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# SIRT1 overexpression in the rheumatoid arthritis synovium contributes to proinflammatory cytokine production and apoptosis resistance

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## ABSTRACT

**Objective** To analyse the expression of SIRT1 in synovial tissues and cells of patients with rheumatoid arthritis (RA) and to study the function of SIRT1 in inflammation and apoptosis in RA.

**Methods** Levels of SIRT1 expression were analysed in synovial tissues and cells from patients with RA by real-time PCR and western blotting before and after stimulation with toll-like receptor ligands, tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) and interleukin 1 $\beta$  (IL-1 $\beta$ ). Immunohistochemistry was used to study the localisation of SIRT1. Fluorescence activated cell sorting analysis was performed to investigate the effect of SIRT1 on apoptosis. Peripheral blood monocytes and rheumatoid arthritis synovial fibroblasts (RASFs) were transfected with wild-type or enzymatically inactive SIRT1 expression vectors or with siRNA targeting SIRT1. Cytokine analysis of IL-6, IL-8 and TNF $\alpha$  were performed by ELISA to study the role of SIRT1 on proinflammatory mediators of RA.

**Results** SIRT1 was found to be constitutively upregulated in synovial tissues and cells from patients with RA compared to osteoarthritis. TNF $\alpha$  stimulation of RASFs and monocytes resulted in further induced expression levels of SIRT1. Silencing of SIRT1 promoted apoptosis in RASFs, whereas SIRT1 overexpression protected cells from apoptosis. Inhibition of SIRT1 enzymatic activity by inhibitors, siRNA and overexpression of an enzymatically inactive form of SIRT1 reduced lipopolysaccharide-induced levels of TNF $\alpha$  in monocytes. Similarly, knockdown of SIRT1 resulted in a reduction of proinflammatory IL-6 and IL-8 in RASFs.

**Conclusion** The TNF $\alpha$ -induced overexpression of SIRT1 in RA synovial cells contributes to chronic inflammation by promoting proinflammatory cytokine production and inhibiting apoptosis.

## INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterised by the destruction of joint cartilage and bone. Synovial hyperplasia and persistent synovial inflammation with infiltration of inflammatory immune cells into the synovial lining are hallmarks of RA.<sup>1</sup> Innate immunity was shown to be important for the development of chronic arthritis. Activation of toll-like receptors (TLRs) and NOD-like receptors in synovial cells leads to the expression of several proinflammatory genes, such as interleukin 6 (IL-6) and tumour necrosis factor  $\alpha$  (TNF $\alpha$ ).<sup>2–3</sup> We have previously reported the induction of the adipokine PBEF (pre-B cell colony enhancing factor), also called

visfatin, upon stimulation of TLRs.<sup>4</sup> PBEF/Visfatin catalyses the rate-limiting step in the biosynthesis of nicotinamide-adenine-dinucleotide (NAD<sup>+</sup>).<sup>5</sup> By regulating intracellular NAD<sup>+</sup> levels, PBEF/visfatin influences the activity of a variety of NAD<sup>+</sup> consuming enzymes, including the sirtuins (SIRT).<sup>6–7</sup>

SIRT1s are a conserved family of NAD<sup>+</sup> dependent histone deacetylases and mono-ADP-ribosyltransferases that target histones, transcription factors and coregulators to adapt gene expression to the cellular energy state.<sup>8–10</sup> In mammals seven sirtuin genes—SIRT1 to SIRT7—have been identified. Among them, SIRT1 is best characterised so far and has been shown to regulate transcription factors such as p53,<sup>11</sup> members of the forkhead transcription factor FOXO family,<sup>12</sup> the DNA repair factor Ku70,<sup>13</sup> nuclear factor- $\kappa$ B (NF- $\kappa$ B)<sup>14</sup> and the transcriptional coactivator p300.<sup>15</sup>

In lung cancer cell lines, SIRT1 was found to physically interact with, and deacetylate, the RelA/p65 subunit of NF- $\kappa$ B, thereby inhibiting its ability to interact with promoter regions of target genes to enhance transcription.<sup>14</sup> Hyperacetylation of lysine 310 of RelA/p65 rendered NF- $\kappa$ B highly active, resulting in increased transcription of proinflammatory cytokines such as TNF $\alpha$  and IL-1 in a myeloid SIRT1 knockout mouse model.<sup>16</sup> However, nicotinamide has been shown to reduce the production of proinflammatory cytokines as well as IL-10 in primary human macrophages, possibly by inhibiting sirtuins.<sup>17</sup>

In recent years, SIRT1 has gained much attention since its expression was shown to mediate longevity.<sup>18</sup> SIRT1 deacetylates the DNA repair factor Ku70 leading to inhibition of apoptosis. In addition, SIRT1 protected epithelial cells from p53-mediated apoptosis.<sup>11–19</sup> In contrast to these findings, in HEK293 epithelial cells, SIRT1 augmented apoptosis in response to TNF $\alpha$ ,<sup>14</sup> highlighting the possibility that SIRT1 may have different biological outcomes depending on the apoptotic stimuli.

So far, SIRT1 expression and function in the RA synovium has not been analysed. Therefore, in view of the controversial results on the influence of sirtuins on inflammation and apoptosis, we analysed the expression, regulation and function of SIRT1 in RA. We showed overexpression of SIRT1 in synovial tissues from patients with RA. In addition, SIRT1 is shown to decrease apoptosis in synovial cells and to promote proinflammatory cytokine production.

