Tofacitinib regulates synovial inflammation in psoriatic arthritis, inhibiting STAT activation and induction of negative feedback inhibitors

W Gao, T McGarry, C Orr, J McCormick, D J Veale, U Fearon

ABSTRACT

Background Psoriatic arthritis (PsA) is a chronic inflammatory disease, characterised by synovitis and destruction of articular cartilage/bone. Janus-kinase and signal transducer and activator of transcription (JAK-STAT) signalling pathway is implicated in the pathogenesis of PsA.

Objectives To examine the effect of tofacitinib (JAK inhibitor) on proinflammatory mechanisms in PsA.

Methods Primary PsA synovial fibroblasts (PsAFLS) and ex vivo PsA synovial explants were cultured with tofacitinib (1 μM). PhosphoSTAT1 (pSTAT1), phosphoSTAT3 (pSTAT3), IL12p40, interleukin (IL)-6 and IL-17, 3 5 several of which signal through the Janus-Kinase (JAK) family of receptor-associated tyrosine kinases. Activated JAKs recruit and activate signal transducer and activator of transcription (STATs), which in turn drive gene transcription. The specific JAK-STAT activated depends on the cytokine signal which includes the interferons (IFNs) and IFN-related cytokines, the common γ-chain cytokines, and the IL-6-type cytokines. Several studies have demonstrated a key role for JAK-STAT signalling in the pathogenesis of rheumatoid arthritis (RA). 9 10 Previous studies have shown increased pSTAT1 and pSTAT3 expression in PsA synovium or fibroblasts. Tofacitinib is a drug of the JAK inhibitor class and is currently approved for the treatment of RA in 27 countries. A recent study in patients with RA demonstrated that tofacitinib downregulated inflammatory pathways in RA synovial tissue; 11 however, a direct effect of tofacitinib on PsA synovial inflammation has not been shown to date.

METHODS

Patient demographics, arthroscopy and culture of synovial fibroblasts

See online supplementary file 1.

INTRODUCTION

Psoriatic arthritis (PsA) is a chronic inflammatory arthritis associated with psoriasis (Ps) and characterised by synovitis and progressive destruction of articular cartilage and bone. One of the earliest events is new vessel formation and invasion resulting in a self-perpetuating and persistent infiltration of leucocytes, transforming the synovium into an aggressive tumour-like ‘pannus’. Previous studies have demonstrated distinct macroscopic vascularity in the PsA joint characterised by elongated, tortuous vessels which is associated with increased expression of cytokines, angiogenic growth factors and decreased cell apoptosis. This facilitates PsA synovial fibroblasts (FLS) to invade adjacent cartilage and bone resulting in joint destruction.

PsA synovium is the target of interplay for many proinflammatory cytokines and growth factors, with key regulating mediators controlling cell traffic from the blood stream into the underlying tissue. Recently developed agents for PsA target IL12p40, interleukin (IL)-6 and IL-17, 3 5 several of which signal through the Janus-Kinase (JAK) family of receptor-associated tyrosine kinases. Activated JAKs recruit and activate signal transducer and activator of transcription (STATs), which in turn drive gene transcription. The specific JAK-STAT activated depends on the cytokine signal which includes the interferons (IFNs) and IFN-related cytokines, the common γ-chain cytokines, and the IL-6-type cytokines. Several studies have demonstrated a key role for JAK-STAT signalling in the pathogenesis of rheumatoid arthritis (RA). 9 10 Previous studies have shown increased pSTAT1 and pSTAT3 expression in PsA synovium or fibroblasts. Tofacitinib is a drug of the JAK inhibitor class and is currently approved for the treatment of RA in 27 countries. A recent study in patients with RA demonstrated that tofacitinib downregulated inflammatory pathways in RA synovial tissue; 11 however, a direct effect of tofacitinib on PsA synovial inflammation has not been shown to date.
Explants were cultured with 1 μM tofacitinib or dimethyl sulfoxide (DMSO) vehicle control for 72 h. Following culture, biopsy wet-weights were obtained and supernatants analysed for cytokines. Tissue morphology and cell viability of PsA explants following culture are described in online supplementary file 1.

