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Tofacitinib restores psoriatic arthritis fibroblast-like synoviocytes function via autophagy and mitochondrial quality control modulation☆

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ABSTRACT

Objectives: To evaluate the *in vitro* effect of tofacitinib on autophagy activity of psoriatic arthritis (PsA) fibroblast-like synoviocytes (FLS), and to confirm its activity on inflammatory and invasive properties of FLS and synovial cells, deepening the impact on mitochondrial function.

Methods: FLS, peripheral blood mononuclear cells (PBMCs), and synovial cells from active PsA patients were cultured with tofacitinib 1 μM or vehicle control for 24 h. Autophagy was measured by Western blot and by fluorescence microscopy. Chemokines/cytokines released into culture supernatants were quantified by ELISA, while invasive properties of FLS by migration assays. Specific mitochondrial probes were adopted to measure intracellular reactive oxygen species (ROS), mitochondrial potential, morphology, turnover and mitophagy. Oxygen consumption rate (OCR), reflecting oxidative phosphorylation, was quantified using the Seahorse technology. Differences were determined by adopting the non-parametric Wilcoxon signed rank test.

Results: 18 patients with moderately-to-severely active PsA were enrolled. Tofacitinib significantly increased the levels of the autophagy markers LC3-II and ATG7 in PsA FLS compared to vehicle control, suggesting an increase in spontaneous autophagy activity; no effect was highlighted in PBMCs and synovial cells cultures. Tofacitinib reduced migration properties of PsA FLS, and reduced MCP-1 and IL-6 release into FLS and synovial cells cultures supernatants. Furthermore, tofacitinib decreased intracellular ROS production, increased basal OCR, ATP production and maximal respiratory capacity, and enhanced mitophagy and mitochondrial turnover.

Conclusions: The JAK inhibitor tofacitinib reduces the pro-invasive and pro-inflammatory properties of PsA FLS. Autophagy induction and mitochondrial quality control modulation by tofacitinib might contribute to FLS function restoration.

1. Introduction

The management of psoriatic arthritis (PsA) involves the adoption of different drugs, each with distinct mechanism of action [1,2]. Since the rules driving the correct therapeutical choice in each patient are not completely defined, several studies are now focusing on biomarkers

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looking for drug response prediction, and synovial membrane can be considered a good target tissue [3–6].

Janus kinases (JAK) inhibitors (JAKis) are able to reduce synovial inflammation, and tofacitinib, a JAK1/3 inhibitor, has been approved for the systemic management of this condition [7,8]. Through interfering with the intracellular domain of JAK/Signal Transducer and Activator of Transcription (STAT)-coupled receptors, tofacitinib modulates the signal transduction mediated by a variety of type I/II cytokines, like interleukin (IL)-6, IL-22, IL-23, interferon-gamma (IFN-g), and others [9]. Preliminary evidence suggests an effect of tofacitinib on fibroblast-like synoviocytes (FLS) from PsA patients, reducing their pro-invasive and pro-inflammatory properties [10]. Moreover, data obtained applying tofacitinib *in vitro* on rheumatoid arthritis (RA) FLS and synovial explants have shown that this drug is able to regulate mitochondrial function, decreasing the extracellular acidification rate (ECAR) oxygen consumption rate (OCR) ratio (ECAR:OCR), a measure of how cells generate energy, thus enhancing oxidative phosphorylation over glycolysis [11]. These data were partially confirmed with other JAKis (upadacitinib, baricitinib, filgotinib, peficitinib) in PsA FLS following exogenous stimuli with oncostatin-M, a known inducer of JAK/STAT pathway [12].

Despite this, the exact links between JAK inhibition and FLS function improvement are not yet fully understood. Among the most conserved pro-survival mechanisms of eukaryotic cells, autophagy permits the removal of damaged or dysfunctional self-components and organelles, exploiting a structured machinery, that allows the fusion of autophagosomes with lysosomes to finalize the degradation process. Autophagy deregulations are extensively studied in several human pathologies, like cancer, neurological disorders, and cardiovascular diseases [13–15]. In RA, autophagy is thought to play a role in proteins citrullination and carbamylation, apoptosis resistance, and bone damage progression [16]. Only one study assessed the effect of tofacitinib on RA FLS, showing a reduction in spontaneous autophagy activity [17]. Data related to PsA are scarce, and, to our knowledge, no clear information is available with respect to FLS [18,19].

The hypothesis under which our research took place was that tofacitinib could act on pro-survival and metabolic mechanisms in synovial cells, with a wider effect than a pure anti-inflammatory action. Thus, the primary objective of this *in vitro* study was to evaluate the effect of tofacitinib on spontaneous autophagy activity of PsA FLS. Secondary objectives were (i) to confirm the effect of tofacitinib on mitochondrial function of PsA FLS, and (ii) to evaluate if such interference drives anti-inflammatory and anti-migratory changes in PsA FLS and synovial cells.

2. Materials and methods

2.1. Ethics approval, patients and public involvement

The study protocol was approved by the Ethics Committee of Area Vasta Emilia Centro-Emilia-Romagna (279/2019/Sper/AOUFe, approval May 14, 2019). All procedures were in accordance with the ethical standards of the responsible committee on human experimentation and with the Declaration of Helsinki. All subjects provided signed informed consent and were asked to assess the burden of the intervention.

