLS, providing evidence that therapies targeting HDAC activity may be useful in suppressing inflammation in RA. We also identify acceleration of mRNA degradation, possibly mediated by regulation of ARE binding proteins, as an important mechanism contributing to HDACi suppression of a subset of inflammatory mediators expressed in activated FLS. However, kinetics analysis of other HDACi-sensitive genes also suggests the presence of additional molecular mechanisms underlying the anti-inflammatory effects of HDACi, which remain to be characterized.

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