Western blot analysis
Protein isolation from Psoriatic Arthritis synovial fibroblasts (PsAFLS) and synovial explants is described in online supplementary file 1. Protein (20–50 μg) was resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (5% stacking, 10% resolving), gels were then transferred onto nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK) prior to 1 h blocking in wash buffer containing 5% non-fat milk. Membranes were incubated with rabbit polyclonal anti-pSTAT3 (Cell-Signaling Technology, UK), total-signal transducer and activator of transcription (tSTAT)3, pSTAT1, tSTAT1, pSTAT2, suppressor of cytokine signaling-3 (SOCS3), protein inhibitor of activated Stat3 (PIAS3; Cell Signaling Technology) and nuclear factor kappa B cells (NFκBp65) (Millipore, California, USA) diluted in 5% non-fat milk containing 0.1% Tween 20 at 4°C overnight. β-Actin (Sigma-Aldrich) was used as a loading control. Membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 3 h at RT. Signal was detected using SuperSignal West-Pico Chemiluminescent Substrate (Amersham Biosciences, UK) and quantified using EDAS-120 system (Kodak, Rochester, New York, USA).

PsAFLS invasion, migration, cytokine/matrix metallopeptidase quantification and statistics
See online supplementary file 1.

RESULTS
Tofacitinib regulates STAT signalling in primary PsAFLS
Initial experiments demonstrated increased expression of pSTAT1 and pSTAT3 in PsA synovium when compared with osteoarthritis (OA) (figure 1A). Tofacitinib inhibited pSTAT3 and pSTAT1 expression in PsAFLS when compared with vehicle control (figure 1B, D). In parallel, tofacitinib induced negative inhibitors of STATs, SOCS3 and PIAS3 compared with vehicle control (figure 1C, D).

Tofacitinib inhibited PsAFLS invasion and migration
Figure 2A shows representative images, demonstrating the inhibitory effect of tofacitinib on PsAFLS migration, in comparison with DMSO control where repopulation of wound margins was observed. Representative images of decreased PsAFLS invasion following culture with tofacitinib are shown in figure 2B, an effect that was significant compared with vehicle control (p<0.05; figure 2C). Furthermore, tofacitinib significantly inhibited PsAFLS invasion formation (figure 2D, E; p<0.05).

Tofacitinib regulates STAT signalling in whole tissue PsA synovial explant cultures
To further examine the effect of tofacitinib, PsA synovial explants were cultured with tofacitinib for 72 h. Figure 3A(i–ii) demonstrates intact tissue morphology and cell–cell contact following culture, with lining-layer, blood vessels and synovial infiltrate clearly visible. Figure 3Ciii demonstrates calcein uptake into the nucleus of the cells following culture indicating viability of cells. Also evident are tortuous elongated dilated vessels which are a hallmark of PsA synovium (figure 3Aiv), with a mean (±SEM) vascularity of 75±5.3. Tofacitinib inhibited pSTAT1 and pSTAT3, with no effect on tSTAT1 or tSTAT3