## MATERIAL AND METHODS

### Patients and tissue preparation

Synovial tissue biopsy specimens were obtained from patients with RA and osteoarthritis (OA), after informed consent had been obtained (Department of Orthopedic Surgery, Schulthess Clinic, Zurich, Switzerland). All patients with RA fulfilled the American College of Rheumatology criteria for classification of RA.<sup>20</sup> Synovial tissue specimens were digested and synovial fibroblasts (SFs) were grown as previously described.<sup>21</sup> The patient characteristics used in this study are shown in table 1.

### Preparation of monocytes from peripheral blood

Isolation of peripheral blood mononuclear cells from the whole blood of healthy donors or patients with RA was performed using standard Ficoll density-gradient centrifugation (GE Healthcare, Otelfingen, Switzerland). Monocytes were separated by positive selection with CD14 MACS MicroBeads or, when used for stimulation or transfection experiments, by negative selection with MACS MicroBeads Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany).

### Stimulation assays

Rheumatoid arthritis synovial fibroblasts (RASFs) and primary peripheral monocytes from healthy individuals were plated in 12- and 24-well plates ( $5 \times 10^4$  and  $3\text{--}5 \times 10^5$  cells/well) in 500  $\mu$ l supplemented Dulbecco's modified Eagle's medium and RPMI, respectively, and stimulated for the indicated time points with the following agents: 300 ng/ml Pam3CSK4, 10  $\mu$ g/ml poly(I:C) (both from InvivoGen, San Diego, California, USA), 10 ng/ml lipopolysaccharide (LPS from *Escherichia coli* J5; List Biologicals, Campbell, California, USA), 10 ng/ml TNF $\alpha$ , 1 ng/ml IL-1 $\beta$  (both from R&D Systems, Abingdon, UK), 30  $\mu$ M Sirtinol (Sigma, Buchs, Switzerland) or 9  $\mu$ M EX-527 (Tocris Bioscience, Bristol, UK).

### Transfection experiments

Monocytes from healthy volunteers and RASFs were transfected using AMAXA nucleofection kits VPA-1007 and VVPI-1002 (both from Lonza, Cologne, Germany), respectively. For silencing experiments, 100  $\mu$ M SIRT1 validated stealth siRNA or stealth control high GC siRNA (both from Invitrogen, Basel, Switzerland) were used. For overexpression experiments, 1  $\mu$ g of SIRT1 wild-type, SIRT1 mutant (H363Y), empty pcDNA3.1(-) (mock) or no nucleic acid (untransfected) was used. pcDNA3.1-SIRT1-MYC/HIS wild-type and the catalytically inactive mutant (H363Y) have been described previously.<sup>22</sup> Transfected monocytes were further incubated at 37°C for 18–20 h before stimulation with LPS for 8 h, in presence or absence of 50  $\mu$ M sc-514 (Sigma). RASFs were transfected for 48 h and subsequently stimulated with LPS or TNF $\alpha$  for 24 h (siRNA) or 40 h (vectors). Successful transfection was confirmed by real-time PCR using SIRT1 mRNA-specific primers.

### Real-time PCR

Total RNA was isolated with the RNeasy Mini kit including treatment with RNase-free DNase (Qiagen, Hombrechtikon, Switzerland) and reverse transcribed using random hexamers and multiscribe reverse transcriptase (both from Applied Biosystems, Rotkreuz, Switzerland). Non-reverse transcribed samples were used as negative controls. Quantification of SIRT1 and IL-6 mRNA was performed by TaqMan RT-PCR using the ABI Prism 7700 Sequence Detection system (Applied Biosystems). The primer sequences are shown in table 2. The endogenous control 18S rRNA was used for correcting the

**Table 1** Characteristics of the study patients

Characteristics	Patients with RA (n=39)	Patients with OA (n=10)	Healthy controls (n=6)
Age (years, (mean (range)))	62 (29–86)	76 (64–98)*	43 (29–64) <sup>†</sup>
Sex (n (female/male))	31/8	5/5	4/2
Disease duration (years, (mean (range)))	25 (3–50)	NA	NA
Medications (n (taking/assessed))			
NSAIDs	12/39	3/10	0/6
DMARDs	31/39	0/10	0/6
RF+ (>20 IU) (n (taking/assessed))	29/29	NA	NA
CRP (mg/l, (mean (range)))	14.4 (1.0–52.5)	6.9 (0.9–39.5)	NA

\*Patients with OA were significantly older than patients with RA and healthy controls.

<sup>†</sup>Healthy controls were significantly younger than patients with OA and RA.

CRP, C-reactive protein; DMARDs, disease-modifying antirheumatic drugs; NA, not assessed; NSAIDs, non-steroidal anti-inflammatory drugs; OA, osteoarthritis; RA, rheumatoid arthritis; RF, rheumatoid factor.

**Table 2** SYBR green primers used for real-time PCR

SIRT1 forward	5'-GCG GGA ATC CAA AGG ATA AT-3'
SIRT1 reverse	5'-CTG TTG CAA AGG AAC CAT GA-3'
IL-6 forward	5'-CTC TTC AGA ACG AAT TGA CAA ACA A-3'
IL-6 reverse	5'-GAG ATG CCG TCG AGG ATG TAC-3'

IL-6, interleukin 6.

results with the comparative threshold cycle method for relative quantification as described by the manufacturer.

### ELISA

Protein in cell supernatants was detected by ELISA with OptEIA Kits (BD Pharmingen, San Diego, California, USA) for TNF $\alpha$ , IL-6 and IL-8 according to the manufacturer's instructions. Absorption was measured at 450 nm and data were analysed using Revelation v4.22 software (Dynex Technologies, Denkendorf, Germany).

