

## EXTENDED REPORT

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

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► Additional data (supplementary methods and supplementary table 1) are published online only at (<http://ard.bmj.com>).

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**ABSTRACT**

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

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

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

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

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

**INTRODUCTION**

Excessive production of inflammatory mediators pivotally contributes to pathology in many chronic immune-mediated diseases (IMIDs), including rheumatoid arthritis (RA).<sup>1</sup> In RA, activated immune cells infiltrating the synovial tissue secrete large quantities of tumour necrosis factor (TNF) $\alpha$ , interleukin 1 (IL-1), IL-8 and IL-6, among other cytokines and chemokines. These secreted

products, as well as cell–cell contacts, activate stromal fibroblast-like synoviocytes (FLS), which are potent effector cells in RA, generating enzymes that degrade cartilage and bone, and serving as a primary source of inflammatory cytokines in the synovium.<sup>2,3</sup>

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

While some studies have indicated that decreased expression of HDACs in synovial tissue may contribute to pathology in RA,<sup>6,7</sup> analyses of murine and human monocytes revealed that HDAC inhibitors (HDACi) are potent anti-inflammatory agents, which suppress lipopolysaccharide (LPS)-induced and TNF $\alpha$ -induced cytokine production.<sup>8–10</sup> Also, HDACi uniformly ameliorate inflammation and prevent joint destruction in prophylactic and therapeutic protocols in animal arthritis models.<sup>11–16</sup> These findings are relevant to RA as we have previously demonstrated that HDACi suppress IL-6 and TNF $\alpha$  production by RA synovial macrophages and synovial tissue explants.<sup>17</sup> Moreover, RA FLS proliferation and survival *in vitro* is suppressed by HDACi.<sup>15, 18, 19</sup> The exact mechanisms by which HDACi alleviate inflammation in acute and chronic inflammatory diseases remain unclear, but could be related to regulation of histone acetylation. Alternatively, HDACi may target some 1700 structural and signal transduction proteins, many of which are relevant to RA, including components of the mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription (STAT) pathways, transcription factors such as p53, nuclear factor  $\kappa$ B (NF $\kappa$ B) p65 and c-Jun, as well as regulators of mRNA stability, protein degradation and secretion.<sup>20–22</sup> Further understanding of



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the molecular mechanism(s) contributing to anti-inflammatory effects of HDACi may facilitate hypothesis-driven decisions as to the suitability of HDACi in the treatment of RA, especially now that one HDACi, ITF2357 (givinostat; Italfarmaco, Cinisello Balsamo, Italy), has demonstrated initial clinical efficacy in the treatment of systemic onset juvenile idiopathic arthritis (SOJIA).<sup>23 24</sup>