Figure 1 Tofacitinib regulates Janus-kinase/signal transducer and activator of transcription (JAK/STAT) pathways components in PsAFLS. (A) Representative western blot showing phosphoSTAT3 (pSTAT3)/phosphoSTAT1 (pSTAT1) and tSTAT3/tSTAT1 protein in psoriatic arthritis (PsA) vs osteoarthritis (OA) synovial biopsy lysates. (B) Representative western blot showing pSTAT3/pSTAT1 and tSTAT3/tSTAT1 protein in dimethyl sulfoxide (DMSO) control vs tofacitinib (TOFA) (1 μM) treated PsA synovial fibroblasts (PsAFLS) for 24 h. Densitometry quantification of pSTAT3/pSTAT1 in PsAFLS (n=7). Data were normalised to tSTAT1/tSTAT3. (C) Representative western blot showing the expression of Protein inhibitor of activated Stat3 (PIAS3) and suppressor of cytokine signaling-3 (SOCS3) protein in DMSO control vs TOFA (1 μM) treated PsAFLS for 24 h. Densitometry quantification of PIAS3 and SOCS3 in PsAFLS (n=7). Data are expressed as mean±SEM. *p<0.05 vs DMSO control; β-actin was used as loading control.
compared with DMSO control (figure 3B, D), pSTAT2 was undetectable. In contrast, tofacitinib induced expression of SOCS3 and PIAS3 (figure 3C, D).

**Inhibition of proinflammatory mediators from PsA explant cultures**

Tofacitinib significantly inhibited spontaneous secretion of IL-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1) from PsA explant cultures (*p<0.05; figure 3E) with no effect observed for IFN-gamma-inducible protein 10 (IP-10) or IL-10 (figure 3E). IL-17 levels were undetectable. Furthermore, tofacitinib inhibited NFκBp65 in PsA explants and PsAFLS, a key transcriptional factor in the inflamed joint (figure 3E). Tofacitinib significantly inhibited matrix metallopeptidase (MMP)3 expression from PsA synovial explants (p<0.05; figure 3F) and inhibited the MMP3/TIMP3 ratio (figure 3F). Finally, tofacitinib inhibited MMP2 and MMP9 activity as observed by gelatine zymography (figure 3F).

**DISCUSSION**

To date, no study has examined JAK-STAT or the effect of tofacitinib in primary cells isolated from PsA synovial biopsies. In this study, tofacitinib significantly decreased pSTAT1, pSTAT3 in PsAFLS and PsA synovial explant cultures ex vivo. In parallel, tofacitinib increased SOCS3 and PIAS3 expression demonstrating negative feedback inhibition. Functionally, tofacitinib significantly decreased PsAFLS invasion, migration and network formation. Finally, tofacitinib significantly decreased spontaneous secretion of key proinflammatory cytokines, the MMP/TIMP ratio and NFκBp65 expression. Thus tofacitinib inhibits proinflammatory and invasive mechanisms which are critically involved in the pathogenesis of PsA.

This is the first study to demonstrate regulation of pSTAT3 and pSTAT1 in primary cells isolated from PsA synovial tissue. Only one previous study in PsA has examined STAT signalling, and this was in synovial fluid (SF) T cells where increased expression of JAK1, pSTAT3 and pSTAT1 was demonstrated compared with peripheral blood (PB) of healthy controls (HC), suggesting activation of JAK-STAT signalling at the site of inflammation.12 PsA studies have shown that increased pSTAT expression is associated with epidermal hyper-proliferation, expression of which was modulated by IFNγ, IL-6 and IL-22.13–16 This is consistent with RA studies, showing that pSTAT3 is associated with synovial inflammation, lining-layer hyperplasia and synovial pO2 levels,13–14 and that STAT3 modulates Th17 differentiation in RA SF and induces RAFLS survival.13

In this study, tofacitinib inhibited pSTAT1, pSTAT3 and NFκBp65 in PsAFLS and PsA explants, an effect that was paralleled by induction of both SOCS3 and PIAS3. SOCS inhibits cytokine signalling by acting as kinase inhibitors to JAKs or competitive binding for docking sites with STAT. PIAS3 binds to Gao W, et al. Ann Rheum Dis 2016;75:311–315. doi:10.1136/annrheumdis-2014-207201
the STAT3 DNA-binding domains and thus prevents physical binding of STAT to target genes thus inhibiting transcriptional activity. In parallel, tofacitinib inhibited PsAFLS invasion, migration and network formation, all associated with progressive and destructive joint disease. While the precise mechanisms by which tofacitinib inhibits invasion/migration is unclear, it may involve (i) inhibition of key cytokines such as IL-6 which results in negative feedback inhibition, (ii) blockade of RhoGTPases (cdc42, Rac1 and RhoA) or growth factors such as platelet-derived growth factor (PDGF), which are critical for cell movement, migration and invasion or (iii) through the observed effects of STAT signaling on NF\(\kappa\)B which is known in other cell types to mediate proliferation and invasive mechanisms via phosphoinositide 3-kinase (PI3K)/serine/threonine kinase or protein kinase B (AKT) signalling pathway.15