### Immunohistochemistry

Synovial tissues were fixed in paraformaldehyde and embedded in paraffin. Tissue sections were deparaffinised and pretreated with 10 mM citrate buffer, pH 6. After blocking endogenous peroxidase and non-specific binding, slides were incubated with rabbit IgG (Jackson, Suffolk, UK) or rabbit-anti-human SIRT1 antibody (E104, 1:40; Abcam, Cambridge, UK) over night at 4°C. For double staining, 2  $\mu$ g/ml mouse-anti-vimentin, mouse-anti-CD68 antibodies (both from Dako, Glostrup, Denmark) or mouse IgG (Jackson) were added. Sections were incubated with biotinylated goat-anti-rabbit IgG and AP-conjugated goat-anti-mouse antibodies (Jackson), followed by incubation with horseradish peroxidase-conjugated streptavidin complex (ABC Kit; Vector Laboratories, Peterborough, UK). SIRT1-positive cells were visualised using DAB-Nickel (Vector Laboratories). Vimentin or CD68-positive cells were visualised using Vector Red reagent. SIRT1 single stained and IgG control slides were counterstained with eosin.

### Immunoblotting

For protein preparation, tissues and cells were lysed in RIPA buffer, boiled and mixed with Laemmli buffer.<sup>23</sup> Proteins were separated on sodium dodecyl sulphate (SDS)-polyacrylamide gels and transferred to Protran nitrocellulose membranes (Schleicher and Schüll, Dassel, Germany). Membranes were probed with anti-SIRT1

## Extended report

antibodies (E104; Abcam) and anti- $\alpha$ -tubulin (Sigma), respectively, and detected with horseradish peroxidase-conjugated secondary antibodies using the ECL western blotting detection system (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA).

### Fluorescence activated cell sorting

RASFs were transfected with siRNA or vectors as described above for 48 h. Medium was changed and 24 h later, cells were detached with Accutase (PAA Laboratories, Pasching, Austria) and stained for annexin V and propidium iodide (PI) with the annexin V-FLUOS Staining Kit (Roche, Mannheim, Germany). Cells were subsequently analysed on a FACSCalibur flow cytometer. Data were processed using CellQuest software (BD Biosciences, San Jose, California, USA).

### Statistical analysis

Unpaired and paired t tests or one-way analysis of variance followed by Dunnett's post-test were used, where appropriate, for statistical evaluation of the data by GraphPad Prism 5.0 software.

Values are presented as mean (SEM). p values <0.05 were considered as significant.

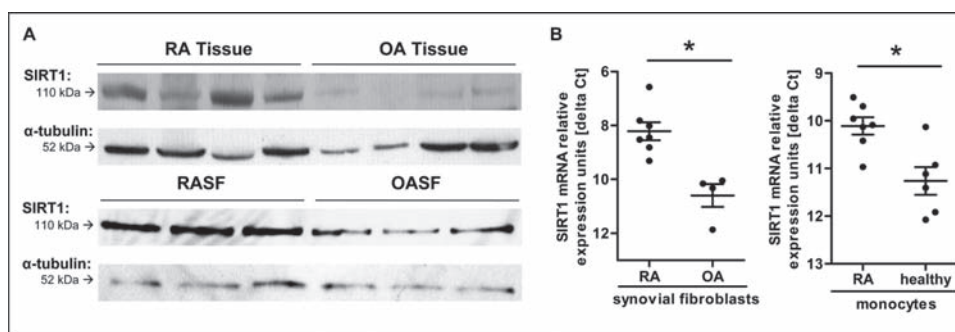
## RESULTS

### Elevated expression of SIRT1 in RA synovial tissues

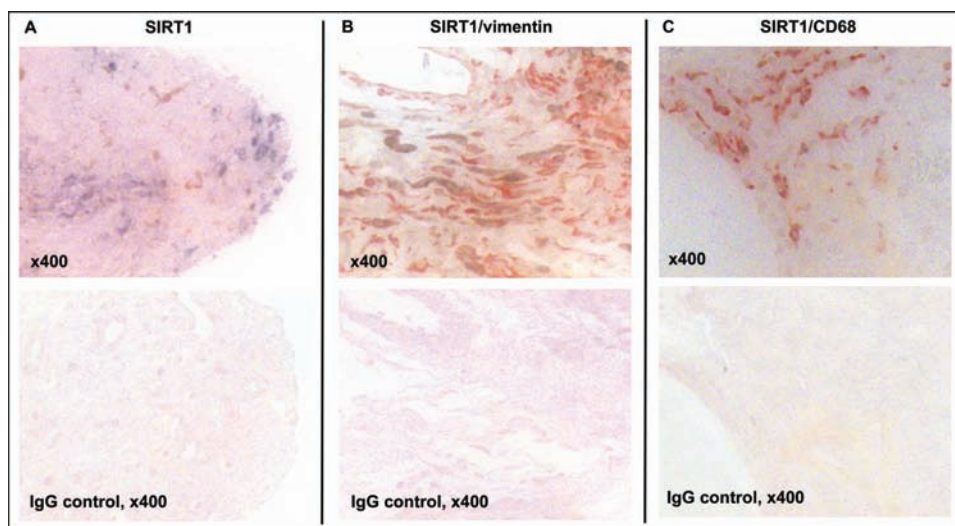
To study the association of SIRT1 expression with chronic joint inflammation in RA, we compared SIRT1 protein levels in RA with non-inflammatory OA synovial tissues. Western blot analysis revealed higher expression of SIRT1 protein in whole synovial tissue and cultured SFs from joints of patients with RA compared with OA (figure 1A). Real-time PCR analysis showed 5.2-fold higher levels of SIRT1 mRNA in SFs from RA compared with patients with OA ( $p<0.01$ ). Additionally, levels of SIRT1 mRNA were 2.2 times higher in peripheral blood monocytes from patients with RA compared with healthy volunteers ( $p<0.01$ ; figure 1B).