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

## MATERIALS AND METHODS

### Cell culture and stimulation

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

### Measurement of cell viability

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

### Quantitative measurement of IL-6 protein and mRNA expression

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

### Protein extraction and immunoblotting

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

## Measurement of transcription factor DNA-binding and transcriptional activity

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

### Statistical analyses

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

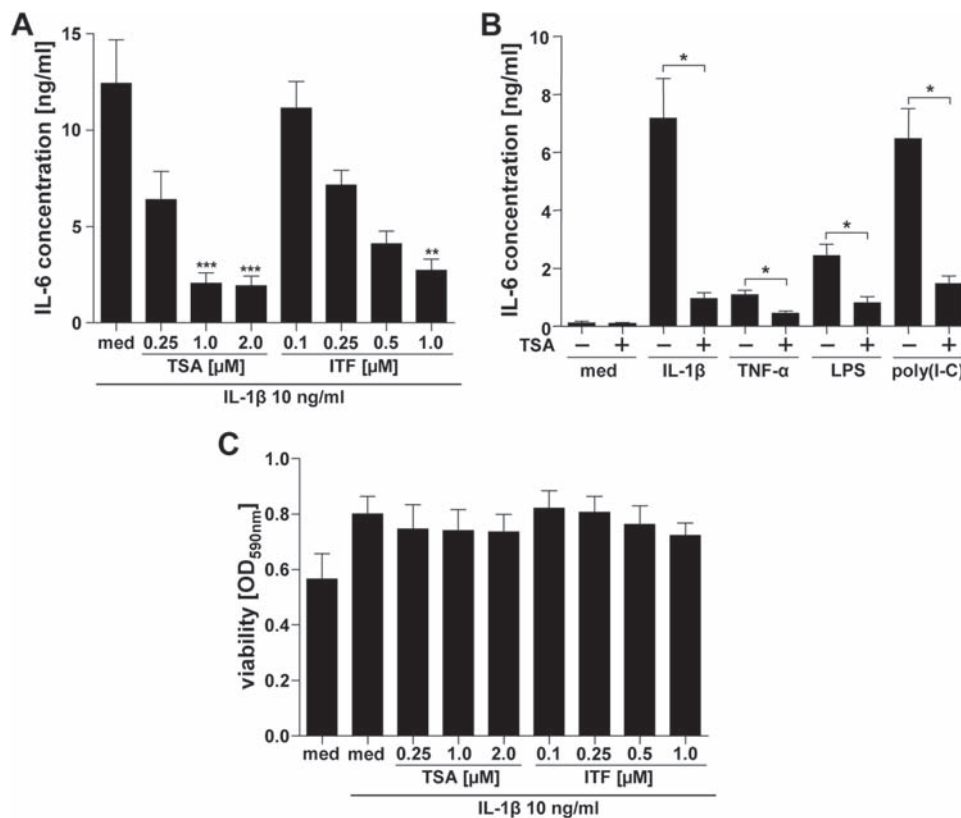
## RESULTS

### HDACi suppress RA FLS IL-6 production

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

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

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



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

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

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

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

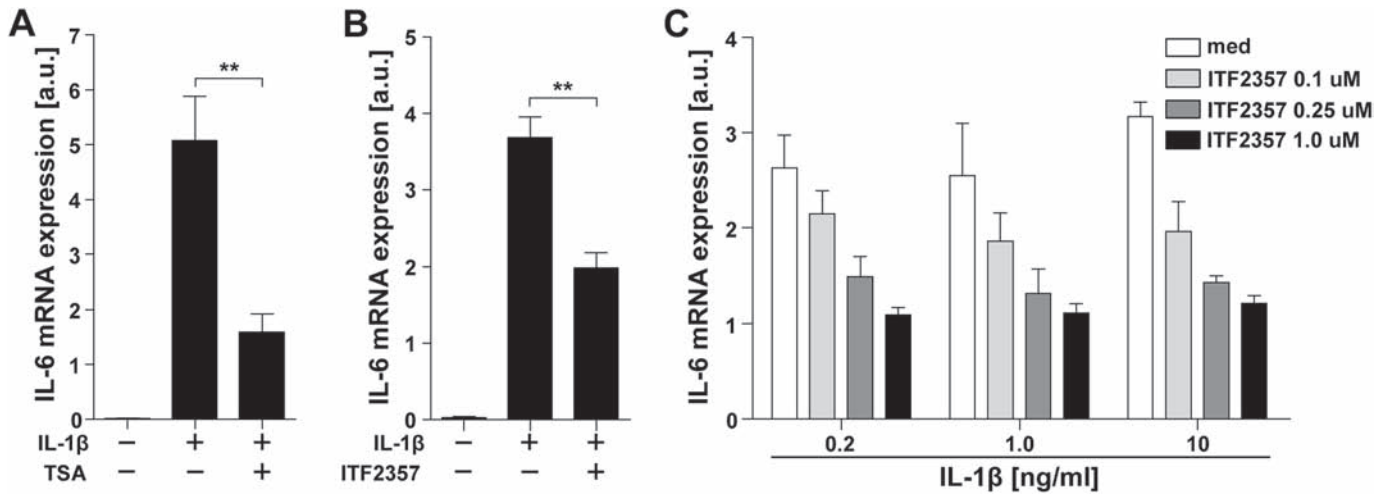
Phosphorylation of c-Jun N-terminal kinase (JNK), activation of the c-Jun component of the AP-1 transcription factor and

subsequent rapid induction of c-Jun expression are essential prerequisites for optimal IL-6 gene transcription, and are sensitive to acetylation.<sup>30 31</sup> However, similarly to p38 and ERK, IL-1 $\beta$ -stimulated JNK phosphorylation in FLS was unaffected by TSA (figure 4B). Also, IL-1 $\beta$  induced a transient fivefold increase of c-Jun mRNA levels which was unaffected by TSA (figure 4C). Stimulation with IL-1 $\beta$  caused a 2.5-fold increase in p-c-Jun DNA binding compared to unstimulated FLS (figure 4D). However, FLS exposure to TSA did not suppress the induction of c-Jun binding activity (figure 4D) and had no influence on DNA binding of Jun family members JunB and JunD (data not shown). Collectively, these results suggest that HDACi-dependent IL-6 suppression in RA FLS is not mediated by direct regulation of MAPK and AP-1 signalling pathways.