2.2. Study population

This is an *in vitro* study. Patients with a diagnosis of PsA, fulfilling the Classification Criteria for Psoriatic Arthritis (CASPAR) [20], attending the Rheumatology Unit, Department of Medical Sciences, University of Ferrara and Azienda Ospedaliero - Universitaria S.Anna, Cona (FE), with active peripheral joint disease, underwent baseline ultrasound (US)-guided synovial biopsy of one of the most inflamed accessible joints within two weeks of receiving new treatment with the designed conventional synthetic (cs)/biologic (b)/targeted synthetic (ts) disease modifying anti-rheumatic drug (DMARD) for the systemic management of PsA (Supplementary Materials). None of the patients had previously received JAKis.

2.3. Histology analysis and immunohistochemistry

Histopathological evaluation was scored according to Krenn's synovitis score (KSS) (Fig. S1) [21]. For immunohistochemistry (IHC), the detection of B cells ($CD20^{pos}$), T cells ($CD3^{pos}$), and macrophages ($CD68^{pos}$) was performed using a BenchMark XT automated immunostainer, in accordance to product data sheet. Staining was semi-quantitatively quantified using an Olympus AX60 microscope at 20× magnification, scored using a modified scoring system derived from Canete et al. [22](Fig. S2).

2.4. Samples handling and cells cultures

For primary *FLS cultures*, synovial tissue samples were cut into small pieces and directly placed in culture medium (complete Dulbecco's modified Eagle's medium (DMEM)) enriched with foetal bovine serum (FBS) (Axenia BioLogix, Dixon, CA, USA). Cells were allowed to grow out of the tissue lump, and trypsinized at confluence, according to literature [23,24]. Synovial cells were then cultured at 37 °C in humidified atmosphere 5 % CO2 incubator, and FLS were used for experiments between the 3rd and 8th passages [10]. FLS were seeded at 150 × 10^3 cells/well in a 6-well plate and treated with dimethyl sulfoxide (DMSO) vehicle control or tofacitinib 1 μM for 24 h [10,17,25]. For specific autophagy tests, ammonium chloride (NH_4Cl) 10 μM for 6 h or bafilomycin A1 (BafA1) 100 nM (Sigma-Aldrich) for 2 h were used before the end of the culture to test autophagy flux, while rapamycin 1 μM for 12 h was utilized as an autophagy inducer. To test reactive oxygen species (ROS) reduction, the antioxidant N-Acetyl-L-Cysteine (NAC, Merck Life Science S.r.l.) at 1-3-5 mM for 48 h was used [26]. For *synovial cells cultures*, synovial tissue samples were cut into small pieces and digested with Liberase Research Grade TM, in RPMI (Roswell Park Memorial Institute, 150 mcg/ml, Roche) with rotation at 37 °C for 1.5 h. Digested tissue was passed through a 100 mm tissue-strainer, then cells were suspended in DMEM at 0.2 × 10^6 cells in 96-well plates [27], treated *in vitro* with DMSO or tofacitinib 1 μM for 24 h. *Peripheral blood mononuclear cells (PBMCs)* were isolated [28], cultured in RPMI medium supplemented with 10 % FBS and 1 % P/S. 1 × 10^6 cells were placed in a 24-well plate and treated with DMSO or tofacitinib.

2.5. Western blot (WB) analysis

Total cell lysates from FLS, PBMCs and synovial cells were lysed in a buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 % Triton X-100, and 0.2%SDS supplemented with protease and phosphatase inhibitor cocktails. Protein extracts were quantified by the Lowry method and 10 μg of proteins were loaded and separated on a Novex NuPage Bis-Tris 4–12 % gel (Life Technologies) and transferred onto a nitrocellulose membrane. Membranes were incubated with the following primary antibodies: anti-phosphorylated (p)STAT3 (Tyr705) anti-total (t)STAT3, anti-ATG7, anti-*p*-mTOR (Ser2448), anti-P70S6K (Cell-Signaling Technology, UK), anti-PGC-1a, anti-NRF2, anti-p62, anti-LC3-II (microtubule-associated protein light chain 3; Sigma-Aldrich, Merck), diluted in 5 % non-fat milk containing 0.1 % Tween 20 at 4 °C overnight. For the specific autophagy testing, we considered the levels of the autophagy markers ATG7, p62 and the conversion of the non-lipidated form of LC3 (LC3-I) to the lipidated, autophagosome-associated form (LC3-II). GAPDH (Cell-Signaling Technology, UK) was used as a loading control. The revelation was assessed by specific horseradish peroxidase-labeled secondary antibodies (Thermo Fisher Scientific), followed by detection by chemiluminescence (Thermo Fisher Scientific), using ImageQuant LAS 4000 (GE Healthcare).

2.6. LC3 vacuoles count

Cells were transduced with a green fluorescent protein (GFP)-LC3 plasmid and then treated with DMSO or tofacitinib 1 μM. After 24 h, FLS were fixed and loaded for 10 min with a DAPI solution. After three washing, images were acquired at 63× magnification using a Zeiss LSM510 fluorescence confocal microscope equipped with a back-illuminated CCD camera (Roper Scientific, Tucson, AZ) and processed by ImageJ software.