### Localisation of SIRT1 expression in RA synovial tissue sections

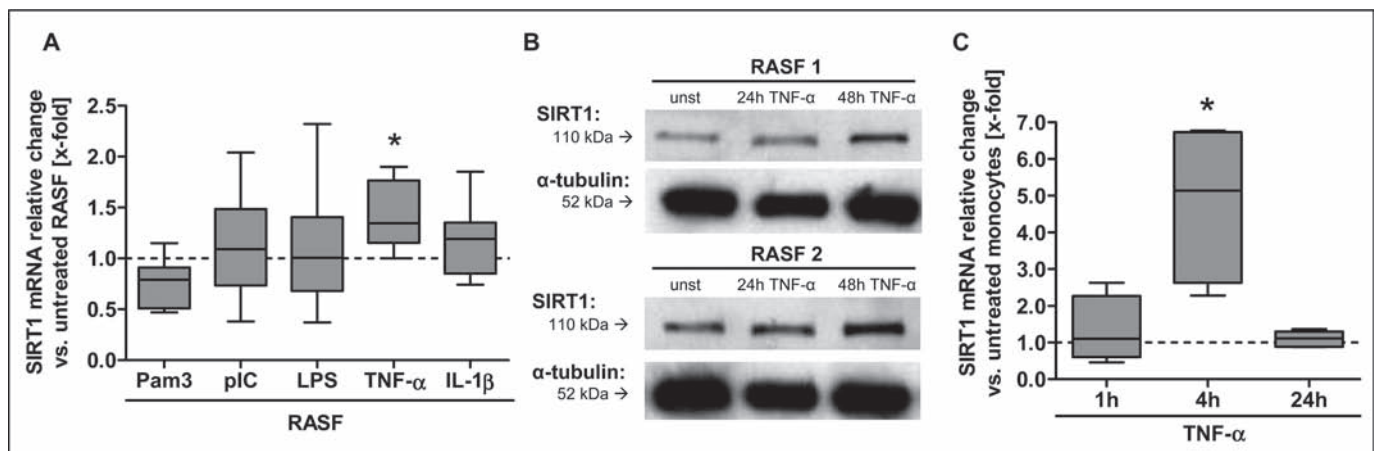
To further analyse the expression of SIRT1 in synovial tissue sections from patients with RA, we performed



**Figure 1** High basal protein and mRNA levels of SIRT1 in tissues, synovial fibroblasts and monocytes from patients with rheumatoid arthritis (RA). (A) Western blot showing the basal expression of SIRT1 protein from patients with RA and osteoarthritis (OA) in synovial tissue samples ( $n=4$ , each) or in synovial fibroblasts (SFs;  $n=3$ , each).  $\alpha$ -Tubulin served as loading control. (B) Relative expression units of SIRT1 mRNA in synovial fibroblasts from patients with RA ( $n=7$ ) or OA ( $n=4$ ) and in monocytes from patients with RA ( $n=7$ ) or healthy controls ( $n=6$ ). Results are presented as the difference in threshold cycle ( $\Delta$  Ct), relative to 18S rRNA. See table 1 for patient characteristics. \* $p<0.01$ , unpaired t test.



**Figure 2** SIRT1 expression in synovial tissues from patients with rheumatoid arthritis (RA). (A) Representative sections of RA synovial tissue specimens stained for SIRT1 or IgG control. Positive staining of SIRT1 appears as dark grey. Sections were counterstained with eosin. (B) Representative sections of RA synovial tissue specimens double-labelled for SIRT1 and vimentin or IgG control. SIRT1 appears as dark grey, and vimentin as red. (C) Representative sections of RA synovial tissue specimens double-labelled for SIRT1 and CD68 or IgG control. SIRT1 appears as dark grey, and CD68 as red. One representative section is shown ( $n=4$ ). Original magnification  $\times 400$ .



**Figure 3** SIRT1 is induced upon stimulation with TNF $\alpha$ . (A) RASFs were treated with different toll-like receptor ligands (300 ng/ml Pam3CSK4 (Pam3), 10  $\mu$ g/ml poly (I:C) (pIC), 10 ng/ml lipopolysaccharide (LPS)) and two major rheumatoid arthritis cytokines (10 ng/ml TNF $\alpha$ , 1 ng/ml interleukin 1 $\beta$  (IL1 $\beta$ )) for 24 h (n=7–9). Changes in SIRT1 mRNA levels relative to untreated RASFs are shown (x-fold). (B) Representative western blots confirming induction of SIRT1 protein upon stimulation of RASFs with TNF $\alpha$  for 48 h (n=3). (C) Monocytes from healthy individuals (n=5–8) were treated with 10 ng/ml TNF $\alpha$  for the indicated time points and SIRT1 mRNA levels relative to untreated cells are shown (x-fold). \*p<0.05, by analysis of variance followed by Dunnett's post-test. TNF $\alpha$ , tumour necrosis factor  $\alpha$ . RASFs, rheumatoid arthritis synovial fibroblasts.

immunohistochemistry. Pronounced expression of nuclear SIRT1 protein was found in the lining and sublining layers, but also in perivascular areas (figure 2A). Double staining with SIRT1 and vimentin or CD68 showed the expression of SIRT1 in vimentin-positive SFs as well as in CD68-positive monocytes/macrophages (figure 2B,C).

