Toll-like receptor 4 signalling is specifically TGF-beta-activated kinase 1 independent in synovial fibroblasts

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Abstract

Objective. Activated synovial fibroblasts are key players in the pathogenesis of RA by driving inflammation and joint destruction. Numerous molecules including cytokines and Toll-like receptor (TLR) ligands induce pro-inflammatory signalling and gene expression through a hierarchical network of kinases. Upstream mitogen-activated protein kinase kinase kinases (MAP3Ks) represent an attractive target for RA treatment. In this study, we sought to determine the role of the MAP3K TGF-β-activated kinase 1 (TAK1) in cytokine and TLR-mediated signalling.

Methods. TAK1 activity was inhibited using either a small molecule inhibitor or lentivirally overexpressed kinase-inactive TAK1-K63W mutant in murine embryonic and human dermal and synovial fibroblasts. Fibroblasts were stimulated with IL-1, TNF, TLR2 or TLR4 agonists and responses were evaluated using transcriptional reporters, western blotting and analysis of gene expression of collagens (MMP3 and MMP13), cytokines (IL-1β and IL-6) and chemokines (IL-8 and MCP-1).

Results. TAK1 inhibition abrogated cytokine- and TLR-induced nuclear factor-κB (NF-κB) and Saa3-promoter reporter activation in murine and human dermal fibroblasts. In synovial fibroblasts, TAK1 regulated IL-1 and TNF-mediated NF-κB, but not Saa3-promoter reporter activation. Inducible mRNA expression of cytokines, collagenases and chemokines, except MCP-1, was TAK1 dependent for IL-1, TNF and TLR2 signalling. Unexpectedly, TLR4-mediated NF-κB reporter activation and inducible mRNA expression was fully TAK1 independent. Accordingly, NF-κB p65 and p38 MAPK phosphorylation was unaffected by TAK1 inhibition.

Conclusion. In general, TAK1 crucially regulates IL-1 and TNF signalling in fibroblasts. Interestingly, TLR4 signalling is specifically TAK1 independent in synovial fibroblasts. Consequently, therapeutic TAK1 inhibition in arthropathies may not dampen the damage-associated molecular pattern-mediated TLR4 activation of synovial fibroblasts.

Key words: TGF-β-activated kinase 1, Signal transduction, Synovial fibroblast, Transcriptional reporter, Toll-like receptor, Nuclear factor-κB, Rheumatoid arthritis.

Introduction

RA is a systemic chronic autoimmune disease that mainly affects the synovial joints that ultimately leads to joint destruction. Before and during joint inflammation, the synovial lining tissue, consisting of synovial fibroblasts and macrophages, becomes activated and hyperplastic resulting in invasion and degradation of adjacent cartilage and bone. In both experimental and human arthritis, synovial fibroblasts have been
identified as cells that actively drive inflammation and joint destruction [1, 2]. Moreover, transmigration of activated RA synovial fibroblasts has been implicated in mediating the spreading of destructive arthritis to unaffected joints [3]. Due to their key role in RA pathogenesis, synovial fibroblasts are major target cells for treatment of disease.

Synovial fibroblasts are potently activated by cytokines such as IL-1 and TNF [4–6], and Toll-like receptor (TLR) ligands [7–9]. TLR2 and TLR4 are predominantly expressed in synovial fibroblasts and their expression is increased in RA patients [8, 10]. Although TLRs are primarily activated by exogenous pathogens, they also recognize endogenous damage-associated molecular patterns (DAMPs) that are abundantly present in arthritic joints [7, 11]. In experimental arthritis, it has been demonstrated that TLR4 activation promotes the onset and severity of disease [12]. Moreover, DAMP-mediated activation of TLR4 specifically in synovium has been found to be crucially involved in joint destruction [13].

After ligation of their respective receptors, expression and secretion of pro-inflammatory mediators including cytokines, chemokines and MMPs are induced through multiple signalling cascades including nuclear factor-κB (NF-κB) and the mitogen-activated protein kinase (MAPK) families p38, c-Jun-N-terminal kinase (JNK) and extracellular-signal-regulated kinase (ERK) (reviewed in [14]). These kinases are regulated through phosphorylation by their upstream kinases, IkB kinase (IKK) and MAPK kinase (MAPKK), respectively. In turn, MAPKK kinases (MAP3Ks) control the activation of IKK and MAPKK and are activated through interactions with receptor-associated proteins, such as IL-1R-associated kinases (IRAKs) and TNF Receptor-associated factors (TRAFs). The MAP3K family comprises numerous members of which MAP-ERK kinase (MEK) kinase-1, -2 (MEKK1 and MEKK2) and TGF-β-activated kinase 1 (TAK1) are most abundantly expressed in RA synovial fibroblasts [15].