2.7. Quantification of intracellular reactive oxygen species (ROS) production

To determine intracellular ROS in FLS, 1×10^5 cells/well were treated in the presence of DMSO or tofacitinib 1 μM (24 h), as well as NAC 1-3-5 mM for 48 h. Cells were washed in PBS buffer, and MitoSOX™ Red Mitochondrial Superoxide Indicator 5 μM was added for 30 min. Images were acquired at 63× magnification using a Zeiss LSM510 fluorescence confocal microscope and processed by ImageJ software [29]. To determine the most suitable concentration of NAC to be used for further experiments, FLS were treated with NAC 1-3-5 mM for 48 h and stained with MitoSOX™ Red Mitochondrial Superoxide Indicator 5 μM for 30 min. Next, cells were counted using a Tali image-based cytometer (Life Technologies).

2.8. Cellular bio-energetic function analysis

OCR, reflecting oxidative phosphorylation, was measured using the Agilent Seahorse XF Cell Mito Stress Test Kit, Agilent Technologies. The OCR was measured before and after treatment with the ATP synthase inhibitor oligomycin (1 μM; Agilent Technologies), the mitochondrial uncoupler carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) (1 μM), and the complex I/III inhibitors rotenone/antimycin A (1 μM) using an XF24 analyzer (Agilent Technologies) [30]. FLS were cultured with tofacitinib 1 μM or DMSO for 24 h. Basal oxidative phosphorylation was calculated by the average of 3 baseline measurements of OCR, obtained during 15 min before injection of specific metabolic inhibitors. To determine the number of vital cells, FLS were washed with PBS, fixed in 4 % paraformaldehyde, and stained with 0.1 % crystal violet. Crystal violet was dissolved with 1 mol/l acetic acid, and absorbance at 595 nm was measured (SPECTROstar Nano Microplate Reader, BMG LABTECH).

2.9. Cellular mitophagy assessment

MitoTracker™ Green FM and LysoTracker™ Red DND-99 assays (Invitrogen™) were used to determine the co-localization of mitochondria and lysosomes, respectively, in the presence of tofacitinib or DMSO vehicle control for 24 h. Cells were stained with 0.5 μM MitoTracker™ Green FM for 15 min at 37 °C and 5 % CO2. Then, the cells were washed twice with complete DMEM, and stained with 0.5 μM LysoTracker™ Red DND-99 for 15 min at 37 °C and 5 % CO2. Images were acquired at 63× magnification using a Zeiss Axiovert 200 fluorescence microscope and processed by ImageJ software. Mander's overlap coefficients (M1 and M2) were used to quantify the degree of co-localization between fluorophores.

2.10. Sample size calculation

Given the pilot design of the study, sample size calculation was based on feasibility. A study population of 15–20 patients was judged sufficient for both practical and ethical reasons.

Cytokines quantification (ELISA), migration analysis, cell survival assay, mitochondrial membrane potential, morphology and turnover analysis, statistical analysis: see Supplementary Materials.

3. Results

3.1. Demographic, clinical, histopathological and IHC data

18 patients with PsA were enrolled (20th June 2019–13th April 2023). Mean (standard deviation, SD) age was 60.1 (7.9), and females were 6 (33.3 %); all patients were Caucasian. DAPSA assessment (mean 27.6, SD 9.3, range 15–54) depicted a moderately-to-severely active peripheral joints disease, while cutaneous involvement was less relevant (mean body surface area, BSA 1.2 %). Mean (standard deviation, SD) KSS was 4.5 (1.8). The mean (SD) IHC semiquantitative score was 1.9 (1.0) for $CD3^{pos}$, 1.6 (1.1) for $CD20^{pos}$ and 1.8 (0.9) for $CD68^{pos}$ cells (Table 1-S1, Supplementary Results).

3.2. Tofacitinib increases autophagy levels and reduces cytokines release in PsA FLS

Compared to the vehicle control DMSO, tofacitinib (1 μM for 24 h) increased LC3-II ($p = 0.0002$) and ATG7 ($p = 0.0001$) levels, with a non-significant increase in p62 levels ($p = 0.0654$), suggesting an activation

Table 1
Baseline demographic, clinical, histopathological and IHC data of included patients (N = 18).