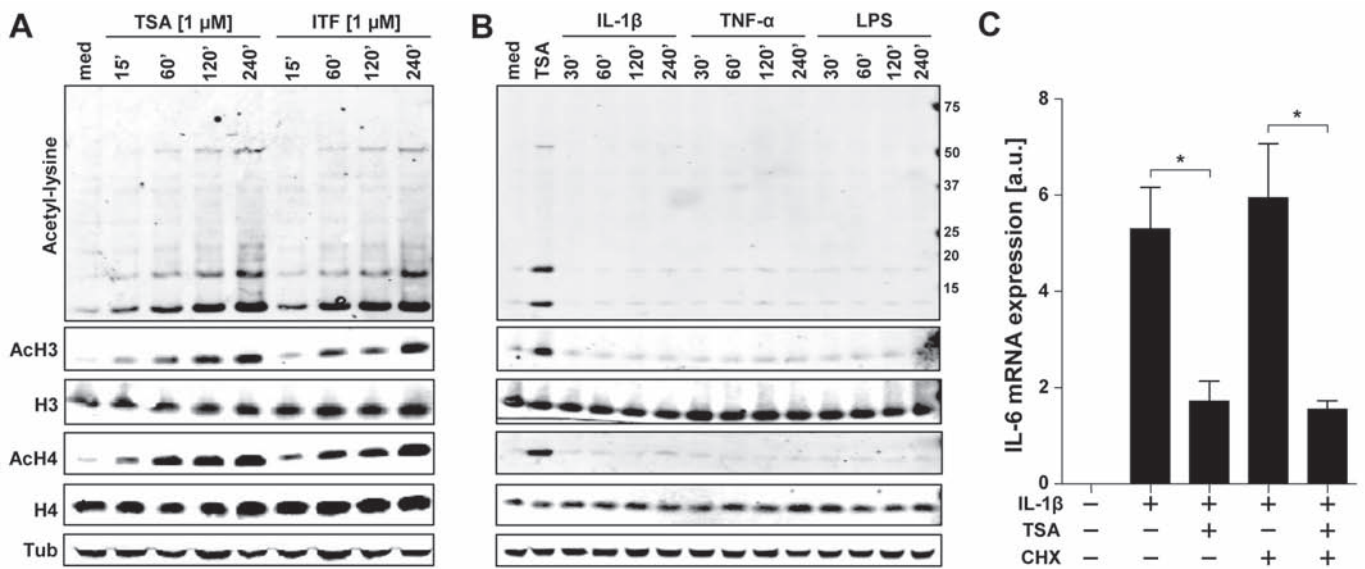
#### HDACi modulate NF $\kappa$ B signalling in RA FLS

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





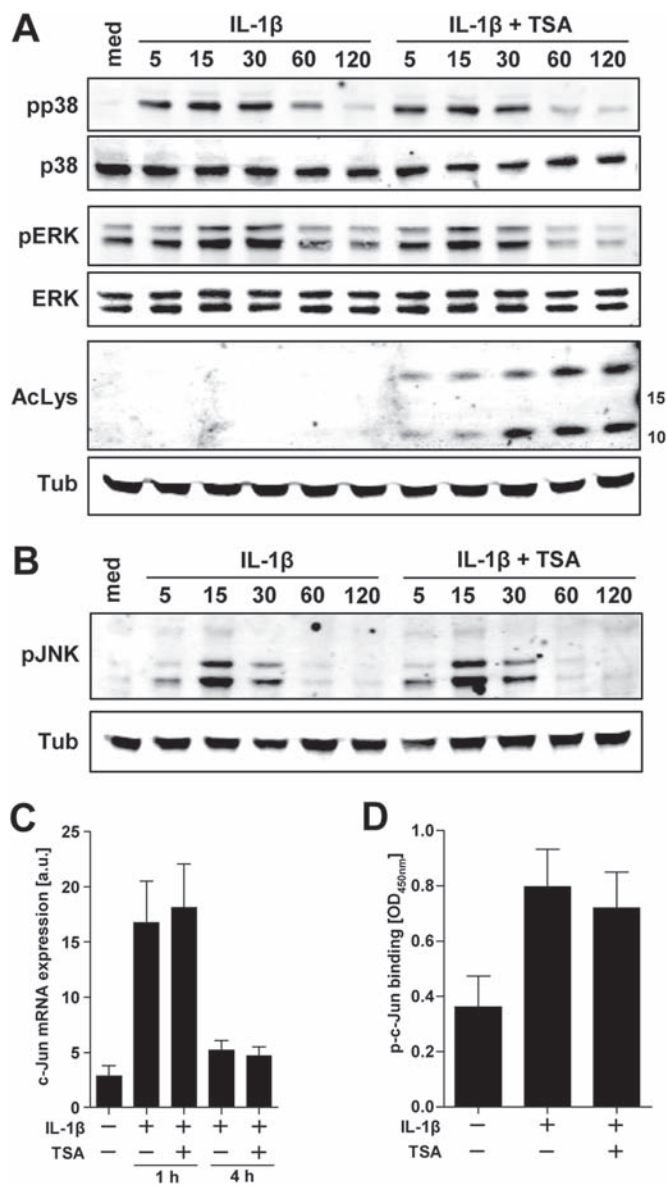
**Figure 2** Histone deacetylase inhibitors reduce interleukin 6 (IL-6) mRNA accumulation in rheumatoid arthritis fibroblast-like synoviocytes (FLS). FLS were left unstimulated, or were stimulated with IL-1β (10 ng/ml) with or without 1 μM trichostatin A (A), or with IL-1β (1 ng/ml) in the presence or absence of 0.25 μM ITF2357 (B) for 4 h, total RNA was extracted, reverse transcribed and IL-6 mRNA accumulation analysed by quantitative PCR (qPCR). Results are presented as mean ± SEM IL-6 expression relative to 18S of five independent experiments. \*\*p < 0.01, Mann-Whitney U test. Alternatively (C), FLS (n=3) were stimulated as above with increasing concentrations of IL-1β (0.2–10 ng/ml) in the absence (med) or presence of increasing concentrations of ITF2357 (0.1–1.0 μM) and IL-6 mRNA expression assessed as in (A) and (B).



