The Janus kinase inhibitor tofacitinib inhibits TNF-α-induced gliostatin expression in rheumatoid fibroblast-like synoviocytes

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Abstract

Objective

Gliostatin (GLS) is known to have angiogenic and arthritogenic activity, and GLS expression levels in serum from patients with rheumatoid arthritis (RA) are significantly correlated with the disease activity. Tofacitinib is a novel oral Janus kinase (JAK) inhibitor and is effective in treating RA. However, the mechanism of action of tofacitinib in fibroblast-like synoviocytes (FLSs) has not been elucidated. The purpose of this study was to investigate the modulatory effects of tofacitinib on serum GLS levels in patients with RA and GLS production in FLSs derived from patients with RA.

Methods

Six patients with RA who had failed therapy with at least one TNF inhibitor and were receiving tofacitinib therapy were included in the study. Serum samples were collected to measure CRP, MMP-3 and GLS expression. FLSs derived from patients with RA were cultured and stimulated by TNFα with or without tofacitinib. GLS expression levels were determined using reverse transcription-polymerase chain reaction (RT-PCR), EIA and immunocytochemistry, and signal transducer and activator of transcription (STAT) protein phosphorylation levels were determined by western blotting.

Results

Treatment with tofacitinib decreased serum GLS levels in all patients. GLS mRNA and protein expression levels were significantly increased by treatment with TNF-α alone, and these increases were suppressed by treatment with tofacitinib, which also inhibited TNF-α-induced STAT1 phosphorylation.

Conclusion

JAK/STAT activation plays a pivotal role in TNF-α-mediated GLS up-regulation in RA. Suppression of GLS expression in FLSs has been suggested to be one of the mechanisms through which tofacitinib exerts its anti-inflammatory effects.

Key words

fibroblast-like synoviocytes, Janus kinase, gliostatin, rheumatoid arthritis, STAT, tofacitinib

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterised by persistent synovitis and systemic inflammation leading to joint destruction (1). Fibroblast-like synoviocytes (FLSs) are a key component of this invasive synovitis and play a major role in initiating and perpetuating destructive joint inflammation. The pathologic effects of FLSs in patients with RA are dependent on their enhanced ability to secrete a variety of cytokines, chemokines, and proangiogenic factors compared with FLSs in healthy individuals (2). Cytokine-mediated pathways are central to the pathogenesis of RA, and the successful inhibition of cytokines, especially interleukin (IL)-6 and tumour necrosis factor (TNF)-α, plays a significant role in the treatment of RA (3). One inflammatory cascade that plays a key in role in RA development and progression is characterised by TNF-α overproduction and overexpression (4). In RA FLSs, TNF-α induces the expression of IL-8, matrix metalloproteinase (MMP)-3 (5), and proinflammatory cytokines, such as IL-6 and IL-1β (6). Macrophages are associated with transient inflammatory responses, whereas TNF-α-stimulated FLSs are associated with prolonged inflammatory responses (7). Thus, blocking TNF-α signalling in RA FLSs is regarded as an important approach to reducing damage in tissues affected by RA.

Gliostatin (GLS) is identical to platelet-derived endothelial cell growth factor (PD-ECGF) and has thymidine phosphorylase (TYMP) activity (8). TYMP expression is up-regulated in a variety of solid tumors, a phenomenon that has been shown to be positively correlated with a poor prognosis (9, 10). TAS-102, a combination of the thymidine-based nucleoside analog trifluridine and the thymidine phosphorylase inhibitor tipiracil, significantly improved overall survival and progression-free survival in metastatic colorectal cancer (11). GLS concentrations in the synovial fluid and sera of patients with RA were higher than those of patients with osteoarthritis or normal control subjects (12). We previously reported that serum GLS levels are decreased in responsive patients with RA who were treated with conventional synthetic DMARDs (csDMARDs) (13) and surgeries, such as synovectomy and total joint arthroplasty (14). Therefore, suppressing GLS activity may also be effective approach to treating RA.

Tofacitinib is a novel oral Janus kinase (JAK) inhibitor developed to function as a targeted immunomodulator that serves as a disease-modifying therapy for RA (15). A recent clinical trial demonstrated that tofacitinib is effective in treating RA and that its administration results in rapid and significant attenuation of RA signs and symptoms (16, 17). Tofacitinib has also been shown to be effective in patients who displayed inadequate responses to TNF inhibitors in a phase III trial (18). Since tofacitinib was initially thought to target JAK3, which is expressed mainly in immune cells, such as T cells (19), the ability of tofacitinib to inhibit immune cell activity *in vivo* and *ex vivo* is well characterised (15, 20). It is now clear that this compound inhibits mainly JAK3, as well as JAK1 and JAK2 (21). In a recent study, tofacitinib was shown to inhibit the expression of several lymphocyte-attracting chemokines secreted by TNF-stimulated RA FLSs (22). In the present study, we evaluated the modulatory effects of tofacitinib on serum GLS levels in patients with RA and GLS production in FLSs derived from patients with RA.