Demographic variables	Frequency
Age (years), mean (SD)	60.1 (7.9)
Female, N (%)	6 (36.3 %)
Caucasian ethnicity, N (%)	18 (100 %)
Height (cm), mean (SD)	165.7 (24.1)
Weight (kg), mean (SD)	87.5 (23.2)
BMI (kg/m^2), mean (SD)	28.3 (4.3)
Actual smokers, N (%)	2 (11.1 %)
Actual alcohol users, N (%)	6 (33.3 %)
Regular physical activity, N (%)	2/15 (13.33 %)
Clinical variables	Frequency
Disease duration (years), mean (SD)	8.7 (8.0)
Skin psoriasis, N (%)	16 (88.9 %)
First-degree relative with psoriasis, N (%)	7 (38.9 %)
Nail psoriasis, N (%)	4 (22.2 %)
Inflammatory bowel disease (IBD), N (%)	0 (0.0 %)
Previous uveitis, N (%)	1 (5.6 %)
ESR (mm), mean (SD)	36.6 (32.2)
CRP (mg/dl), mean (SD)	3.5 (6.6)
Clinimetrics	Frequency
Pain joint to be biopsied (0–100), mean (SD)	59.7 (27.6)
Stiffness joint to be biopsied (0–100), mean (SD)	55.6 (27.5)
Swelling joint to be biopsied (0–100), mean (SD)	60.3 (31.4)
Patient Global Pain (0–100), mean (SD)	68.8 (21.6)
Patient Global Activity (0–100), mean (SD)	74.2 (16.0)
Physician Global Activity (0–100), mean (SD)	57.8 (17.6)
Tender joint count (0-68), mean (SD)	6.4 (5.1)
Swollen joint count (0-66), mean (SD)	3.4 (1.8)
DAPSA, mean (SD)	27.6 (9.3)
BSA, mean (SD)	1.2 (1.7)
LEI, mean (SD)	0.4 (0.5)
Mean number of painful entheseal points, mean (SD)	0.7 (0.8)
HAQ (0–3), mean (SD)	0.9 (0.5)
Histopathological and IHC data	Frequency
Biopsied joint: right knee, N (%)	4 (22.2 %)
Biopsied joint: left knee, N (%)	14 (77.8 %)
Krenn's synovitis score (0–9), mean (SD)	4.5 (1.8)
Krenn's lining layer enlargement (0–3), mean (SD)	1.6 (0.9)
Krenn's resident cells density (0–3), mean (SD)	1.2 (0.6)
Krenn's inflammatory infiltrate (0–3), mean (SD)	1.8 (0.9)
$CD3^{pos}$ semiquantitative score (0–4), mean (SD)	1.9 (1.0)
$CD20^{pos}$ semiquantitative score (0–4), mean (SD)	1.6 (1.1)
$CD68^{pos}$ semiquantitative score (0–4), mean (SD)	1.8 (0.9)

Abbreviations: IHC, immunohistochemistry; SD, standard deviation; BMI, body mass index; ESR, erythrocyte sedimentation rate; CRP, C reactive protein; DAPSA, Disease Activity Index for Psoriatic Arthritis; BSA, body surface area; LEI, Leeds Enthesitis Index; HAQ, Health Assessment Questionnaire.

of autophagy in FLS cultures (Fig. 1A, Table S2). We confirmed the increase in autophagy by counting autophagic vesicles in FLS transfected with a recombinant plasmid expressing LC3 protein linked to GFP (GFP-LC3). Such GFP-LC3 punctae were more frequent in FLS treated with tofacitinib ($p = 0.0313$) (Fig. 1B, Table S2).

To discriminate whether the increase in the autophagic process was due to an effective activation of the autophagic flux or to an inhibition of the autophagosome-lysosome fusion, we treated FLS with NH_4Cl or BafA1. Accordingly, NH_4Cl and BafA1, which both abolish the acidification of lysosomes, caused an increased abundance of LC3-II in both FLS cultured with DMSO or tofacitinib, (Fig. 1C–D). These findings confirm that FLS treated with tofacitinib have more sustained autophagic levels than untreated FLS, and that this feature was not due to alteration in the autophagic flux.

We next sought to investigate if the concentrations and timings of tofacitinib used for *in vitro* experiments [10,11] permitted the correct functioning of the drug. In the cellular homogenates, the levels of pSTAT3 by WB were reduced by tofacitinib after 24 h ($p = 0.0313$), with no effect over tSTAT3 ($p = 0.2500$) (Fig. 1E, Table S2).

We sought to evaluate its anti-inflammatory effects on FLS. Tofacitinib reduced monocyte chemoattractant protein (MCP)-1 and IL-6 levels in FLS supernatants ($p < 0.0001$ and $p = 0.0009$, respectively) (Fig. 1F, Table S2). This effect was more evident on MCP-1 rather than IL-6, since the standardized mean difference of tofacitinib effect over