**Figure 3** New protein synthesis is not required for fibroblast-like synoviocyte (FLS) interleukin 6 (IL-6) suppression by histone deacetylase inhibitors. A. FLS were left untreated (med) or were treated with trichostatin A (TSA) (1 μM) or ITF2357 (1 μM) for the indicated time (min). Protein extracts were prepared and analysed by immunoblotting for acetylated lysine (Ac-Lys), Ac-H3, H3, Ac-H4, H4 and tubulin (Tub) content. B. FLS were left unstimulated in medium (med) or were stimulated with IL-1β (10 ng/ml), tumour necrosis factor (TNF)α (10 ng/ml) or lipopolysaccharide (1 μg/ml), lysed and examined by immunoblotting with antibodies recognising Ac-Lys, Ac-H3, H3, Ac-H4, H4 and Tub. FLS treated with 1 μM TSA for 4 h were used as a positive control. Results are representative of three independent experiments. C. FLS were left untreated or were treated with IL-1β (10 ng/ml) with or without 1 μM TSA for 4 h. To block protein translation, cells were preincubated with 1 μg/ml cycloheximide (CHX) for 30 min prior to stimulation. IL-6 mRNA levels were assessed by quantitative PCR (qPCR) and presented as relative IL-6 expression ± SEM (n=4). \*p < 0.05, Mann-Whitney U test.

nuclear accumulation of p50 and p65 NFκB subunits assessed by immunoblotting. TSA had little effect on nuclear p50 and p65 in the absence of inflammatory stimulation, but slightly increased the amounts of both proteins 1 h after IL-1β stimulation. However, nuclear retention of these subunits 24 h post stimulation was strongly reduced (figure 5B). In DNA-binding assays we observed a trend towards elevated p50 and p65 binding 1 h after IL-1β stimulation in the presence of TSA, while a

significant reduction of DNA binding was observed only after 24 h (figure 5C). To determine if p65 retained transcriptional activity in the presence of TSA, we cotransduced RA FLS with adenoviral vectors encoding 4×NFκB-minimal promoter (MLP)-luciferase reporter, and evaluated the effects of TSA on luciferase enzymatic activity. TSA did not inhibit, and clearly increased, the transcriptional activity of NFκB early after stimulation with IL-1β (figure 5D). Together, these findings indicate that HDACi-



**Figure 4** Histone deacetylase inhibitors (HDACi) fail to affect mitogen-activated protein kinase (MAPK) signalling and activator protein 1 (AP-1) activation in rheumatoid arthritis fibroblast-like synoviocytes (FLS).

**A.** FLS were left unstimulated (med) or were stimulated with 10 ng/ml of interleukin 1 $\beta$  (IL-1 $\beta$ ) for the indicated time (min) in the presence or absence of 1  $\mu$ M trichostatin A (TSA), proteins were extracted and MAPK activation analysed by immunoblotting with antibodies specific for phospho(p)-p38, p38, p-extracellular signal regulated kinase (ERK), ERK and tubulin (Tub). Anti-acetylated lysine (Ac-Lys) antibody was used to confirm TSA HDACi activity in this experiment. **B.** FLS were subjected to the same treatment as in (A) and c-Jun N-terminal kinase (JNK) activation determined in cellular lysates by immunoblotting for p-JNK and Tub content. **C.** HDACi do not modulate c-Jun induction by an inflammatory stimulus. FLS were stimulated with medium alone (med) or with IL-1 $\beta$  (10 ng/ml) with or without 1  $\mu$ M TSA for 1 h or 4 h, total RNA was reverse transcribed, and changes in c-Jun mRNA levels were determined by quantitative PCR (qPCR). Results of four independent experiments are shown as relative expression  $\pm$ SEM. **D.** HDACi have no effect on c-Jun DNA binding activity. FLS were left unstimulated in medium (med) or were treated with IL-1 $\beta$  (10 ng/ml) for 4 h in the presence or absence of 1  $\mu$ M TSA, nuclear fractions were extracted and levels of active p-c-Jun were determined using an ELISA-based DNA-binding assay. Data represent the mean  $\pm$ SEM optical density at 450 nm for five independent experiments.