TAK1 has been identified as the key regulator of IL-1, TNF and TLR-induced activation of NF-κB and MAPK pathways in mice [16–18]. Inhibition of TAK1 catalytic activity prevents chemical-induced inflammation in mice [19]. However, studies using cell-type-specific TAK1-deficient mice have also revealed that TAK1 is a crucial regulator of homeostasis in cartilage, skin, epithelium and liver [20–23]. Therefore, insight into the cell-specific regulation of signalling pathways by TAK1 is indispensable for developing a treatment based on its inhibition.

Until now, it has been shown that TAK1 regulates the IL-1-induced JNK pathway and activator protein-1 (AP-1) transcription factor in synovial fibroblasts [24]. Here, we investigated the role of TAK1 in TNF-, TLR2- and TLR4-mediated signal transduction and induction of pro-inflammatory gene expression in murine embryonic and primary human dermal and synovial fibroblasts. The present study reveals that TLR4 signalling is specifically TAK1 independent in synovial fibroblasts.

**Materials and methods**

**Patients and samples**

Synovial tissue was obtained from RA (n = 4) and OA (n = 2) patients undergoing open joint replacement surgery or arthroscopic synovectomy at the Clinic of Orthopedics, Waldkrankenhaus Rudolf Elle, Eisenberg, Germany (kindly provided by R.W. Kinne, University of Jena, Jena, Germany). Informed patient consent was obtained and the study was approved by the Ethics Committee of University Hospital Jena, Jena, Germany. RA and OA patients were classified according to the ACR criteria.

Clinical characteristics of patients are described in Huber et al. [25]. Synovial fibroblasts were purified from synovial tissue as previously published [26]. Briefly, the tissue samples were minced, digested with trypsin/collagenase p (Sigma, St Louis, MO, USA), and the resulting single-cell suspension was cultured for 7 days. Non-adherent cells were removed by medium exchange. Fibroblasts were obtained by negative isolation using Dynabeads M-450 CD14 Magnetic Activated Cell Sorting purification. Human dermal fibroblasts were obtained from skin biopsies of healthy volunteers at the Radboud University Nijmegen Medical Centre (kind gift from J. Schalkwijk, Department of Dermatology, Nijmegen, The Netherlands).

**Cell culture**

Mouse embryonic fibroblasts (NIH-3T3) were maintained in DMEM supplemented with 1 mM pyruvate, 40 μg/ml gentamicin and 5 or 10% fetal calf serum (FCS), respectively. Stable transcriptional reporter cell-line NIH-3T3-5xNF-κB-luciferase was described previously [27]. Human dermal and synovial fibroblasts were cultivated in DMEM supplemented with 1 mM pyruvate, 80 μg/ml gentamicin and 10% FCS. Cells were kept at 37°C in a humid atmosphere containing 5% CO₂.

**Plasmids**

For generation of recombinant lentiviral vectors, we used the third-generation self-inactivating transfer vector pRLL-cPPT-PGK-mcs-PRE-SIN (PGK empty) containing the human phosphoglycerate kinase (PGK) promoter (kind gift from J. Seppen, AMC Liver Center, Amsterdam, The Netherlands). For cloning, we used cloned *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA) and T4 DNA ligase (New England Biolabs, Ipswich, MA, USA) and T4 DNA ligase (New England Biolabs, Ipswich, MA, USA). All generated constructs were verified by sequencing. A lentiviral transcriptional luciferase reporter containing four tandemly arranged NF-κB binding sites (pTRH2-NF-κB-Luc) was purchased from System Biosciences (Mountain View, CA, USA). Construction of the Saa3-promoter luciferase reporter is described previously [27]. The cDNA sequences of a non- and enhanced Green Fluorescent Protein (EGFP)-tagged kinase-inactive mutant of TAK1 (K63W) were PCR cloned from pEGFP-C1-TAK1-K63W (kind gift from M. Kracht, Rudolf-Buchheim-Institute for Pharmacology, Giessen, Germany) into *Nhel/Nsil* sites of PGK empty using the
following primers: RV 5’-ATGCATTGATGAAGTGACTG-3’, FW 5’-GCTAGGCCGACATGCGAAGCCGTCGGC-3’ (non-tagged, Kozak sequence for enhanced translation introduced) and FW 5’-GCTGGTTTATGAGACGCTAGC-3’ (EGFP tagged).