Materials and methods

Reagents

Recombinant human TNF-α was purchased from R&D Systems (Minneapolis, MN, USA) and was dissolved in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin. The JAK inhibitor tofacitinib was obtained from Selleck Chemicals (Houston, TX, USA) and was dissolved in dimethyl sulfoxide (DMSO) and stored at -80°C. The final concentration of DMSO was below 0.003% in culture conditions. Anti-vimentin antibody (Ab) was purchased from Cell Signaling Technology (Danvers, MA, USA), anti-CD14 Ab was purchased from Abcam (Cambridge, UK), anti-human von Willebrand factor Ab was purchased

from Dako (Glostrup, Denmark), and anti-TYMP/GLS Ab was purchased from Acris Antibodies (San Diego, CA, USA). Phospho-specific and pan antibodies against signal transducer and activator of transcription (STAT)-1 (Tyr701), STAT-3 (Tyr705), and STAT-5 (Tyr694) were purchased from Cell Signaling Technology, and anti-actin Ab was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Patients

Six patients with RA who met the 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) criteria for the diagnosis of RA (23) were recruited for this study. The clinical characteristics of these patients are shown in Table I. All the patients enrolled in the study had failed therapy with csDMARDs and at least one or more TNF inhibitors and had received tofacitinib therapy (10 mg/day) for more than 12 weeks. Serum samples from all the patients were collected at baseline and after 4, 8 and 12 weeks of treatment and were stored at -80°C until assayed. Synovial specimens were obtained from 13 patients with RA at the time of total joint replacement or synovectomy. The clinical characteristics of these patients are shown in Table II. This study was approved by the Nagoya City University Ethics Committee, and informed consent was obtained from all the patients upon their enrolment in the study.

Table I. Clinical characteristics of the patients receiving tofacitinib therapy at baseline.

Case	1	2	3	4	5	6
Gender	female	male	female	female	female	female
Age, years	53	61	65	69	85	46
Disease duration, years	42	21	5	18	12	15
CRP, mg/dl	8.37	7.73	4.69	4.04	1.62	0.7
ESR, mm/hour	53	87	24	55	44	22
MMP-3, ng/ml	359.6	407.1	489.5	448.9	285.3	183.3
ACPA, U/ml	negative	N.T.	30.4	N.T.	N.T.	negative
RF, U/ml	negative	471	265	154	30	negative
DAS28-4 [ESR]	3.69	5.23	3.58	4.52	4.37	4.35
Steinbrocker stage/class	IV/3	IV/2	IV/2	IV/2	IV/3	I/1
MTX dose (mg/week)	10	8	12	12	4	12
Prior TNF inhibitor	CZP	IFX	ETN	ETN	CZP	ETN

CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; ACPA: anti-cyclic citrullinated protein; RF: rheumatoid factor; N.T.: not tested; MTX: methotrexate; CZP: certolizumab pegol; IFX: infliximab; ETN: etanercept.

Table II. Characteristics of the patients with RA who donated synovial specimens for this study.

	Synovial tissues
Gender (female/male)	13 (12/1)
Age, mean (range), years	66.8 (52-82)
Disease duration, mean (range), years	19.0 (1.9-55.2)
CRP, mean (range), mg/dl	1.70 (0.03-7.86)
ESR, mean (range), mm/hour	28.2 (4-62)
MMP-3, mean (range), ng/ml	256.7 (87.8-706.6)
Seropositive/seronegative	12/1
ACPA positive/negative/not tested	4/2/7
RF positive/negative	12/1
ACPA negative, RF negative	1
Steinbrocker stage, II/III/IV	1/6/6
Patients using DMARDs	
Methotrexate	9
Bucillamine	2
Salazosulfapyridine	1
Mizoribine	2
Tacrolimus	2
TNF inhibitor	4
Patients using oral steroids	6

CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; ACPA: anti-cyclic citrullinated protein; RF: rheumatoid factor; DMARDs: disease-modifying anti-rheumatic drugs.

Preparation of FLSs

Human FLSs were obtained from the superficial layer of synovial tissues with synovitis and minced synovial tissue specimens, according to our standard protocol (13, 24). The FLSs were maintained in Dulbecco's modified Eagle medium (DMEM; Wako Pure Chemical Industries, Osaka, Japan) supplemented with penicillin (100 units per ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD, USA) at 37°C in a humidified atmosphere with 5% CO_2. Cells from passages 3 through 8 were used in the experiments. The cell population was homogeneous and displayed typical FLS morphology (24). The cultures were completely free of lymphocytes, monocytes and endothelial cells, as determined by immunostaining studies utilising anti-vimentin, anti-CD14, and anti-human von Willebrand factor Abs. The cultures displayed strong vimentin immunoreactivity but negative CD14 and von Willebrand factor immunoreactivity (data not shown).

Cell viability assay

FLSs (4×10^4/well) were incubated with tofacitinib and TNF-α in 96-well plates in 100 μl of medium for 72 hours, after which they were treated with 10 μl of WST-8 (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan). The mixture was then incubated for 4 hours at 37°C, after which the absorbance was measured at 450 nm.