Tofacitinib significantly inhibited IL-6, IL-8, MCP-1, MMP3 and MMP2/9 spontaneous secretion from PsA explants, with no significant effect on IP-10 or IL-10. The PsA biopsies used to establish the explant model were obtained from the site of inflammation under direct visualisation and closely reflect the patients’ inflammatory activity in vivo. Previous studies have shown that tofacitinib inhibits tumour necrosis factor (TNF)-\(\alpha\) and IL-6-induced osteoclastogenesis and bone destruction,16 an effect mediated by receptor activator of nuclear factor kappa-B ligand (RANKL).17 Tofacitinib decreases the T-cell stimulatory capability of dendritic cells through suppression of type-I-IFN signalling.18 Furthermore, tofacitinib reduces MMP and IFN-regulated gene expression in RA synovium, with clinical improvement correlating with reductions in pSTAT1 and pSTAT3.8 While JAK1-mediated IFN and IL-6 signalling likely play a key role in the synovial response, more recent data have shown that CP690550 inhibits TNF-induced expression of IP10, RANTES and MCP1 in RAFLS,11 inhibits IL-4-dependent Th2 cell differentiation and Th17 cell differentiation and

**Figure 3** Janus-kinase/signal transducer and activator of transcription (JAK/STAT) pathway components were modulated by tofacitinib in synovial tissues. (A) Representative images of psoriatic arthritis (PsA) explants following cultures. Histological images of explant tissue following culture at low (i) and high power (ii) showing intact tissue morphology and cell–cell contact with lining layer (black arrows), blood vessels (red arrows) and synovial infiltrate clearly visible. (iii) Calcein uptake into the nucleus of the cells in PsA whole mounts following culture indicating viability of cells, also evident are the tortuous elongated dilated vessels which are a hallmark of PsA synovium (iv). Representative western blot showing pSTAT3/pSTAT1 and tSTAT3/tSTAT1 protein in dimethyl sulfoxide (DMSO) control vs TOFA (1 \(\mu\)M) treated PsA synovial explants for 72 h (n=7; B). Representative western blot showing the expression of PIAS3 and suppressor of cytokine signaling-3 (SOCS3) protein DMSO control vs TOFA (1 \(\mu\)M) treated PsA synovial explants for 72 h (n=7; C). Densitometry quantification of phosphoSTAT3 (pSTAT3)/phosphoSTAT1 (pSTAT1) and protein inhibitor of activated Stat3 (PIAS3)/SOCS3 (D). Data were normalised to tSTAT3 or \(\beta\)-actin. Data are expressed as the mean±SEM of replicate experiments (n=7). *\(p<0.05\) vs DMSO vehicle control; \(\beta\)-actin was used as loading control. TOFA (1 \(\mu\)M) inhibited spontaneous secretion of interleukin (IL)-6, IL-8, MCP-1, with no effect on IP-10 or IL-10 (E; n=7–11). Representative western blot showing inhibition of NF\(\kappa\)Bp65 in PsA synovial explants and PsA synovial fibroblasts (PsAFLS) following culture with TOFA (1 \(\mu\)M) compared with DMSO control (1 \(\mu\)M; E). \(\beta\)-actin was used as loading control. TOFA (1 \(\mu\)M) inhibited spontaneous secretion of MMP3, TIMP3 from PsA synovial explants and inhibited the MMP3/TIMP3 (F) ratio (n=6). DMSO was used as vehicle control. Representative in-gel zymography of MMP9/2 activities in PsA synovial explant cultured with TOFA (1 \(\mu\)M) for 72 h (F). DMSO was used as vehicle control (n=7). Data are expressed as mean±SEM. *\(p<0.05\) vs DMSO control.
decreases cartilage destruction through suppression of IL-17 and IFNγ-producing CD4+ T cells.19