#### SIRT1 expression is induced upon stimulation with TNF $\alpha$

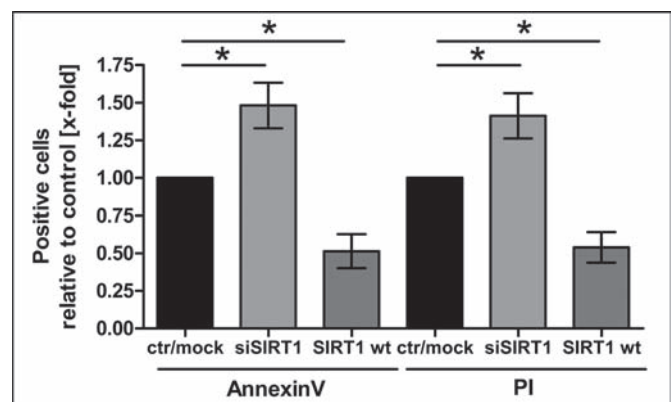
To analyse the influence of activated TLR pathways and IL-1 $\beta$  on SIRT1 expression, RASFs were treated with TLR ligands and IL-1 $\beta$  for 24 h. Real-time PCR analysis showed no changes in the levels of SIRT1 mRNA. However, stimulation of RASFs with TNF $\alpha$  revealed a modest induction of SIRT1 mRNA (figure 3A). Time course analysis showed increased levels of SIRT1 mRNA already after 1 and 4 h upon stimulation with TNF $\alpha$  (online supplementary figure 1). Western blot analysis of TNF $\alpha$ -stimulated RASFs confirmed induction of SIRT1 protein expression after 48 h (figure 3B). Similarly to RASFs, activation of monocytes by TNF $\alpha$  resulted in a 4.8-fold increase of SIRT1 mRNA levels after 4 h of stimulation (p<0.05), whereas at 24 h SIRT1 mRNA levels had returned to the baseline level (figure 3C).

#### SIRT1 mediates apoptosis resistance in RASF

As SIRT1 has been shown to prolong cellular lifespan and as spontaneous apoptosis is known to be reduced in activated RASFs, we assessed the effect of SIRT1 on apoptotic cell death. RASFs were transfected with siRNA and expression vectors as described above. SIRT1-silenced RASFs showed an increase of 48% and 41% in the number of annexin V-positive (p<0.02) and PI-positive (p<0.03) cells, respectively. When RASFs were transfected to overexpress a vector encoding SIRT1, cells were less susceptible to apoptosis, displayed by a decrease in annexin V-positive (49%, p<0.05) and PI-positive (46%, p<0.05) RASFs (figure 4). These results suggest that the constitutive overexpression of SIRT1 in RASFs may lead to a prolonged lifespan of these cells.

#### SIRT1 positively affects production of the proinflammatory mediators IL-6 and IL-8 in RASFs

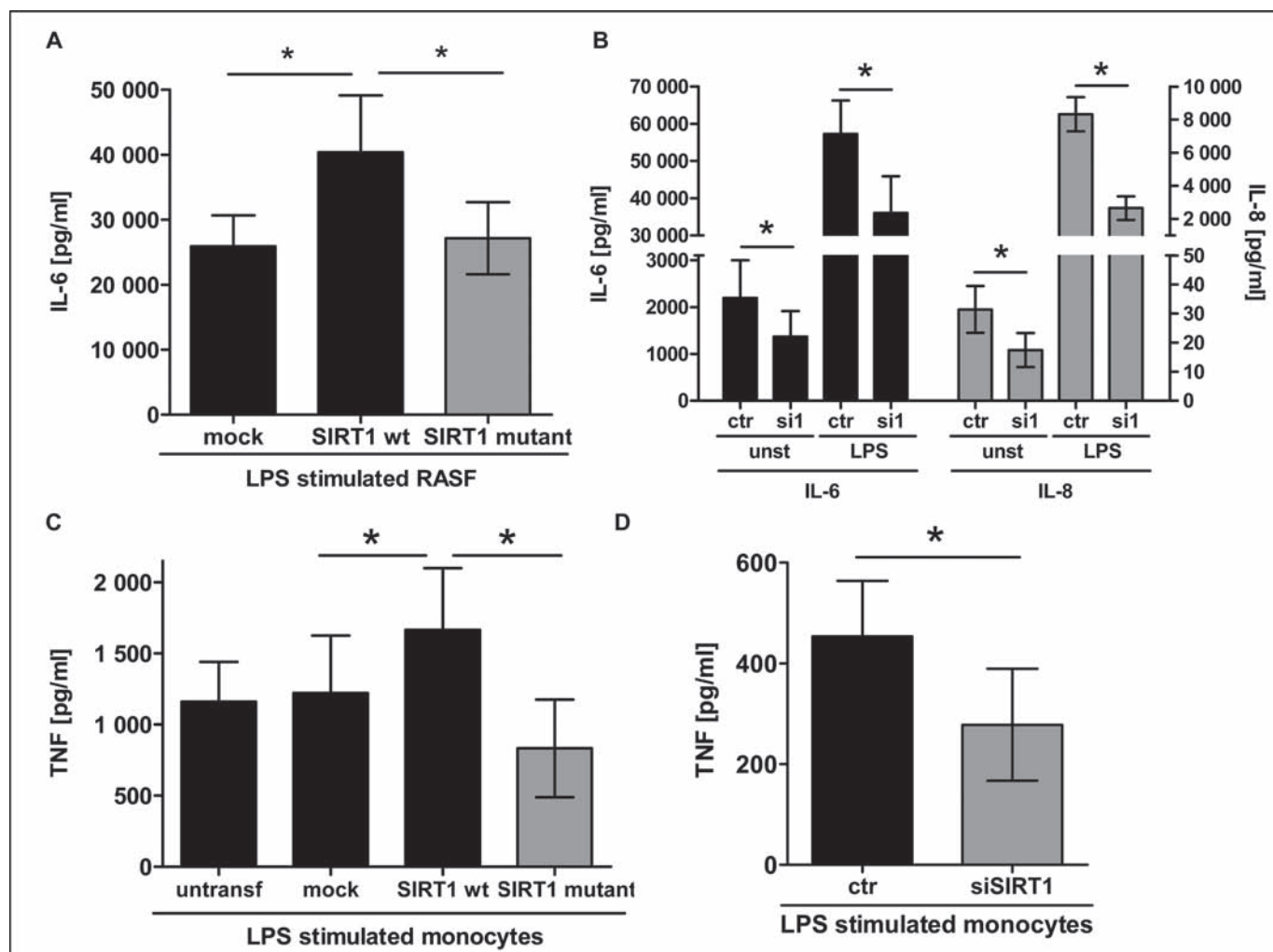
We next assessed whether SIRT1 also has a direct impact on the inflammatory phenotype of RASFs. Therefore, the production