**Lentiviral vector production**

Packaging of vesicular stomatitis virus G (VSV-G) pseudotyped recombinant lentiviruses was performed by transient transfection of 293T cells. One day before transfection, 293T cells were seeded in a T75 flask at 1 x 10^6 cells/cm² in DMEM supplemented with 10% FCS, 1 mM pyruvate, 40 μg/ml gentamicin and 0.01 mM water-soluble cholesterol (Sigma). Cells were co-transfected with 19 μg of transfer vector, 14 μg of gag/pol packaging plasmid (pMDL-g/p-RRE), 4.7 μg of rev expression plasmid (RSV-REV) and 6.7 μg of VSV-G expression plasmid (pHIT-G) by calcium phosphate precipitation. Transfections were performed in 6 ml of DMEM without antibiotics and cholesterol and proceeded for 16 h. Thereafter, medium was replaced with fully supplemented DMEM and supernatant harvested after 24 and 48 h. Cell debris was removed by centrifugation at 300g for 5 min at 4°C, followed by passage through a 0.45 μm pore polyvinylidene fluoride Durapore filter (Millipore, Bedford, MA, USA). For concentration by ultracentrifugation, 28 ml of supernatant was overlaid on 4 ml of 20% sucrose solution and centrifuged at 40000g for 4 h in a SureSpin 630 rotor (Thermo Fisher Scientific, Waltham, MA, USA). Pelleted viruses were resuspended in sterile PBS and stored at −80°C. Viral titres were determined by assaying p24 values with a commercial ELISA kit (Abbott Diagnostics, Hoofddorp, The Netherlands) and expressed as ng p24/mL.

**Luciferase measurements**

For in vitro reporter studies, cells were seeded at 5 x 10^4 cells per well in a Krystal 2000 96-wells plate (Thermo Labystems, Brussels, Belgium) and transduced for 4 h with 25 ng of p24 equivalents lentivirus in 50 μl of medium supplemented with 8 μg/ml polybrene (Sigma). Transduction efficiency was 80–100% and comparable between donors (data not shown). Consecutively, cells were serum starved (1% FCS) for 2 days, pre-incubated for 1 h with indicated concentrations of TAK1 inhibitor (11,12-dihydro-5Z-7-oxozeaenol; AnalytiCon Discovery, Potsdam, Germany) or dimethyl sulphoxide (DMSO) as vehicle control and stimulated with recombinant murine or human IL-1β (1 ng/ml; R&D Systems Europe, Oxford, UK), TNF-α (10 ng/ml; Abbcam, Cambridge, UK), Escherichia coli lipopolysaccharide (LPS) (1 μg/ml; Sigma), ParnCys (1 μg/ml; EMC Microcollections, Tübingen, Germany) for indicated hours. Cells were lysed in ice-cold lysis buffer [0.5% nonidet-P 40 (NP-40), 1 mM 1,4-dithiothreitol (DTT), 1 mM EDTA, 5 mM MgCl₂, 100 mM KCl, 10 mM Tris–HCl pH 7.5, 1 × protease inhibitor cocktail (Roche, Mannheim, Germany)]. Luciferase activity was quantified using the Bright-Glo luciferase assay system (Promega, Madison, WI, USA) by adding an equal volume of Bright-Glo to the cell lysate. Luminescence was quantified in a luminometer (Lumistar; BMG, Offenburg, Germany), expressed as relative light units (RLU) and normalized to total protein content of the cell extracts.