induced suppression of IL-6 mRNA, detectable 4 h after inflammatory stimulation, precedes inhibitory effects of HDACi on p50 and p65 DNA binding and nuclear retention.

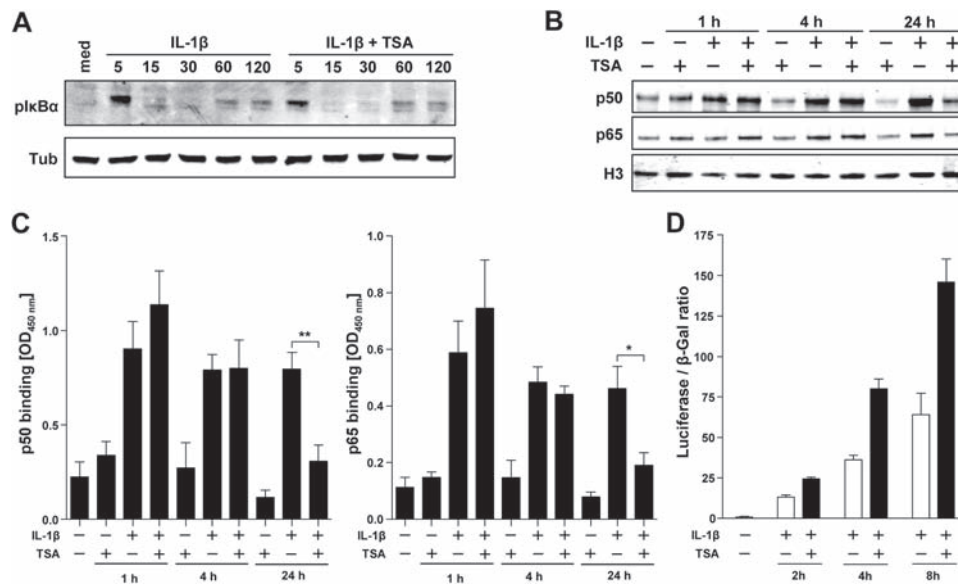
#### HDACi modulate IL-6 mRNA stability in RA FLS and HD macrophages

To gain more insight into the kinetics of IL-6 suppression by HDACi in RA FLS, we treated the cells with IL-1 $\beta$  for 1–8 h with or without TSA, and measured changes in IL-6 mRNA levels over time. IL-1 $\beta$  induced a 20-fold increase in IL-6 mRNA levels within 1 h, which remained stable until 4 h before further increasing. TSA failed to affect IL-6 mRNA accumulation 1 h after stimulation, but a strong reduction was observed at 4 h and became more pronounced at 8 h ( $p < 0.05$ ) (figure 6A). As we had previously ruled out a need for de novo repressor synthesis in HDACi-mediated IL-6 suppression, we examined if differences might be attributable to effects on mRNA stability. FLS were stimulated with IL-1 $\beta$  for 4 h in the presence or absence of TSA, and after inhibiting transcription with actinomycin D (ActD), we analysed time-dependent IL-6 mRNA decay. Accelerated degradation of IL-6 mRNA in TSA-treated FLS was detectable within 1 h of ActD treatment, becoming more pronounced at later time points ( $p < 0.05$  for AUC) (figure 6B). Similar effects were observed in the presence of 0.25  $\mu$ M ITF2357 (figure 6C). The presence of TSA also significantly accelerated IL-6 mRNA decay in LPS-stimulated macrophages (figure 6D).<sup>17</sup> Together, these results indicate that modulation of mRNA stability is a common mechanism by which IL-6 production is suppressed by HDACi in macrophages and RA FLS.