Clinical assessments

Serum C-reactive protein (CRP) and MMP-3 levels were assessed. Serum GLS levels were measured by an enzyme immunoassay (EIA) system, as previously described (25). Overall disease activity was assessed in 28 joints in all the patients with RA with the disease activity score, which was calculated based on the erythrocyte sedimentation rate (DAS28-4 [ESR]) (26), before treatment and after 4, 8 and 12 weeks of treatment. Clinical responsiveness

was determined according to the criteria of the European League of Association Against Rheumatism (EULAR) in this study (27).

Reverse transcription-polymerase chain reaction (RT-PCR)

GLS gene expression was assessed by RT-PCR. Total RNA was extracted from the FLSs using an RNeasy® mini kit (Qiagen, Hilden, Germany), and cDNA was prepared with PrimeScript™ RT Master Mix (Takara Bio Inc., Otsu, Shiga, Japan). The cDNA was then subjected to real-time PCR, which was performed with a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with GoTaq® qPCR Master Mix (Promega, Madison, WI, USA) and the appropriate primers. The PCR protocol consisted of the following steps: an initial denaturation for 10 minutes at 95°C, followed by 40 cycles of 5 seconds at 95°C and 1 minute at 60°C. The relative expression levels of GLS were normalised to those of β-actin, which served as an endogenous control, after the GLS and β-actin cDNA was confirmed to have been amplified with the same efficiency. The following primers were used to amplify the GLS and β-actin cDNA: GLS, 5'-GAGGCACCTTGGATAAGCTGGA-3' and 5'-GCTGTCACATCTCTGGCTGCATA-3'; and β-actin, 5'-TGGCACCCAGCACAATGAA-3' and 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'.

Immunocytochemistry

Confluent FLSs on chambered slides coated with BD Matrigel Matrix (BD Biosciences, Franklin Lakes, NJ, USA) were fixed in 3% paraformaldehyde for 30 minutes, perforated with 0.2% Triton X-100 for 5 minutes, washed with PBS, and blocked with blocking solution comprising 3% bovine serum albumin, 0.1% glycine and 0.1% sodium azide in PBS for 60 minutes at room temperature. After being washed, the cells were labelled with the appropriate primary antibody (1:100 anti-TYMP/GLS Ab) overnight at 4°C, after which they were washed and labelled with an Alexa Flour 594-labelled (red) goat anti-mouse IgG (1:1,000; Invitrogen, Carlsbad, CA, USA) secondary antibody. After being washed, the sections were mounted on glass slides containing ProLong Gold Antifade with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). The stained cells were then visualised by a confocal laser-scanning microscope (Nikon, Tokyo, Japan), and the total intensity of the immunostaining in five random fields was quantified by ImageJ software, which is available at http://imagej.nih.gov/ij/ (September 10, 2017). The numbers of cells in the fields were counted. The data were presented as the mean staining intensity per cell.

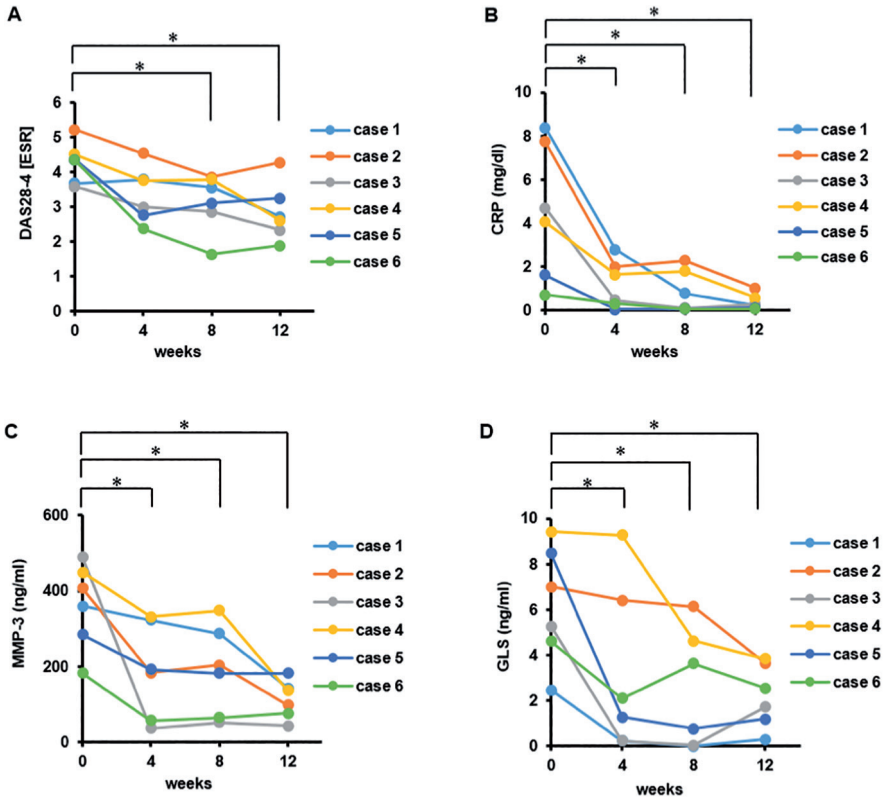

Fig. 1. Treatment with tofacitinib (TOF) decreased serum gliostatin (GLS) levels and facilitated clinical improvement in six patients with RA.
Serum samples were collected from six patients with RA at baseline and after 4, 8, and 12 weeks of treatment with TOF. DAS28 was calculated using the ESR (*A*). Serum CRP (**B**), MMP-3 (**C**) and GLS (**D**) levels were significantly down-regulated at 8 weeks after treatment initiation compared with baseline in all patients. The mean differences in the expression levels of the above proteins between baseline and 4 weeks later were evaluated with the Wilcoxon signed rank test. *$p<0.05$ was considered significant.