**Western blot analysis**

Synovial fibroblasts were seeded at 1 x 10^6 cells per well in 6-well plates and serum starved (1% FCS) for 48 h. Cells were pre-incubated for 1 h with 500 nM TAK1 inhibitor or DMSO, followed by stimulation with human IL-1β (1 ng/ml) or LPS (1 μg/ml) for 5 and 30 min, as indicated. Lysis was performed in 10 mM Tris–HCl (pH 7.05), 50 mM NaCl, 30 mM sodium pyrophosphate, 1% Triton X-100, 2 mM Na₃VO₄, 50 mM NaF, 20 mM β-glycerophosphate, 1 μg/ml pepstatin, 10 μg/ml leupeptin, 1 mM phenylmethanesulphonylfluoride and 1 μM microcystin. Cell lysates were subjected to SDS–PAGE on 8% gels and western blotting was performed as described [28]. Proteins of interest were detected by antibodies against phospho(S536)-p65 (Cell Signalling, Danvers, MA, USA), p65 (C-20; Santa Cruz, CA, USA), phospho (TY180/2)-p38 (Invitrogen, Carlsbad, CA, USA) and p38 [29].

**RNA isolation**

Cells were seeded at 90% confluency in 24-well plates and serum starved (1% FCS) for 48 h. Consecutively, cells were pre-incubated with TAK1 inhibitor or DMSO for 1 h and stimulated as mentioned above. Thereafter, cells were washed in ice-cold PBS and total RNA was extracted using TRI reagent (Sigma). Isolated RNA samples were treated with RNase-free DNase I (Qiagen, Venlo, The Netherlands) for 15 min. Synthesis of cDNA was accomplished by reverse transcription PCR using an oligo(dT) primer and Moloney murine leukaemia virus RT (Invitrogen).

**Quantitative PCR**

Quantitative polymerase chain reaction (QPCR) was performed using SYBR Green PCR Master mix and the ABI 7000 Prism Sequence Detection system (Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer’s instructions. Primers were designed over exon–exon junctions in Primer Express (Applied Biosystems Inc.) and used at 300 nM in the PCR reaction. PCR conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Gene expression (cycle threshold, Ct) values were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene (ΔCt = Ct gene − Ct GAPDH). Primers sequences are listed in supplementary data, available at *Rheumatology* Online.

**Statistics**

Data are represented as means (s.e.m.) and significant differences were calculated using a one-way analysis of variance (ANOVA) or repeated measures ANOVA followed by Bonferroni’s multiple comparison test (GraphPad Prism, San Diego, CA, USA), where appropriate. P-values of <0.05 were considered statistically significant.
Fig. 1 TAK1-dependent NF-κB activation in murine fibroblasts. TAK1 inhibition by an inhibitor and LV-TAK1-K63W (a). NIH-3T3-5xNF-κB-luciferase cells were either transduced with indicated p24<sup>eq</sup> equivalent of LV-K63W (△) and control lentivirus (PGK empty) or pre-treated with indicated concentrations of inhibitor (▲) and vehicle. Two days post-transduction or 1 h after pre-treatment, respectively, cells were stimulated with IL-1β (1 ng/ml) for 6 h. (b) NIH-3T3-5xNF-κB-luciferase fibroblasts were pre-treated with 500 nM inhibitor or vehicle and stimulated for 6 h with murine IL-1β (1 ng/ml), TNF-α (10 ng/ml), LPS (TLR4, 1 μg/ml) and Pam3Cys (TLR2, 1 μg/ml). (a and b) NF-κB activation was measured by luciferase assay and data are expressed as the percentage of activity compared with controls [mean (s.e.m.), n = 3–5]. (c) Induction of Saa3 mRNA expression by IL-1β, TNF-α, LPS (TLR4) and Pam3Cys (TLR2) in NIH-3T3 stably transduced with LV-TAK1-K63W or PGK empty. Data are represented as fold induction (mean ± S.E.M., n = 3) over untreated cells. *P < 0.05; ***P < 0.001 by ANOVA.