#### DISCUSSION

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

RA FLS treatment with HDACi, as previously observed with RA synovial macrophages,<sup>17</sup> rapidly induced acetylation of histone and non-histone proteins, raising the possibility that HDACi-induced expression of transcription corepressors might explain the suppressive effects of HDACi on IL-6 production. However, treatment of cells with CHX, to prevent translation of putative repressors, failed to rescue IL-6 production in the presence of HDACi. Consistent with this, even though HDACi induce H4 hyperacetylation in the IL-6 promoter region in murine bone marrow-derived macrophages, IL-6 production is downregulated,



**Figure 5** Histone deacetylase inhibitors (HDACi) increase nuclear factor  $\kappa$ B (NF $\kappa$ B) transcriptional activity early after stimulation, but inhibit NF $\kappa$ B nuclear retention at later time points. A. Fibroblast-like synoviocytes (FLS) were left untreated (med) or were treated with interleukin 1 $\beta$  (IL-1 $\beta$ ) (10 ng/ml) for 5–120 min in the presence or absence of 1  $\mu$ M trichostatin A (TSA), and total cell lysates examined by immunoblotting for phospho(p)-inhibitor of  $\kappa$ B $\alpha$  (I $\kappa$ B $\alpha$ ) and tubulin (Tub) content. Alternatively, FLS were cultured in the presence or absence of 1  $\mu$ M TSA and then stimulated with medium alone or 10 ng/ml of IL-1 $\beta$  for 1 h, 4 h or 24 h. Nuclear extracts (n=4) were either separated by electrophoresis and nuclear accumulation of NF $\kappa$ B subunits analysed by immunoblotting with antibodies recognising p50, p65 and histone 3 (H3) (B), or were used for determination of p50 and p65 DNA-binding activities by an ELISA-based DNA binding assay (C). Values representing changes in p50 (left panel) and p65 (right panel) are shown as mean  $\pm$  SEM optical density at 450 nm. \* $p$ <0.05; \*\* $p$ <0.01, Mann–Whitney U test. D. FLS were cotransduced with a 4 $\times$ NF $\kappa$ B-minimal promoter (MLP)-luciferase and control cytomegalovirus  $\beta$ -galactosidase (CMV- $\beta$ -gal) adenoviral vectors. At 48 h after transduction, cells were serum-starved for 24 h and then left untreated or stimulated with IL-1 $\beta$  (10 ng/ml) with or without 1  $\mu$ M TSA for 2 h, 4 h or 8 h. Cellular lysates were prepared and the luciferase (Luc) and  $\beta$ -gal enzymatic activities determined by luminometry and spectrophotometry, respectively. Data are presented as relative luciferase activity normalised to  $\beta$ -gal activity  $\pm$ SD and a representative of two independent experiments is shown.

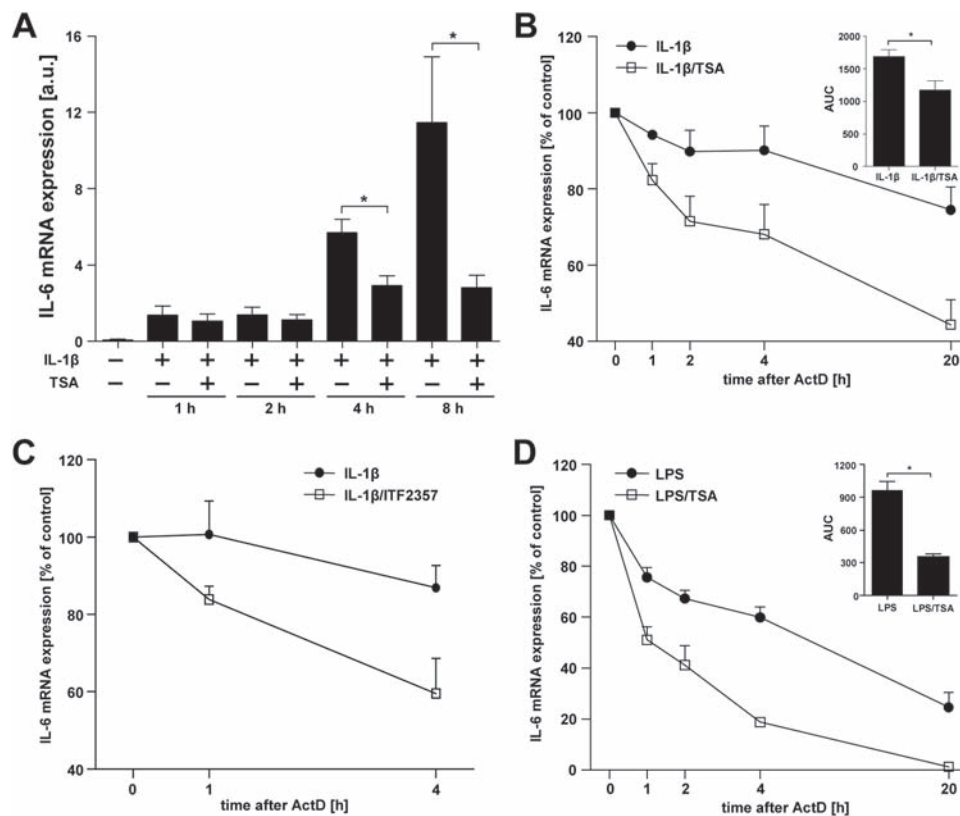
arguing against involvement of changes in histone acetylation status in HDACi regulation of IL-6.<sup>33</sup> Surprisingly, we also found little evidence supporting a role for HDACi in modulating acute signal transduction events downstream of IL-1 $\beta$  stimulation. Previous reports have indicated that HDACi might regulate inflammatory gene expression via blockade of MAPK signalling,<sup>34</sup> suppression of c-Jun induction and activation,<sup>31</sup> or reduction of NF $\kappa$ B nuclear retention.<sup>19</sup> In murine macrophages, HDACi treatment causes acetylation and activation of MAPK phosphatase-1 which prevents p38 phosphorylation, ultimately leading to inhibition of TLR signalling.<sup>34</sup> However, we observed no effect of TSA on IL-1 $\beta$ -induced activation of p38, ERK and JNK MAPKs, *c-Jun* gene induction, or DNA binding activity of Jun family members. Our negative results highlight the difficulties of extrapolating effects of HDACi in various cell types to primary cells from RA synovial tissue in the absence of formal studies.