Western blotting

FLSs were incubated with 1 ng/ml TNF-α in the presence or absence of tofacitinib in a 6-well plate. After being treated, the FLSs were harvested and gently homogenised on ice in 10 mM Tris-HCl (pH 6.8) containing 0.5% SDS and a protease inhibitor cocktail (Sigma-Aldrich). Protein content was estimated by a BCA protein assay regent kit (Thermo Fisher Scientific). Equal amounts of total protein were fractionated on 10% polyacrylamide gels containing sodium dodecyl sulfate and then electrotransferred to polyvinylidene difluoride (PVDF) membranes (Immobilion-P; Millipore, Billerica, MA, USA), which were blocked with 5% skimmed milk in Tris-buffered saline with Tween 20 (TBS-T: 20 mM Tris-HCl, pH 7.6; and 137 mM NaCl; 0.1% Tween 20) overnight at 4°C before being incubated with primary antibodies against phospho-STAT1, phospho-STAT3, and phospho-STAT5 or antibodies against the corresponding total STATs diluted in TBS-T overnight at 4°C. Actin was used as a gel loading control. The blots were developed with the appropriate horseradish peroxidase-conjugated secondary antibodies (1:3000 dilution;

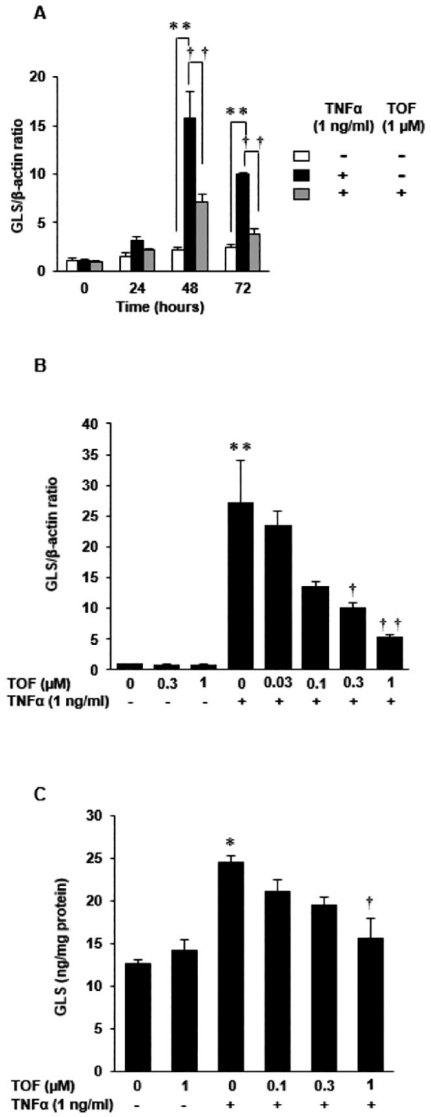

Fig. 2. Tofacitinib (TOF) suppressed TNF-α-induced gliostatin (GLS) expression in RA FLSs. Confluent cells were incubated in the presence (*grey bars*) or absence (*black bars*) of 1 μM TOF for 24 hours before being incubated with TNF-α (1 ng/ml), as indicated. Control FLSs were cultured without additional agents (*white bars*). GLS mRNA expression levels were normalised to those of β-actin (**A**). Confluent FLSs were incubated in the presence or absence of 0.03 to 1 μM TOF for 24 hours before being incubated with TNF-α (1 ng/ml) for 48 hours. GLS mRNA expression levels were normalised to those of β-actin (**B**). Confluent FLSs were incubated in the presence or absence of 0.1 to 1 μM TOF for 24 hours before being incubated with TNF-α (1 ng/ml) for 48 hours. GLS immunoreactivity was determined by EIA (**C**). Control FLSs were cultured without additional agents. The results are presented as the mean ± SEM of experiments performed in triplicate. The statistical significance of the differences between the groups was calculated by ANOVA and Tukey's post hoc test; compared with controls *p<0.05, **p<0.01; compared with samples treated with TNFα alone $^{\dagger}p$<0.05, $^{\dagger\dagger}p$<0.01.

GE Healthcare UK Ltd, Little Chalfont, UK), and the bands were visualised by enhanced chemiluminescence (ECL) (Amersham Biosciences Corp, Piscataway, NJ, USA). The band densities were measured by LI-COR® Image Studio Lite v. 5.2 (M&S Technosystems, Osaka, Japan), which is available at https://www.licor.com/bio/products/software/image_studio_lite/ (September 10, 2017).