**Figure 4** SIRT1 mediates apoptosis resistance in RASFs. RASFs were transfected with SIRT1-specific siRNA (n=7) or with a vector encoding wild-type SIRT1 (n=3) for 72 h. Flow cytometry was used to measure levels of annexin V and propidium iodide (PI)-positive cells relative to control siRNA (ctr) or mock (empty vector) transfected RASFs. Values are relative changes in the number of annexin V or PI-positive cells compared with control transfected cells (x-fold). \*p<0.05, by paired t test. RASFs, rheumatoid arthritis synovial fibroblasts.

of the proinflammatory cytokine IL-6 was analysed in LPS-stimulated RASFs after transfection with a SIRT1 expression vector. Interestingly, overexpression of SIRT1 increased the production of LPS-induced IL-6 compared with mock transfected RASFs by 56% (p<0.05). This enhancing effect on IL-6 production was not seen when cells were transfected with a mutant form of SIRT1 (H363Y, figure 5A). Similar results were obtained by stimulation with TNF $\alpha$  instead of LPS (online supplementary figure 2). Analysis of SIRT1 mRNA levels revealed successful overexpression in both wild-type and mutant SIRT1-transfected RASFs (p<0.05, online supplementary figure 3A).

Accordingly, when RASFs were transfected with control or SIRT1 specific siRNA for 48 h, there was a significant reduction of both basal and LPS-stimulated expression of IL-6 and IL-8 after knockdown of SIRT1. Basal IL-6 and IL-8 production was reduced by 37 $\pm$ 11% and 47 $\pm$ 21% (p<0.05), respectively, whereas



**Figure 5** Increased levels of proinflammatory cytokines in RASFs and monocytes overexpressing SIRT1. (A) RASFs were transfected with expression vectors encoding wild-type (n=8) and mutant (n=8) forms of SIRT1. Interleukin 6 (IL-6) protein concentrations were measured in culture supernatants at 40 h after transfection. (B) RASFs (n=5) were transfected with control siRNA (ctr) or SIRT1-specific siRNA (si1) for 48 h and stimulated with 10 ng/ml lipopolysaccharide (LPS) for another 24 h. Basal and LPS-induced production of IL-6 and IL-8 was measured in culture supernatants. (C) Primary peripheral monocytes from healthy individuals were transfected to overexpress wild-type or mutant SIRT1 (n=8), and LPS-induced TNF $\alpha$  production in the supernatants was measured. (D) Monocytes (n=6) were transfected with control siRNA (ctr) or with SIRT1 siRNA (siSIRT1). LPS-induced production of TNF $\alpha$  in culture supernatants is shown. Values are means  $\pm$  SEM. \*p<0.05, by paired t test. TNF $\alpha$ , tumour necrosis factor  $\alpha$ . RASFs, rheumatoid arthritis synovial fibroblasts.

LPS induced IL-6 and IL-8 levels were reduced by 40 $\pm$ 15% and 70 $\pm$ 14% (p<0.05), respectively (figure 5B). Also, IL-6 and IL-8 mRNA levels were reduced after SIRT1 knockdown, arguing against purely translational effects of SIRT1 (online supplementary figure 4). Successful knockdown of SIRT1 was confirmed by real-time PCR (online supplementary figure 3B).

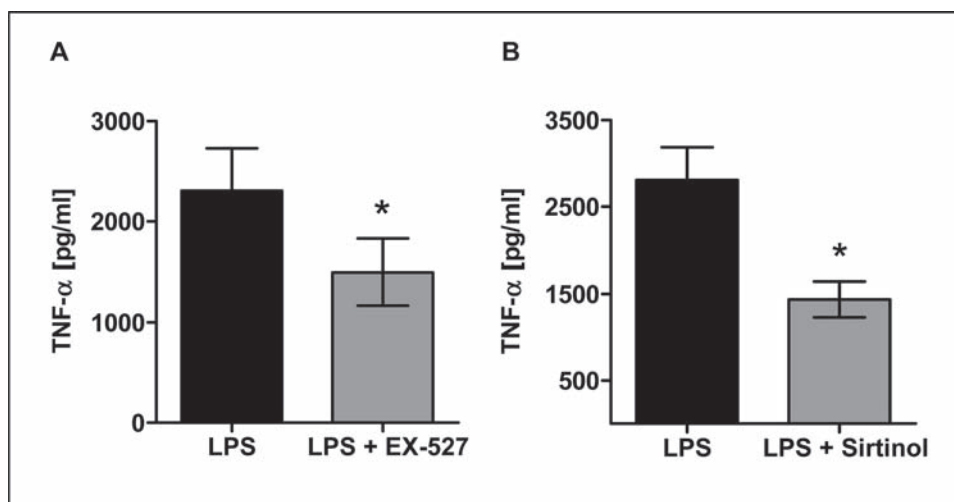
#### SIRT1 increases production of TNF $\alpha$ in monocytes