In conclusion, this is the first study to demonstrate the effect of tofacitinib in PsAFLS and PsA explant cultures. Tofacitinib differentially regulated JAK-STAT signalling, inhibiting pSTAT1, pSTAT3 and inducing SOCS3/PIAS3 in vitro and ex vivo parallel by inhibition of invasive mechanisms. These data further support a role for blockade of JAK-STAT signalling pathways in the treatment strategy for PsA.

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REFERENCES


Supplementary File 1

Methods

Patient Recruitment, demographics and arthroscopy

PsA patients with active arthritis were recruited from the Rheumatology Department, St. Vincent’s University Hospital. All subjects gave fully informed written consent as approved by the institutional ethics committee and all research was performed in accordance with the Declaration of Helsinki. Eleven patients with PsA were recruited, 7 female and 4 male with median age (range) 49 years (35-60). These patients had a disease activity score (DAS28) of 3.8±0.39, macroscopic synovitis of 74±5.3 and macroscopic vascularity of 75±5.3. Thirty six % sixty were naïve to treatment, 63.6% on synthetic disease modifying anti-rheumatic drug methotrexate and 0.9% on methotrexate+biologic. No significant difference in DAS28 was observed between patients on no treatment compared to DMARD or biologic. Synovial tissue biopsies were obtained at arthroscopy under local anaesthetic using a Wolf 2.7 mm telescope (Storz, Tuttlingen, Germany) as previously described.[1] Biopsies were also obtained from OA patients as a disease comparator. Biopsies were either OCT embedded (TissueTek, Zoeterwoude, The Netherlands) for immunohistochemical analysis, protein lysates, established as ex-vivo PsA whole tissue synovial explant cultures or primary PsA synovial fibroblasts.

Tissue Morphology and Cell viability of PsA synovial biopsies following culture.

To examine cell viability and morphology of PsA synovial tissue explants following culture ex vivo, explants were stained with live viability marker calcein. Following PsA explant culture in 96 well plates, media was removed and explants washed with PBS and incubated for a further 15 mins in calcein (1:1000 in PBS). Explants were then removed from culture, and whole mounted on a glass slide and fluorescent viability marker assessed using a fluorescent microscopy. Further biopsies were snap frozen in OCT embedding media and
stored at 80°C for H&E staining. 7µm OCT sections were cut with a cryostat, placed on glass slides coated with 2% 3aminopropyltriethoxysilane (Sigma-Aldrich Ireland Ltd, Dublin, Ireland) and dried overnight at room temperature. Tissue sections were allowed to reach room temperature, fixed in acetone for 10 mins and air-dried. Sections were incubated with haematoxylin for 2 mins, washed, dehydrated through alcohol and xylene and mounted. Section morphology was examined by light microscopy.

**Culture of PsA Synovial fibroblasts.**

Primary Psoriatic Arthritis synovial fibroblasts (PsAFLS) were isolated from PsA biopsies by digestion with 1 mg/ml collagenase type 1 (Worthington biochemical, Freehold, NJ, USA) in RPMI 1640 (Life Technologies, BRL, Paisley, UK) for 4 hours at 37°C in humidified air with 5% CO2. Dissociated cells were plated and cultured in RPMI 1640 (Life Technologies, BRL) supplemented with 10% FCS (Life Technologies BRL), penicillin (100 units/ml; Biosciences, Dublin, Ireland), streptomycin (100 units/ml; Biosciences), fungizone (0.25 µg/ml; Biosciences) and HEPES (20 mM; Life Technologies BRL). PsAFLS were grown to confluence and used between passages 38. Cells were cultured in the presence of tofacitinib (1µM) or DMSO vehicle control. Protein lysates were prepared and supernatants collected for cytokine analysis.