Results

TAK1 regulates cytokine- and TLR-induced NF-κB activation in murine fibroblasts

TAK1 has been identified as the crucial mediator of IL-1- and TNF-induced activation of NF-κB, JNK and p38 MAPK signal transduction pathways in NIH-3T3 fibroblasts [29]. Using a 3T3 NF-κB reporter cell line, we investigated TAK1-inhibitory efficiency of a small molecule inhibitor [19] and lentivirally overexpressed kinase-inactive TAK1-K63W mutant (LV-K63W) (Fig. 1a). Maximal inhibition of IL-1β-induced NF-κB activity was achieved at 500 nM inhibitor [66.2 (8.6%)] or 150 ng p24<sup>eq</sup> equivalent of LV-K63W per 5 × 10<sup>4</sup> cells [65.4 (6.2%)], respectively. There were no significant differences in maximal inhibition between inhibitor and LV-K63W, and aforementioned concentrations were applied throughout the following experiments. Next, we analysed TAK1-mediated regulation of TLR-induced signal transduction (Fig. 1b). Treatment of fibroblasts with inhibitor resulted in a significant reduction in NF-κB activation (~60%, P < 0.05) induced by TLR2 (Pam3Cys) and TLR4 (LPS) agonists. As a control, IL-1- and TNF-induced NF-κB activation were also potently suppressed by inhibitor. Finally, we evaluated the effect of TAK1 inhibition on the inducible expression of serum amyloid A3 (Saa3), which has been identified as a strictly TAK1-dependent TNF target gene [29] (Fig. 1c). Stable transduction with LV-K63W also completely prevented induction of Saa3 expression by IL-1β and LPS. These results confirm the crucial role of TAK1 in mediating cytokine- and TLR-mediated signal transduction in murine fibroblasts.

TAK1-dependent signalling in primary human dermal fibroblasts

Since activated fibroblasts play an important role in the pathogenesis of chronic inflammatory diseases [1, 30], inhibition of inflammatory signal transduction represents a straightforward treatment strategy. Therefore, we assessed the contribution of TAK1 to pro-inflammatory signal transduction in primary human dermal and synovial fibroblasts. Overexpression of TAK1-K63W in dermal fibroblasts completely abrogated IL-1-induced NF-κB activation (Fig. 2a). Also NF-κB activation through TNF [66.2 (2.5%)] and TLR4 [68.5 (4.6%)]] was strongly reduced. Since SAA3 is a pseudogene in humans and TAK1 directly regulates the transcriptional activity of the Saa3 promoter [29], we additionally used a Saa3-promoter luciferase reporter for studying the effect of TAK1 inhibition in human fibroblasts (Fig. 2b). TAK1 crucially regulated IL-1β-, TNF- and TLR4-mediated activation of Saa3-promoter reporter as revealed by ~80% reduction of luciferase activity. Reduction of NF-κB and Saa3-promoter activation through TAK1 inhibition correlated with suppression of IL-1β, IL-6 and IL-8 gene expression (Fig. 2c). These data identify TAK1 as a pivotal regulator of pro-inflammatory signalling in dermal fibroblasts.

Cytokine- and TLR-mediated induction of transcriptional reporters and pro-inflammatory genes in synovial fibroblasts

In order to identify TAK1-dependent signalling pathways in synovial fibroblasts, we first validated whether cytokines and TLR ligands induced NF-κB and Saa3-promoter transcriptional reporters (Fig. 3a and b) and pro-inflammatory gene expression (Fig. 3c–f). We found strong up-regulation of both reporter activities upon IL-1, TNF and TLR4 stimulation, whereas TLR2 stimulation only led to marginal induction. Accordingly, expression of NF-κB target genes IL-1β, IL-6 and IL-8 was strongly up-regulated upon cytokine and TLR stimulation. Additionally, we detected significantly induced expression of AP-1 (MMP3/13) [31] and INF regulatory factor 3 (IRF3) (MCP-1) [32] transcription factor target genes.
TLR4-mediated signalling is TAK1 independent in synovial fibroblasts