NF $\kappa$ B signalling is complexly regulated by reversible acetylation, as p65 can be acetylated on at least five unique lysine residues, each having a distinct effect on p65 function. Acetylation at Lys221 promotes p65 dissociation from I $\kappa$ B $\alpha$  and nuclear import, while acetylation at 310 enhances p65 transcriptional activity.<sup>35</sup> Alternatively, p65 acetylation at Lys122 and Lys123 decreases DNA binding affinity, enhances I $\kappa$ B $\alpha$  association and promotes nuclear export.<sup>37</sup> We observe a similarly complex regulation in RA FLS, as p65 nuclear import, DNA binding activity and transcriptional capacity are all enhanced by HDACi in the initial hours following IL-1 $\beta$  stimulation. However, HDACi promote dissociation from DNA and nuclear export at later time points, consistent with recent observations in an SV40 Tag-transformed RA FLS cell line.<sup>19</sup> The changes we observe in NF $\kappa$ B activity are

consistent with temporal acetylation of NF $\kappa$ B at distinct regulatory sites, but direct studies are needed to address this possibility. However, the kinetics of alterations in NF $\kappa$ B activity cannot explain the acute suppression of IL-1 $\beta$ -induced IL-6 production by HDACi, but may confer a protective effect in the face of chronic inflammatory stimulation.

While many of the HDACi effects on cellular activation have been attributed to epigenetic regulation or influences on signal transduction, studies in cancer biology have provided initial evidence that these compounds can modulate gene expression via regulation of mRNA stability.<sup>38–40</sup> We observe a similar and shared mechanism of action in the suppression of IL-6 production in macrophages and RA FLS. mRNA stability is regulated by hundreds of gene products, some of which have been identified as functionally associated with HDACs.<sup>41–42</sup> Selectivity is also observed in mRNAs targeted by these proteins as, for example, Zc3h12 RNase specifically regulates IL-6 mRNA stability.<sup>43</sup> Alternatively, as observed in cancer biology studies, HDACi might differentially modulate microRNA (miR) expression.<sup>44</sup> IL-6-specific miRs have yet to be identified, but the expression of several miRs associated with inflammatory responses is altered in RA FLS and synovial tissue.<sup>45</sup> Future studies will be needed to elucidate how HDACi enhance IL-6 mRNA degradation, and test how globally this mechanism regulates gene expression in RA synovial tissue. Preliminary experiments indicate that HDACi might also regulate FLS production of IL-8 and matrix metalloproteinase 1 without affecting mRNA stability (data not shown), indicating additional mechanisms by which HDACi modulate gene expression in RA FLS. Intriguingly, HDACi can accelerate decay of DNA methyltransferase 3B mRNA in human





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

endometrial cells, raising the possibility that HDAC-dependent mRNA destabilisation might regulate inflammatory gene expression through crosstalk with epigenetic DNA methylation pathways.<sup>46</sup> The results presented here provide the first mechanistic evidence by which HDACi might suppress inflammatory cytokine production in RA, and, together with the initial proof of principle that has recently been obtained in SOJIA,<sup>23 24</sup> support the notion that HDACi may represent a novel therapeutic approach for the treatment of RA.