Statistical analysis

All data were entered into an electronic database and analysed by GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA, USA). All quantitative data are presented as the mean ± standard error of the mean (SEM) unless otherwise stated. The statistical significance of the differences in the levels of the indicated parameters between baseline and the indicated treatment time points was examined using the Wilcoxon signed rank test, and the significance of the differences between two groups was assessed by one-way analysis of variance (ANOVA) and Tukey's post hoc test. *P*-values less than 0.05 were considered statistically significant.

Results

Serum GLS levels were significantly increased in patients with RA and were significantly decreased in patients with RA who were treated with tofacitinib

Serial data pertaining to GLS levels were obtained from six patients treated with tofacitinib and were analysed to determine their correlations with routinely measured clinical parameters, such as DAS28-4 [ESR] and serum CRP and MMP-3 concentrations. Fig. 1A-D shows the time courses of the six patients, who were followed over 12 weeks. All patients were found to be responsive to therapy, according to the criteria of the EULAR, after 8 weeks of treatment. Serum GLS levels were correlated with other clinical indicators of RA severity, such as CRP and MMP-3 concentrations and DAS28-4 [ESR]. The mean values of the indicated clinical and laboratory parameters were significantly decreased after 12 weeks of treatment with tofacitinib compared with before treatment. Specifically, DAS28-4 [ESR] decreased from 4.29 to 2.85 (Fig. 1A), the CRP concentration decreased from 4.53 to 0.37 mg/dl (Fig. 1B), the MMP-3 concentration decreased from 362.3 to 113.6 ng/ml (Fig. 1C), and the GLS concentration decreased from 6.20 to 2.21 ng/ml (Fig. 1D). The statistical significance of the above changes was calculated using the Wilcoxon signed rank test, p<0.05.

Effects of tofacitinib on TNF-α-induced GLS production in cultured FLSs

To study the time course of GLS-mediated induction of mRNA expression, we pretreated confluent FLSs cultured in 6-well plates with or without 1 μM tofacitinib for 24 hours and then incubated them with 1 ng/ml TNF-α for the indicated times. We found that GLS mRNA expression increased in response to treatment with TNF-α (1 ng/ml) and peaked after 48 hours of treatment (GLS mRNA expression was 15.8-fold higher after 48 hours of treatment than after 0 hours of treatment). These increases in GLS mRNA expression were significantly suppressed by treatment with 1 μM tofacitinib compared with treatment with TNF-α alone (Fig. 2A).

We pretreated FLSs with 0 to 1 μM tofacitinib for 24 hours and then incubated them with 1 ng/ml TNF-α for 48 hours. We found that treatment with TNF-α alone significantly increased GLS mRNA (Fig. 2B) and protein (Fig. 2C) expression levels (GLS mRNA and protein expression levels were 27.1-fold and 1.92-fold higher in the treatment group than in the control group, respectively) and that treatment with tofacitinib suppressed these increases in a dose-dependent manner. GLS expression was not induced in cells treated with tofacitinib alone. These findings indicate that the above increases in GLS mRNA and protein expression were significantly suppressed by treatment with 1 μM tofacitinib. Moreover, these findings are consistent with those of experiments indicating that tofacitinib exerts its GLS-inhibiting effects by inhibiting the JAK/STAT pathway.