As monocytes produce only small amounts of IL-6, we measured the production of TNF $\alpha$ , a major cytokine involved in the pathogenesis of RA. Freshly isolated monocytes were subjected to transfection with SIRT1 expression vectors or siRNA as described above and subsequently stimulated with LPS. Consistent with the results obtained in RASFs, monocytes overexpressing wild-type SIRT1 showed an increase of 36% in TNF $\alpha$  production compared with mock transfected cells (p<0.01). This induction in TNF $\alpha$  levels was not seen when monocytes were transfected with the mutant form of SIRT1 (H363Y, figure 5C). Non-stimulated cells did not produce detectable amounts of

TNF $\alpha$ . Further confirmation that SIRT1 signalling regulates the LPS-induced production of TNF $\alpha$  in monocytes was obtained by specific downregulation of SIRT1 using siRNA. Measurement of LPS-induced TNF $\alpha$  in the cell culture supernatants indicated a significant decrease of 31% (p<0.02) (figure 5D). Real-time PCR analysis was used to verify successful transfections (p<0.02, online supplementary figure 3C,D).

#### Enzymatic sirtuin inhibitors decrease TNF $\alpha$ production in monocytes

As we found a prominent difference in the levels of TNF $\alpha$  between wild-type and mutant SIRT1-transfected monocytes, we tested the effects of commercially available SIRT inhibitors on TNF $\alpha$  production. Monocytes were stimulated with LPS in the presence of the SIRT1-specific enzymatic inhibitor EX-527 and levels of TNF $\alpha$  were measured. Consistent with our previous results, EX-527 reduced the expression of TNF $\alpha$  by 37 $\pm$ 17% (p<0.01, figure 6A). Furthermore, we used the pan



**Figure 6** Inhibition of the activity of SIRT1 by enzymatic inhibitors reduces levels of TNF $\alpha$ . (A) Monocytes from healthy individuals (n=9) were stimulated with 10 ng/ml lipopolysaccharide (LPS) for 24 h in presence or absence of the SIRT1-specific inhibitor EX-527 (9  $\mu$ M). Concentrations of TNF $\alpha$  were measured in the culture supernatants. (B) Monocytes (n=7) were stimulated with 10 ng/ml LPS in presence of the pan sirtuin inhibitor Sirtinol (30  $\mu$ M) for 24 h and the levels of TNF $\alpha$  were measured. Results are shown as means  $\pm$  SEM. \*p<0.05, by paired t test. TNF $\alpha$ , tumour necrosis factor  $\alpha$ .

sirtuin inhibitor Sirtinol to block the activity of all the sirtuins. Interestingly, Sirtinol also reduced the LPS-induced TNF $\alpha$  production by 49 $\pm$ 9% (p<0.01), suggesting that the sirtuin family overall has proinflammatory effects in monocytes (figure 6B).

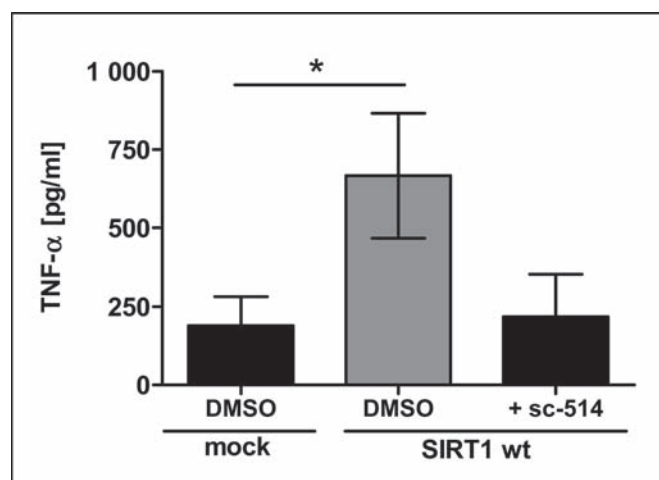
#### The proinflammatory activity of SIRT1 is mediated through NF- $\kappa$ B dependent pathways

NF- $\kappa$ B is known to be essential for cytokine signalling in RA.<sup>24</sup> Additionally, SIRT1 was shown to affect expression of NF- $\kappa$ B-dependent genes, such as p53 and Bcl-2.<sup>14 25</sup> We assessed whether the effects of SIRT1 in monocytes are dependent on NF- $\kappa$ B by using an inhibitor of IKK-2, sc-514. When SIRT1 was overexpressed in monocytes, LPS-induced TNF $\alpha$  levels were increased as shown before. This SIRT1-dependent increase was completely blocked by treatment with the IKK-2 inhibitor sc-514, suggesting that SIRT1 acts via a NF- $\kappa$ B dependent mechanism (figure 7).

#### DISCUSSION

SIRT1, a member of the NAD<sup>+</sup> dependent class III histone deacetylases, deacetylates both histones and non-histone targets.<sup>10</sup> Thereby, SIRT1 controls a broad range of cellular processes, including cell survival and inflammation.<sup>19 26</sup> Interestingly, SIRT1 was shown to be induced by calorie restriction (CR), thereby connecting SIRT1 with the beneficial effects of CR on longevity and age-related disorders such as diabetes.<sup>27</sup> In addition, increased levels of SIRT1 were found in diseases such as neurodegeneration, cancer and experimental autoimmune encephalomyelitis.<sup>28</sup> We have analysed SIRT1 expression and found SIRT1 to be overexpressed in RA synovial tissues as compared with OA. SIRT1 is expressed in SFs and monocytes/macrophages, suggesting that in joints SIRT1 is predominantly expressed in tissue resident cells.