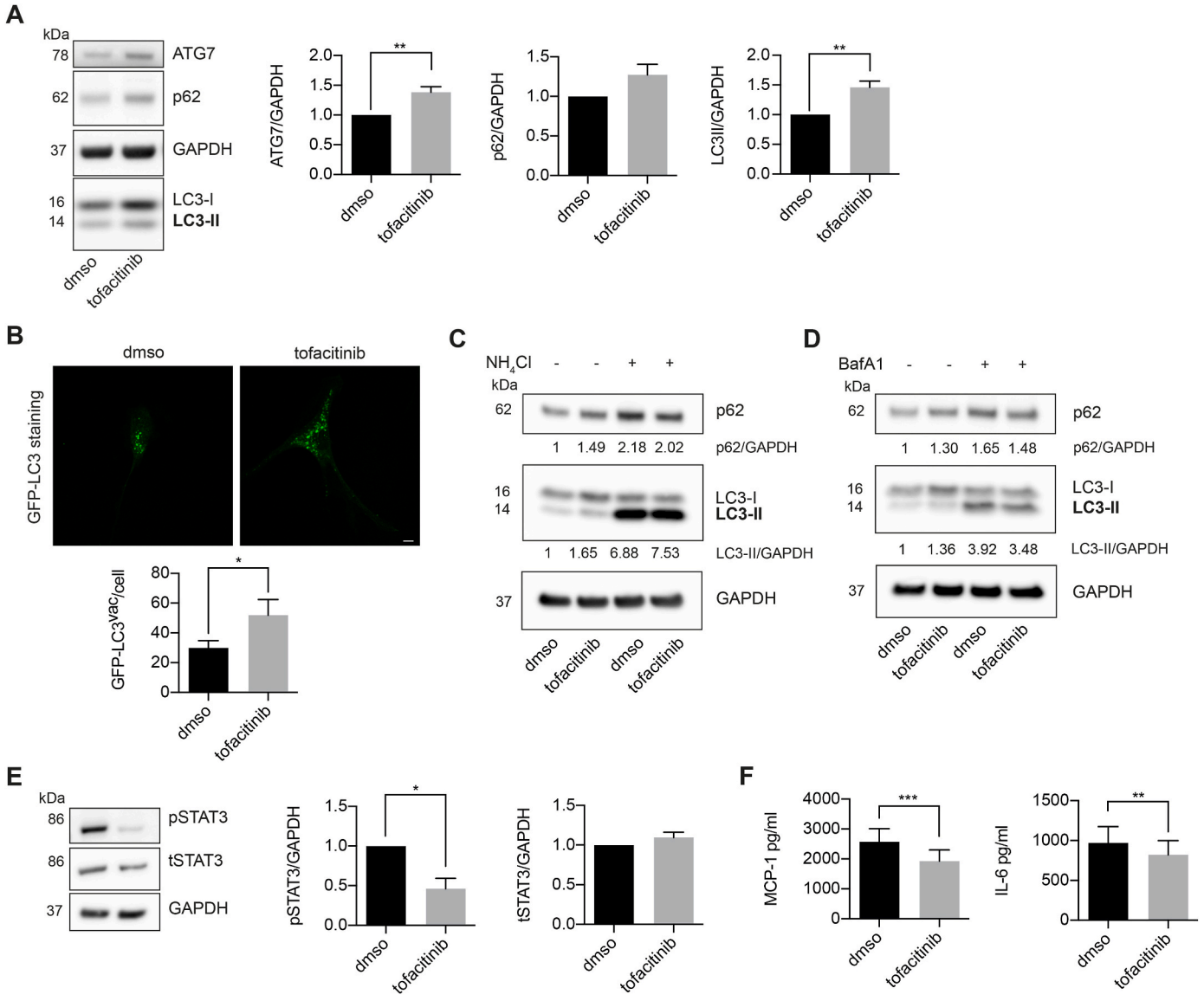

Fig. 1. *Tofacitinib increases autophagic process in PsA FLS and reduces cytokines release.*
(A) Immunoblot detection of autophagy in FLS lysates. GAPDH was used as a loading marker; ATG7, p62 and LC3 as autophagic markers. This is representative of 16 replicates. Bars, SEM. **: $p < 0.01$. n = 16. (B) Representative images of GFP-LC3 punctae in FLS treated with DMSO or tofacitinib 1 μM for 24 h and quantification of autophagic dots/cells. Scale bar, 10 μm. Bars, SEM. *: $p < 0.05$, n = 6. (C–D) Immunoblot detection of autophagic flux in FLS lysates using NH4Cl (D) or BAF-A1 (E). GAPDH was used as a loading marker; LC3 as an autophagic marker. This is representative of 3 replicates. (E) Immunoblot detection of pSTAT3 and tSTAT3 (membrane stripped) in FLS lysates after treatment with tofacitinib. GAPDH was used as a loading marker. Bars, SEM. *: $p < 0.05$, n = 7. (F) FLS were treated with DMSO or tofacitinib 1 μM for 24 h. The MCP-1 and IL-6 levels in the supernatants were determined by ELISA. Bars, SEM. **: $p < 0.01$; ***: $p < 0.001$, n = 15.
Abbreviations: ATG, autophagy-related gene; GAPDH, Glyceraldehyde 3-Phosphate Dehydrogenase; LC3, Microtubule-associated protein; DMSO, dimethyl sulfoxide; GFP, green fluorescent protein; NH4Cl, ammonium chloride; BafA1, bafilomycin a1; pSTAT, phosphorylated signal transducer and activator of transcription, tSTAT, total signal transducer and activator of transcription; MCP-1, MCP-1, Monocyte Chemoattractant Protein-1; IL, interleukin; PsA, psoriatic arthritis; FLS, fibroblast-like synoviocytes, SEM, standard error of the mean.