FLSs were cultured to confluence and then treated with or without 1 μM to-

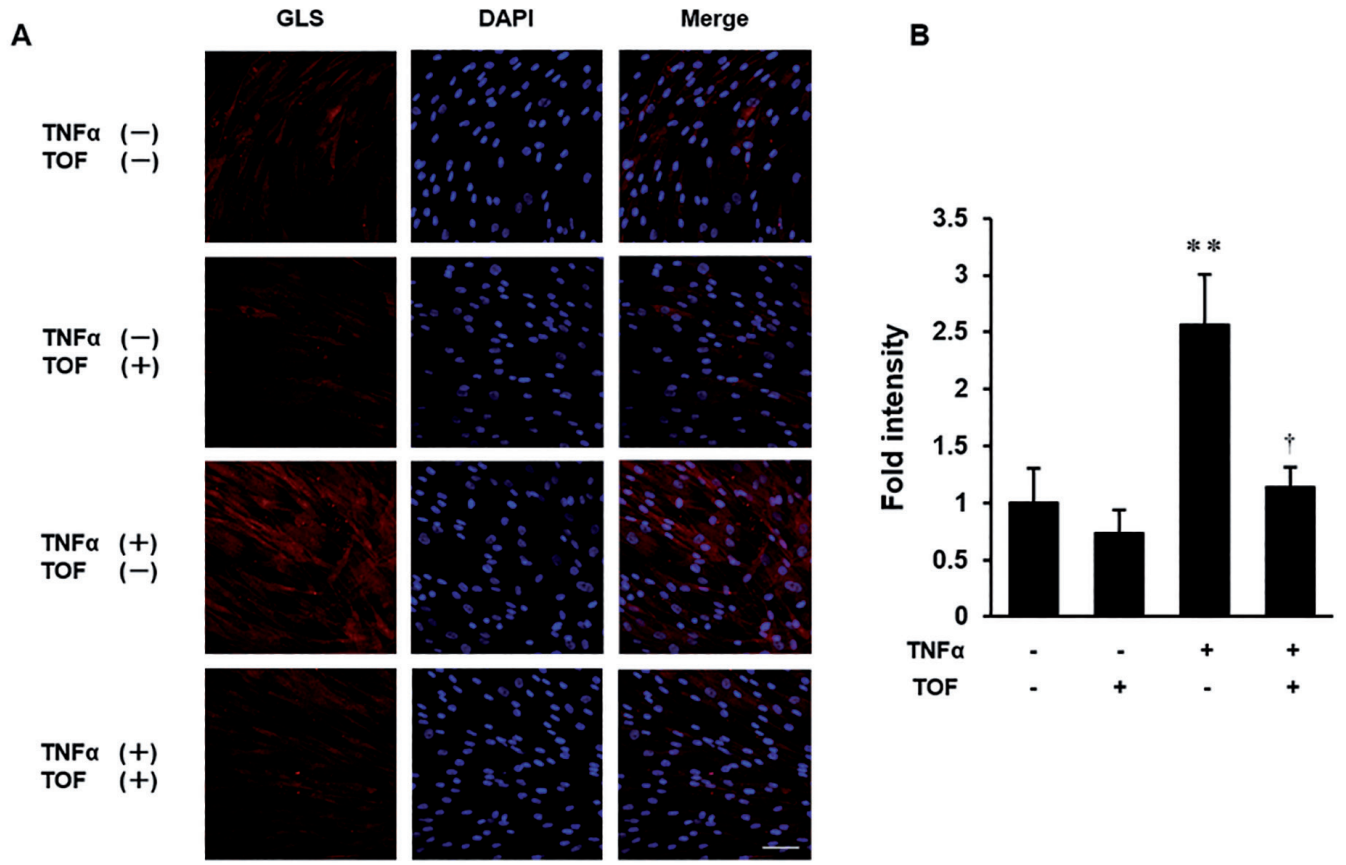

Fig. 3. Immunocytochemical detection of gliostatin (GLS) in RA FLSs.
FLSs were treated with or without 1 μM tofacitinib (TOF) for 24 hours before being incubated with or without TNF-α (1 ng/ml) for 48 hours, after which they were immunostained with GLS antibody (red). The cell nuclei were stained by DAPI (blue). The scale bar represents 100 μm (**A**). Control FLSs were cultured without additional agents. The total intensity of the immunostaining in a random field was quantified by ImageJ, and the numbers of cells in the field were counted. Data (intensity/cell) were presented as the mean ± SEM of five determinations. The statistical significance of the differences between the groups was calculated by ANOVA and Tukey's post-hoc test; compared with controls $^{**}p<0.01$; compared with samples treated with TNF-α alone $^{\dagger}p<0.05$ (**B**).

facitinib for 24 hours, after which they were incubated with or without 1 ng/ml TNF-α for 48 hours. The FLSs were subsequently immunostained with anti-GLS antibodies (red). The immunostaining results showed that untreated cells displayed weakly diffuse staining in the cytoplasm (Fig. 3A). Treatment with tofacitinib alone did not have an effect on GLS staining. GLS protein expression was significantly enhanced by treatment with TNF-α alone (GLS protein expression was 2.6-fold higher in treated cells than in control cells), a change that was significantly suppressed by treatment with tofacitinib (GLS expression was 0.4-fold higher in cells treated with tofacitinib than in cells treated with TNF-α alone) (Fig. 3B). We confirmed that the concentrations of tofacitinib and TNF-α that were used in this study were non-toxic by cell viability assay (data not shown). Regardless of the background of 13 patients (Table II), there was no difference in GLS expression in FLSs induced by TNF-α.

Tofacitinib inhibits TNF-α-mediated induction of phospho-STAT1 expression

FLSs were treated with 0 or 1 μM tofacitinib for 24 hours and then incubated with 1 ng/ml TNF-α for the indicated time. TNF-α induced significant STAT1 phosphorylation (but not STAT3 phosphorylation) at the 4-hour post-treatment time point (STAT1 phosphorylation was 2.6-fold higher in the treated group than in the control group) but not at the 30-minute post-treatment time point (Fig. 4A). STAT5 phosphorylation was not induced by treatment with TNF-α alone (data not shown). Treatment with tofacitinib inhibited TNF-induced STAT1 phosphorylation at the 4-hour post-treatment time point (STAT1 phosphorylation was 0.14-fold higher in the sample treated with tofacitinib than in the sample treated with TNF-α alone) (Fig. 4B).