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## **Supplementary materials and methods**

### **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) and protein content quantitated with a BCA Protein Assay Kit (Pierce, Rockford, IL). Equivalent amounts of lysate or nuclear extract were resolved by electrophoresis and analyzed by immunoblotting using primary antibodies recognizing acetylated lysine, acetylated histone 3 (H3), acetylated histone 4 (H4), H3, phospho (p)-I $\kappa$ B $\alpha$ , p-p38, p-ERK, p-JNK, p38, ERK (all from Cell Signaling Technology, Beverly, MA), H4 (Upstate, Temecula, CA), NF- $\kappa$ B p65 and p50 (both from Santa Cruz Biotechnology, Santa Cruz, CA), and tubulin (Sigma-Aldrich), followed by development with IRDye-680-labelled anti-rabbit or IRDye-800-labelled anti-mouse immunoglobulin secondary antibodies (LI-COR Biosciences, Bad Homburg, Germany) and visualization using an Odyssey infrared imaging system (LI-COR Biosciences).

### **Quantitative measurement of mRNA expression**

FLS were left unstimulated or were stimulated for 1-8 h with IL-1 $\beta$  in the presence or absence of TSA. Alternatively, cells were treated with IL-1 $\beta$  for 4 h with or without TSA and cycloheximide (CHX) (Sigma-Aldrich). Total RNA was extracted using a GenElute RNA isolation kit (Sigma-Aldrich) and reverse transcribed using a First-Strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania). Quantitative (q)PCR was performed on a StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA) using Power SybrGreen PCR Master Mix (Applied Biosystems) and the following primers: IL-6 forward, GACAGCCACTCACCTCTTCA; IL-6 reverse, CCTCTTTGCTGCTTTCACAC; c-Jun forward, GGAAGCTGGAGAGAATCGCC; c-Jun reverse,

TTCTGTTTAAGCTGTGCCACCTG; 18S forward, CGGCTACCACATCCAAGGAA; 18S reverse, GCTGGAATTACCGCGGCT (Invitrogen). For measurement of mRNA stability, FLS or macrophages were incubated in medium alone or IL-1 $\beta$  or LPS, respectively, for 4 h in the presence or absence of TSA. Culture medium was replaced with medium containing 10  $\mu$ g/ml actinomycin D (ActD, Sigma-Aldrich), and RNA isolated at 0, 1, 2, 4 and 20 h following ActD exposure. qPCR reactions were performed in duplicate. Specific PCR product amplification was confirmed by dissociation curve analysis for each primer pair. Relative gene expression was calculated using StepOne Software v2.1 (Applied Biosystems) and expressed as the ratio between the gene of interest and 18S.

### **Measurement of NF- $\kappa$ B transcriptional activity in RA FLS**

Adenoviral 4xNF- $\kappa$ B-MLP-luciferase (Luc) reporter vector was generated using previously described methodologies.[47] A 4xNF- $\kappa$ B enhancer was excised from a 4xNF- $\kappa$ B-TK-SEAP plasmid (Clontech/BD Biosciences) and cloned into pGL3-MLP-Luc in front of a major late adenoviral minimal promoter (MLP). Next, a cassette containing an artificial polyA track, 4xNF- $\kappa$ B enhancer, MLP promoter, Luc ORF and a polyA signal was re-cloned into pShuttle vector and recombined with an Easy-1 adenoviral backbone in BJ1583 bacteria. The linearized cosmid was transfected into HEK-293 cells and amplified as an adenovirus. Adenoviruses encoding 4xNF- $\kappa$ B-MLP-Luc and control CMV- $\beta$ -galactosidase ( $\beta$ -Gal) were amplified and titrated in transcomplemental HEK-293 cells and purified by cesium chloride gradient ultracentrifugation.[48] FLS were co-transduced with 4xNF- $\kappa$ B-MLP-Luc and CMV- $\beta$ -Gal at a multiplicity of infection of 300 and 30, respectively. 48 hours after transduction, cells were serum starved for 24 hours and then left unstimulated or stimulated with IL-1 $\beta$  in the presence or absence of TSA for 2-8 hours. Cells were lysed in a 1x Passive

Lysis Buffer (Promega, Madison, WI). Luc activity was measured with a Luciferase Assay reagent (Promega) using a Victor3 Multilabel Reader (Perkin Elmer, Norwalk, CT).  $\beta$ -Gal activity, measured at OD<sub>405</sub> in 100 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 100 mM  $\beta$ -mercaptoethanol, and 0.67 mg/ml *O*-nitrophenyl- $\beta$ -D-galactopyranoside, was used to correct for transduction efficiency.



**Supplemental table 1.** Clinical features of RA patients (N = 18) included in the study\*

<b>Characteristic</b>	<b>Median (range)</b>
Age (years)	57.5 (48-80)
Male: Female	6:12
Disease duration [months]	84 (1-348)
ESR [mm/hour]	28.5 (2-77)
CRP [mg/l]	7.1 (1.1-119.3)
DAS28	4.11 (2.6-6.75)

\*ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DAS28 = disease activity score