DMSO was −1.28 (p = 0.0002) for MCP-1 and -0.88 (p = 0.0042) for IL-6 (Table S3). To assess if the tofacitinib effect on autophagy was selective on FLS, we analyzed autophagic levels by WB in PBMCs and synovial cells cultures in the presence of DMSO or tofacitinib. Tofacitinib was not able to influence autophagy levels neither in PBMCs nor in synovial cells cultures (Figs. S3A–B, Table S2). Similarly, no effect of tofacitinib on tumour necrosis factor (TNF), IL-17A, MCP-1, and IL-6 release was documented in PBMCs cultures (Fig. S3C), while we observed a reduction in MCP-1 and IL-6 release in synovial cells cultures (Fig. S3D). These data suggest that the effect of tofacitinib on autophagy was selective on FLS, while its anti-inflammatory properties applied also to synovial cells cultures.

3.3. Tofacitinib improves mitochondrial functions of PsA FLS

We next evaluated if tofacitinib was able to affect mitochondrial function in PsA-FLS. To test our hypothesis, MitoSOX™ Red Mitochondrial Superoxide Indicator was used to document the effect of tofacitinib on FLS intracellular ROS production. Tofacitinib reduced the production of mitochondrial ROS (p = 0.0156) (Fig. 2A, Table S2). No effect was retrieved on mitochondrial potential following JC-1 staining (p = 0.3125) (Fig. 2B), while we documented that tofacitinib increased basal mitochondrial oxygen consumption (OCR) (p = 0.0313), as well as adenosine triphosphate (ATP) production (p = 0.0313) and the maximal respiratory capacity (MRC) (p = 0.0156) (Fig. 2C). These data show that tofacitinib treatment inhibits ROS production without affecting the mitochondrial membrane potential; in parallel, tofacitinib administration induces changes in metabolic activity, favouring oxidative phosphorylation.

3.4. Tofacitinib contributes to mitochondria turnover

We assessed whether FLS from patients with PsA exhibit abnormalities in the clearance of damaged mitochondria. We determined the co-localization of mitochondria and lysosomes in the presence of tofacitinib or vehicle control. Mander's overlap coefficients (M1 and M2) were used to quantify the proportion of mitochondria co-localizing with lysosomes (M1) and the proportion of lysosomes co-localizing with mitochondria (M2). M1 coefficient significantly increased following tofacitinib administration (p = 0.0156), suggesting an increase in mitophagy (Fig. 3A, Table S2). In support of the concept that tofacitinib affects mitophagy, we investigated the mitochondrial morphology finding that