Discussion

Serum TNF-α levels are elevated in patients with RA (28), and TNF-α has been identified as a principal cytokine in the pathogenesis of RA; however approximately one-third of patients with RA are non-responsive to TNF-α inhibitors (29). We previously reported that serum GLS levels were decreased in responsive patients with RA who were treated with tacrolimus, a csDMARD (13), or with arthroplasty and synovectomy (14). In the present study, we investigated the effects of tofacitinib on serum GLS levels in patients with RA who displayed an inadequate response to TNF-α inhibitors. We found that tofacitinib reduced serum GLS, CRP and MMP-3 concentrations, which were correlated with rheumatoid disease activity. This study has provided the first

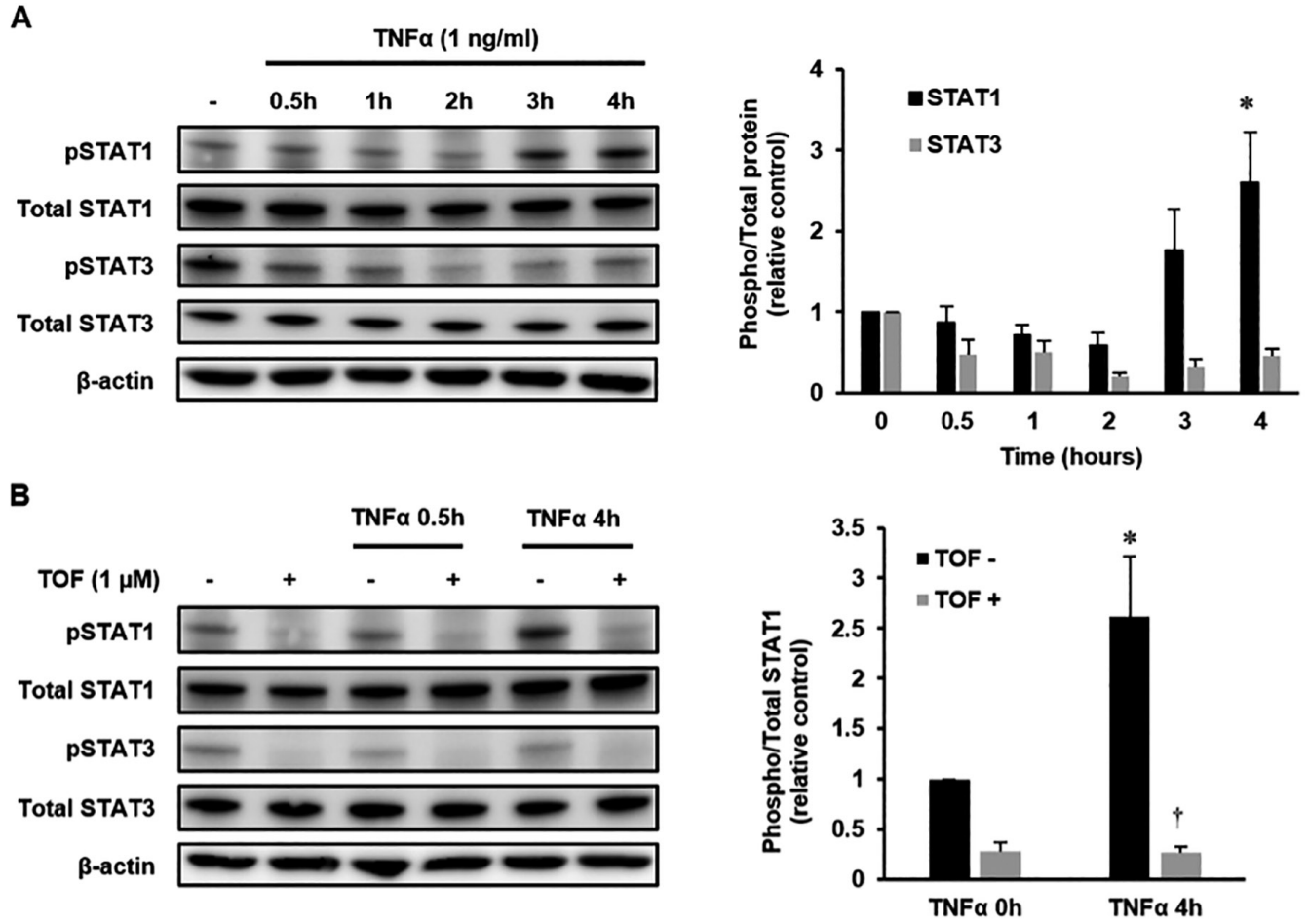

Fig. 4. Tofacitinib (TOF) inhibits late induction of phospho-STAT1 by TNF-α.
Confluent cells were treated with TNF-α (1 ng/ml) for the indicated time. TNF-α induced phospho-STAT1 expression but not phospho-STAT3 expression in RA FLSs (**A**). Confluent cells were incubated in the presence or absence of 1μM TOF for 24 hours before being incubated with TNF-α, as indicated. TOF (1 μM) inhibited TNF-α-induced STAT1 phosphorylation (**B**). The results are expressed as the mean ± SEM of three experiments involving RA FLSs. The statistical significance of the differences between the groups was calculated by ANOVA and Tukey's post hoc test; compared with samples treated for 0 hours *$p<0.05$; compared with samples treated with TNF-α alone †$p<0.05$.

evidence indicating that tofacitinib can effectively regulate serum GLS levels in patients with active RA.