**Protein preparation**

PsA synovial tissue biopsies were powdered using a mikrodismembrator U (B. Braun Biotech International, Melsungen, Germany) as previously described.[12] PsAFLS were trypsinized and collected prior to cell lysis. Icecold RIPA (Radio-Immunoprecipitation Assay) buffer (SigmaAldrich) containing 10µg/ml phosphatase inhibitor cocktail and 10 µg/ml protease inhibitor cocktail (Sigma Aldrich) was used to extract protein from powdered biopsies and PsAFLS pellets. Measurement of protein concentration was performed using a BCA assay.
Transwell Invasion assay

Matrigel Invasion Chambers (Becton Dickinson, UK) were used to assess PsAFLS cell invasion in the presence tofacitinib (0.5\(\mu\)M-1\(\mu\)M) or DMSO (1\(\mu\)M) vehicle control for 24 hours. Cells were seeded at a density of 4\(\times\)10\(^4\) per well in the invasion chamber on 8 \(\mu\)m membranes pre-coated with matrigel. EGM containing tofacitinib or DMSO control was placed in the lower well of the chamber. Cells were allowed to invade for 24 hours in EBM medium containing 1% FCS. Non-invading cells were removed from the upper surface by gentle scrubbing. Invaded cells adherent to the lower surface were fixed in 1% gluteraldehyde (VWR, Dublin, Ireland) and stained using 1% crystal violet solution (Prolab, Merseyside, UK). The quantification of invading cells was assessed by counting five random high powered fields (Magnification 40x).

Matrigel network formation

Matrigel (50\(\mu\)l) (Becton Dickenson) basement membrane matrix was plated in 96well culture plates and allowed to polymerize at 37°C in 5% CO2 humidified for 30 minutes.[15] PsAFLS were removed from culture, trypsinized, and resuspended at 2\(\times\)10\(^4\) cells/ml in RPMI 1640. Cells were cultured with tofacitinib (1\(\mu\)M) or vehicle control for 16 hours. Network formation was examined using phase contrast microscopy and photographed (Mag 20x).

Cell migration

PsAFLS were seeded into 48well plates and serum starved upon confluence in FCS free RPMI 1640 for overnight. A single scratch wound was induced across the middle of each well with a sterile pipette tip. Cells were subsequently cultured in the presence or absence of tofacitinib (1 \(\mu\)M) or vehicle control for 16 hours. Cell migration across the wound margins was assessed using phase contrast microscopy and photographed.
Cytokine Quantification  Cytokines IL-6, IL-8, IP-10, MCP-1, IL-17, IL-10, MMP3 and TIMP3 were quantified by ELISA (R&D Systems, Abingdon, UK) or MSD multiplex (Mesoscale, Rockville, Maryland, USA) according to manufacturer’s instructions. Absorbance was measured in a microtiter plate spectrophotometer (Dynatech MR4000, Alexandria, VA) or using MSD Sector Imager 2400.

Gelatin Zymography
The activities of MMP2/9 secreted in cultured supernatants were measured in supernatants by in-gel zymography. Briefly, 1015 µl of supernatants were added into 7.5% polyacrylamide gels consisted of 1 mg/ml gelatin, following incubation with substrate buffer (50 mM Tris, 5 mM CaCl2, pH 7.5), gels were stained with Coomassie brilliant blue R 250 and destained with distilled water.

Statistical Analysis
SPSS15 system for Windows was used for statistical analysis. Nonparametric Wilcoxon Singed Rank test for analysis of PsAFLS and PsA synovial tissue was performed. p<0.05 were considered statistically significant.