The regulation of SIRT1 expression is incompletely understood. Different effects of cytokines on SIRT1 expression have been described. Both the cytokines IL-1 $\beta$  and interferon  $\gamma$  have been shown to reduce the expression of SIRT1 in rat islets,<sup>29</sup> whereas TNF $\alpha$  induced SIRT1 expression in human vascular



**Figure 7** SIRT1 mediates its proinflammatory effects through NF- $\kappa$ B. Primary human monocytes from healthy volunteers (n=4) were transfected to overexpress wild-type SIRT1 or were mock transfected and stimulated with 10 ng/ml lipopolysaccharide in the presence or absence of an inhibitor of IKK-2, sc-514 (50  $\mu$ M, in dimethyl sulphoxide (DMSO)). The production of TNF $\alpha$  was measured in the culture supernatants. Results are shown as means  $\pm$  SEM. \*p<0.05, by paired t test. NF- $\kappa$ B, nuclear factor  $\kappa$ -light-chain-enhancer of activated B cells; TNF $\alpha$ , tumour necrosis factor  $\alpha$ .

smooth muscle cells.<sup>30</sup> Here we show that the basal overexpression of SIRT1 in RA can be further induced upon stimulation with TNF $\alpha$ , a major cytokine found in joints of patients with RA. However, SIRT1 expression was not influenced by stimulation with TLR ligands and IL-1 $\beta$  in vitro, suggesting that TLR pathways do not directly regulate SIRT1 expression.

SIRT1 has emerged as a key antiageing protein in different experimental models, such as in *Saccharomyces cerevisiae*, in *sirt1*-null mice as well as in various in vitro cell cultures.<sup>11 13 14 31-34</sup> RASFs characteristically show a resistance to apoptosis.<sup>35</sup> We have analysed the effect of SIRT1 on apoptosis in RASFs.

We found that overexpression of SIRT1 protected cells from apoptosis. The antiapoptotic effect of SIRT1 together with its constitutive high expression levels in RA suggest that SIRT1 may contribute to the apoptosis-resistant phenotype seen in RASFs.

Up to now, published data on the effects of SIRT1 on inflammation are controversial. Macrophages from myeloid SIRT1 knockout mice displayed increased NF- $\kappa$ B-mediated inflammation in response to environmental stress, indicating an anti-inflammatory activity of SIRT1.<sup>16</sup> In agreement, a sirtuin activator had an inhibitory effect on LPS-induced inflammation in intraperitoneal murine macrophages.<sup>36</sup> However, the biological effects mediated by SIRT1 seem to differ between cell types. It has been shown that the beneficial effects of CR-induced SIRT1 in white adipose tissue or skeletal muscle cells were not seen in liver tissues.<sup>37</sup>

A previous report using RA synovial biopsy explants showed that treatment with nicotinamide, a non-specific inhibitor of sirtuins, resulted in a reduction of IL-6 and TNF $\alpha$  secretion, suggesting that the sirtuin family may have proinflammatory effects.<sup>17</sup> In line with this finding, it was recently reported that the proinflammatory cytokine HMGB1 stimulated production of TNF $\alpha$  through activation of TLR4 in periodontal cells. This TLR4-dependent enhanced expression of TNF $\alpha$  was blocked when SIRT1 or NF- $\kappa$ B were inhibited.<sup>38</sup> As we have previously shown that TLR activation results in upregulation of NAD<sup>+</sup> producing PBEF/visfatin and as SIRT1 activity is NAD<sup>+</sup> dependent,<sup>4 6 39 40</sup> it is conceivable that TLR4 stimulation of synovial cells during chronic arthritis induces SIRT1 activity and that SIRT1 in a NF- $\kappa$ B-dependent fashion prolongs the lifespan of RASFs and enhances the proinflammatory cytokine production.

Endogenous TLR4 ligands such as heat shock proteins, HMGB1 and others have been described, but it is not known which of these are disease relevant in RA.<sup>41</sup> In our detailed analysis of the effects of SIRT1 on cytokine production in RASFs and monocytes, we found that overexpression of SIRT1 had a modulatory effect on IL-6 and TNF $\alpha$  production in RASFs and monocytes, respectively, resulting in a significant induction of these cytokines in a NF- $\kappa$ B dependent fashion. These enhancing effects on cytokine expression were not seen when cells were transfected with an enzymatically inactive mutant form of SIRT1, which would suggest that higher wild-type SIRT1 expression levels may support the production of proinflammatory cytokines. Interestingly, the pan sirtuin inhibitor Sirtinol also reduced proinflammatory cytokines similar to the SIRT1-specific inhibitor EX-527, suggesting that the overall inhibition of sirtuins may have an anti-inflammatory effect.

In summary, we report the overexpression of SIRT1 in RA synovial tissue and despite evidence for an anti-inflammatory role of SIRT1 in animal models of inflammation we show that SIRT1 directly enhances proinflammatory cytokine production of synovial cells. Cytokine production is further potentiated by an antiapoptotic effect prolonging the lifespan of RASFs. Our results suggest that careful investigation of the cell and disease-specific effects of sirtuins is necessary to delineate possible therapeutic uses of agents targeting these molecules.

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## SIRT1 overexpression in the rheumatoid arthritis synovium contributes to proinflammatory cytokine production and apoptosis resistance

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