Next, we repeated experiments as described above in the presence of TAK1 inhibitor. Consistent with our previous results in murine and human dermal fibroblasts, IL-1β [47.9 (7.9%)] and TNF-mediated [48.4 (6.2%)] NF-κB activation was significantly reduced through TAK1 inhibition (Fig. 4a). In contrast, induction of Saa3-promoter activation was unaffected by treatment with TAK1 inhibitor (Fig. 4b). Surprisingly, both NF-κB and Saa3-promoter activation through TLR4 triggering demonstrated TAK1
independence, as indicated by a complete lack of inhibition of transcriptional reporters upon inhibitor treatment. These results prompted us to investigate phosphorylation of components of the NF-κB and MAPK pathway by western blotting (Fig. 4c and d). Stimulation of synovial fibroblasts with IL-1 and LPS clearly induced phosphorylation of p38 MAPK and the p65 subunit of NF-κB. In the presence of TAK1 inhibitor, IL-1β-induced phosphorylation of both substrates was considerably reduced. Corroborating the data above, TAK1 inhibition failed to affect phosphorylation induced by LPS. To conclude, we analysed the effect of TAK1 inhibition on the induction of pro-inflammatory genes by cytokines (Fig. 5a and b) and TLR agonists (Fig. 5c and d). Indeed, induction of NF-κB target genes IL-1β, IL-6 and IL-8 through IL-1 and TNF was significantly reduced by TAK1 inhibitor treatment. Corresponding with the established regulation of the JNK-AP-1 pathway by TAK1 [24], IL-1- and TNF-induced MMP3 expression was also clearly reduced. The IRF3-target gene MCP-1 demonstrated TAK1 independence for all applied stimuli. Whereas TAK1 inhibition resulted in suppression of TLR2-induced IL-1β, IL-6 and MMP3 expression, TLR4-induced gene expression was completely unaffected by inhibitor treatment. Together these data reveal TAK1-independent TLR4 signaling that is specific for synovial fibroblasts.

**Discussion**

Given the multitude of activating molecules and the redundancy and complexity of MAPK signalling [33, 34], upstream MAP3Ks are of particular interest as therapeutic targets as they potentially couple multiple receptors to downstream signalling pathways. In this study, we investigated the role of the MAP3K TAK1 in mediating pro-inflammatory signalling in several types of fibroblast and revealed specific TAK1-independent TLR4 signalling in human synovial fibroblasts.

Although there is substantial evidence that IL-1 signals through both TAK1 and MEKK3 [35, 36], information on a physiological role of these two kinases in synovial fibroblasts is limited. In two studies, analysis of MAP3K expression at the mRNA and protein level in synovial fibroblasts revealed abundant expression of TAK1 and trace amounts of MEKK3 [15]. Knockdown of MEKK3 did not affect IL-1β-induced MAPK activation and TAK1 was identified as the crucial mediator in JNK, but not ERK or p38 MAPK pathways. siRNA-mediated suppression of...
TAK1 did not significantly inhibit NF-κB nuclear translocation and DNA binding, but suppressed IL-6, a typical NF-κB target gene [24]. Hence, our results using transcriptional reporter systems confirm that a significant portion of NF-κB activation through IL-1R and TNFR is mediated by TAK1.

Unexpectedly, we discovered that TAK1 plays no role in TLR4-induced NF-κB activation and pro-inflammatory gene expression in synovial fibroblasts. Several studies have emphasized a central role of TAK1 in LPS/TLR4-mediated NF-κB activation in murine macrophages, embryonic fibroblasts, B cells and human HEK293 cells [16, 17, 37, 38]. In addition, we have revealed that TAK1 regulates NF-κB in murine NIH-3T3 fibroblasts and primary human dermal fibroblasts. TLR4 activates signal transduction through TRIF- (TIR-domain-containing adapter-inducing INF-β) and MyD88-dependent pathways. Upon ligand binding MyD88 is recruited, which subsequently leads to the recruitment and phosphorylation of IRAK proteins, which then interact with TRAF6. The activated complex activates downstream IKK and MAPKs through an interaction with TAK1. The TRIF-dependent pathway activates the IRF3 pathway through TANK-binding kinase 1 (TBK1). NF-κB and MAPK pathways are activated through interaction with receptor interacting protein-1 (RIP1), which consequentially interacts with TRAF6 and TAK1 [39].

Maximal induction of inflammatory cytokines such as TNF-α and IL-6 is dependent on the activation of both TRIF and MyD88 pathways [40]. Based on these studies, we expect TAK1-dependent regulation of MyD88- and TRIF-dependent NF-κB and MAPK activation, but not the IRF3 pathway. The latter was confirmed by our RT-PCR analysis results, which indicated indeed that regulation of the IRF3 target gene MCP-1 [32] was not inhibited by treatment of synovial fibroblasts with TAK1 inhibitor. Inhibition of IL-1β-induced NF-κB activation by inhibitor treatment suggested a MyD88-TAK1-IKK pathway in synovial fibroblasts. Based on the latter, and our observations that TAK1 does not mediate TLR4 signal transduction in synovial fibroblasts, at least two mechanisms might underlie these remarkable results.