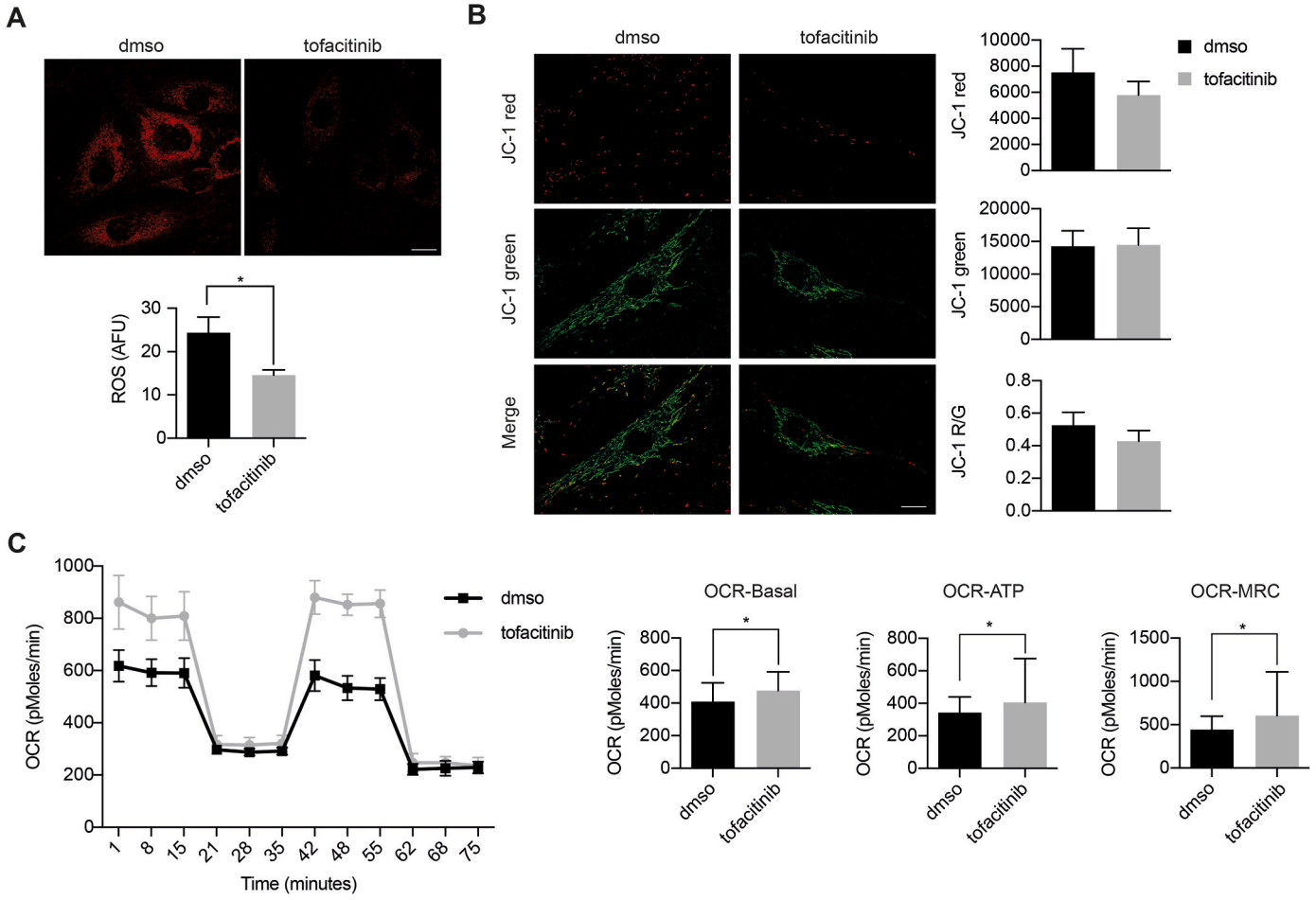

Fig. 2. *Tofacitinib enhances mitochondrial functions of PsA FLS*
(A) Tofacitinib significantly reduced the production of intracellular ROS (DMSO: 24.384 ± 3.583; tofacitinib: 14.606 ± 1.159, *: $p < 0.05$; n = 7). Representative images of FLS after MitoSOX staining, following DMSO and tofacitinib *in vitro* administration, and relative quantification. Results are expressed in AFU/cell. Scale bar: 10 μm (B) No effect of tofacitinib was retrieved on mitochondrial potential. Representative images of FLS after JC-1 staining, following DMSO and tofacitinib *in vitro* administration. n = 8. Scale bar: 10 μm (C) Mitochondrial respiratory function is impaired in FLS cells. Upper panel, OCR was analyzed in real time using the XF96 extracellular flux analyzer. Representative experiments were performed in FLS treated with DMSO or tofacitinib 1 μM for 24 h. Lower panels, basal respiration (OCR-Basal), ATP production (OCR-ATP), and maximal respiration capacity (OCR-MRC) were rescued after tofacitinib treatment. Bars, SEM. *: $p < 0.05$, n = 7.
Abbreviations: DMSO, dimethyl sulfoxide; ROS, reactive oxygen species; AFU, arbitrary fluorescence units; OCR, oxygen consumption rate; ATP, Adenosine triphosphate; MRC, maximal respiratory capacity; PsA, psoriatic arthritis; FLS, fibroblast-like synoviocytes, SEM, standard error of the mean.