TNF-α induced GLS production by FLSs in a dose-dependent manner (30). The results of the present study consistently showed that TNF-α induces GLS production in RA FLSs, and we confirmed that tofacitinib inhibited GLS mRNA and protein expression in a dose-dependent manner. Our immunocytochemical studies revealed that GLS displayed weak and diffuse staining in the cytoplasm of untreated FLSs and that GLS staining intensity was increased by treatment with TNF-α and suppressed by treatment with tofacitinib at concentration of 1 μM. These results strongly suggest that the JAK/STAT pathway is essential for TNF-α-induced GLS production in RA FLSs and that the pathway can be blocked by tofacitinib. Tofacitinib was originally believed to be a JAK3 inhibitor (31); however, it is now clear that in addition to inhibiting JAK3, this compound also inhibits JAK1 and JAK2 at similar concentrations (32). JAK3 is expressed mainly in hematopoietic cells, whereas JAK1, JAK2, and TYK2 are expressed ubiquitously in mammals (33). In RA FLSs, tofacitinib suppressed phosphorylation of the JAK1-, JAK2- and JAK3-induced IL-6-type cytokine oncostatin M (34). Therefore, we investigated whether TNF-α activates STAT1, STAT3 and STAT5, which are downstream of the JAK proteins.

In this study, neither STAT1 nor STAT3 was immediately activated by TNF-α in RA FLSs, and only STAT1 was activated at 4 hours after the proteins were stimulated with TNF-α. Tofacitinib inhibited TNF-α-mediated induction of phospho-STAT1 expression. The delayed effect of TNF-α on STAT1 phosphorylation suggested that TNF-α induced the production of a secondary mediator, which subsequently activated JAK/STAT-dependent GLS expression. The requirement of the synthesis of a secondary protein for the completion of the above process was confirmed in experiments in which the protein synthesis inhibitor cycloheximide significantly decreased TNF-α-induced GLS mRNA production in a dose-dependent manner (35).

We showed that tofacitinib suppressed STAT1 phosphorylation at 4 hours after the protein was stimulated with TNF-α. STAT1 expression and phosphorylation were more abundant in the intimal

lining layer of patients with RA than in that of patients with osteoarthritis (36). Furthermore, the vast majority of FLSs in the intimal lining layer expressed STAT1 at high levels, whereas the T lymphocytes, B lymphocytes and macrophages in the indicated layer expressed STAT1 only to a limited extent (36). van der Pouw Kraan *et al*. suggested that STAT1 plays a role in promoting synovial inflammation by activating inflammatory gene expression (37). The results of the present study demonstrate that tofacitinib inhibits TNF-α-induced GLS expression in RA FLSs. It is thought that inactivation of STAT1 by tofacitinib leads to a decrease in GLS expression in RA FLSs. Our data clearly demonstrated that JAK/STAT activation play a pivotal role in TNF-α-mediated GLS up-regulation in RA FLSs.

GLS expression was found to be up-regulated by inflammatory cytokines, such as IL-1β (38) and TNF-α, in cultured RA FLSs (30, 35). GLS stimulates the expression of angiogenic and metastatic factors in human cancer cells (39). GLS induces angiogenesis through a process driven by endothelial cell proliferation and chemotactic migration (40). GLS regulated vascular endothelial growth factor (VEGF) production and thus induced angiogenesis in RA (24). Angiogenesis is necessary for prolonged inflammation; thus, inhibiting angiogenic factors, such as GLS and VEGF, may represent a means through which the inflammatory cascade can be suppressed in RA synovitis (41). Members of the MMP family, namely, MMP-1, MMP-3, and MMP-13, play an important role in severe cartilage degradation in RA (42, 43). We showed that GLS acted as a cytokine to augment its own synthesis through an autocrine mechanism in FLSs and also induced the extracellular secretion of MMP-1, MMP-3, MMP-9 and MMP-13, thereby triggering cartilage degeneration (44, 45). We also reported that direct injection of GLS into rabbit knees induced RA-like synovitis (46). These findings indicate that GLS is capable of triggering not only angiogenesis but also cartilage degradation. Therefore, suppressing GLS activity may be an effective means of treating RA.

A limitation of this study was that we could not identify the second mediator that directly activates the JAK/STAT pathway. Rosengren *et al*. reported that TNF-α induced early interferon (IFN) β expression and that IFNβ-mediated STAT1 phosphorylation was inhibited by tofacitinib. Furthermore, they revealed that the induction of several lymphocyte-attracting chemokines by TNF-α was blocked by tofacitinib and that the secondary mediator induced by TNF-α was IFNβ (22). However, in our study, we found that IFNβ was not involved in mediating TNF-α-induced GLS expression in RA FLSs (data not shown). Thus, another second mediator may activate the JAK/STAT pathway to induce GLS expression.

In conclusion, our study was the first to demonstrate that tofacitinib down-regulates GLS mRNA and protein expression in RA FLSs. Our data suggest that the beneficial effects of tofacitinib in RA may be at least partially attributable to its antiangiogenic and anti-arthritogenic activity, which is associated with its ability to down-regulate GLS. Suppression of GLS expression in FLSs has been suggested to be the mechanism through which tofacitinib exerts its anti-inflammatory effects.

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