First, the TLR4-MyD88-IKK/MAPK pathway can be regulated by a MAP3K other than TAK1. Recent investigations have unravelled a TAK1-independent and MEKK3-dependent mechanism for TLR8-mediated IKK and JNK activation [41], and a similar pathway was discovered for IL-1-mediated NF-κB activation [42]. As in our study, NF-κB activity is not completely inhibited by the TAK1 mutant or the TAK1 inhibitor, and knowledge of regulation of MEKK3 in synovial fibroblasts is very limited. Therefore, MEKK3 is a likely candidate that accounts for TAK1-independent signalling in synovial fibroblasts. Thus,

**Fig. 5** Effects of TAK1 inhibition on induction of pro-inflammatory gene expression in synovial fibroblasts. Fibroblasts were serum starved for 2 days, pre-treated with 500 nM inhibitor or vehicle for 1 h, and left untreated or stimulated with human IL-1β (1 ng/ml, a), TNF-α (10 ng/ml, b), LPS (TLR4, 1 μg/ml, c) or Pam3Cys (TLR2, 1 μg/ml, d) for 6 h. Expression of indicated genes was measured using RT-PCR. Data are represented as fold reduction (2^-ΔΔCt) compared with DMSO-treated cells (mean ± S.E.M., six donors). Statistical differences were determined using repeated measures ANOVA. *P < 0.05; **P < 0.01.
a divergence of IL-1R/TLR MyD88-dependent pathways into IL-1R-TAK1 and TLR4-MEKK3 routes could be a possibility.

Second, TLR4 signalling through the MyD88-dependent pathway might be less dominant or even ablated in synovial fibroblasts. A possible mechanism for such a phenomenon has been described for LPS-tolerance induction in human monocytes and dendritic cells [43, 44]. Upon restimulation with a TLR4 ligand, tolerized cells show decreased TLR4–MyD88 complex formation, which results in impaired IRAK-1 phosphorylation [45]. Moreover, it has been shown that IRAK-M is up-regulated in RA synovial fibroblasts, which acts as a negative regulator of the MyD88-dependent pathway, and is associated with tolerance induction [46, 47]. If this were to take place in synovial fibroblasts, we would expect predominant signal transduction through TRIF. However, in the case of predominant TRIF signalling we would still expect activation of the TRIF-TRAF6-TAK1 pathway, unless TAK1 can be bypassed for NF-κB and MAPK signalling in this route.

Circumventing TAK1 in TLR4-mediated NF-κB activation can be achieved by TANK, which was shown to slightly induce NF-κB transcription after overexpression [48]. Another pathway without involvement of TAK1-mediated NF-κB activation has been discovered in IL-1 signalling [49]. In this pathway, TRAF6 is associated with p62, which activates atypical protein kinase C and this subsequently leads to NF-κB activation.

Not only TLR4 signalling, but also cytokine-induced Saa3-promoter activation was specifically TAK1 independent in synovial fibroblasts. This promoter is synergistically activated through cooperation of NF-κB and CAAT/enhancer-binding protein (C/EBP) transcription factors [50, 51]. Since IL-1/INF-induced NF-κB activation proved to be TAK1 dependent, the lack of Saa3-promoter activation upon TAK1 inhibition could point towards differential regulation of C/EBP transcription factors in synovial vs dermal fibroblasts.

In conclusion, using a previously characterized dominant-negative mutant or small molecule inhibitor of TAK1, we have found a more restricted role for this MAP3K in mediating pro-inflammatory signalling in synovial fibroblasts. This phenomenon proved independent of disease as it was observed in both RA and OA patients. In the light of the recently established role of TLR4 in experimental arthritis [12, 13, 52], insight into TLR4 signalling in synovial fibroblasts is of particular interest for understanding pathogenesis and treatment. The underlying mechanism for TAK1-independent regulation of TLR4 signalling, potentially TLR4 tolerance or involvement of an alternative MAP3K, remains to be addressed in future research.

**Rheumatology key messages**

- TLR4 signalling is specifically TAK1 independent in synovial fibroblasts.
- TAK1 regulates IL-1 and TNF-induced NF-κB activation in synovial fibroblasts.

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**Supplementary data**

Supplementary data are available at *Rheumatology* Online.

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TAK1-independent TLR4 signalling in synovial fibroblasts