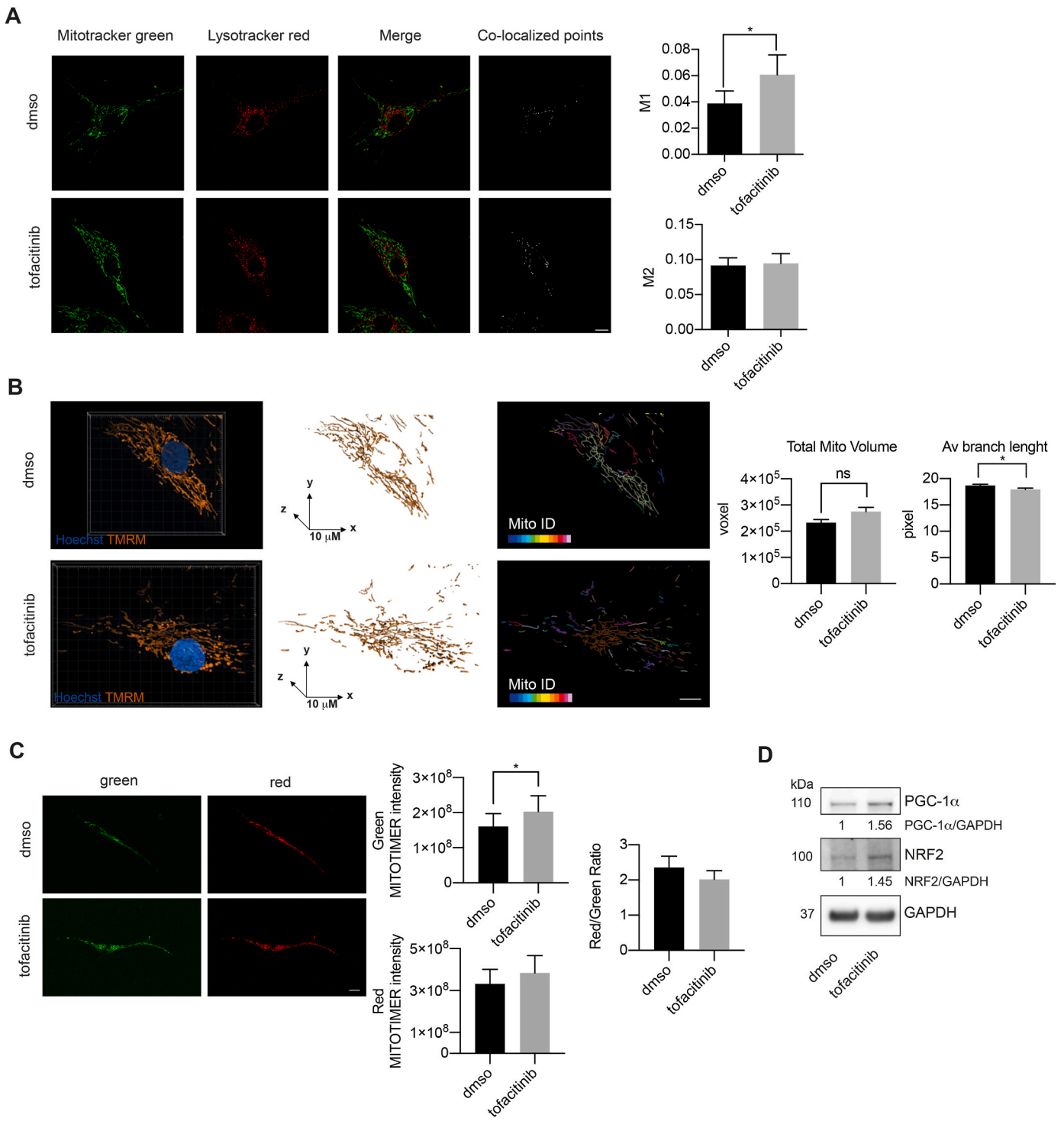

Fig. 3. *Tofacitinib improves mitophagy and mitochondrial quality control.*
(A) Co-localization analysis between LysoTracker Red DND-99 to mark lysosomal structures (red) and MitoTracker Green FM to visualize mitochondria (green) as index of mitophagy. Mander's overlap coefficients (M1 and M2) were used to quantify the co-localization of mitochondria inside lysosomes and lysosomes inside mitochondria. M1 coefficient significantly increased following tofacitinib administration with respect to DMSO (*: $p < 0.05$; n = 7). Scale bar: 10 μm (B) Mitochondrial morphology in FLS treated with DMSO or tofacitinib 1 μM for 24 h. A tetramethyl rhodamine methyl-ester (TMRM) probe was used to perform 3D confocal microscopy tomography of the mitochondrial morphology. Tofacitinib does not significantly alter the total volume of mitochondria (mean number of cells analyzed = 137) but alters mitochondrial fragmentation through reducing average branch length (mean number of cells analyzed = 137, *: $p < 0.05$). The left panel show representative maximum intensity projection of original staining. The middle panel show representative isosurface rendering of mitochondrial network (scale bar: 10 μm). The right panel show representative images of segmented mitochondria. For each segmented mitochondria a numeric ID is assigned (Mito ID) which is then rendered as a color coding. (C) MitoTimer measurement of mitochondrial age of FLS treated with DMSO or tofacitinib 1 μM for 24 h. Representative images of the green and the red channels are shown with statistics. Tofacitinib significantly increased green mitochondria, with a non-significant effect on older ones. The ratio between older and newer mitochondria slightly decreased. *: $p < 0.05$ n = 11. Scale bar: 10 μm. (D) Immunoblot detection of mitochondrial biogenesis markers as PGC-1a and NRF2 (membrane stripped) in FLS lysates after tofacitinib treatment. GAPDH was used as a loading marker. This is representative of 3 replicates. Abbreviations: DMSO, dimethyl sulfoxide; TMRM, tetramethyl rhodamine methyl-ester.

tofacitinib does not alter the total volume of mitochondria (p = 0.0939) but slightly reduces mitochondrial elongation (p = 0.0357) (Fig. 3B).

Next, we decided to explore mitochondrial turnover further and adopted a fluorescent probe (MitoTimer), which labels in green newly synthesized mitochondria, while shifting to red fluorescence when the mitochondria age [31]. Tofacitinib increased green mitochondria (p = 0.0244), with a non-significant effect on older ones (p = 0.5195). The ratio between older and newer mitochondria slightly decreased (p = 0.1230), suggesting tofacitinib influences mitochondrial turnover, reducing mitochondrial stress (Fig. 3C). Accordingly, we documented an increased expression of mitochondrial biogenesis markers, like PGC-1a and NRF2 [32–34], following tofacitinib treatment (Fig. 3D).

Taken together, these data suggest that tofacitinib removes damaged mitochondria but balances with new mitochondria that are more energized, maintaining a "healthy" cohort of mitochondria.

3.5. Induction of autophagy and ROS inhibition reduce pro-migratory properties of PsA FLS

We confirmed that tofacitinib reduced the percentage of cells open area at 24 h and 48 h in a wound-healing assay, confirming a deceleration in FLS migration (p = 0.0024 and 0.0137, respectively), with a tendency towards lower migration enhanced also at 72 h (p = 0.0938) (Fig. S4A, Table S2). This was not due to reduced proliferation of PsA FLS, that was comparable between DMSO and tofacitinib at all the time points assessed (Fig. S4B).

Since our data further suggest that tofacitinib reduces ROS levels and stimulates autophagy and mitophagy, with aged mitochondria that are able to be renewed, we tested the hypothesis that boosting autophagy or blocking ROS production could improve the healthy state of the cells. Rapamycin is a recognized inducer of autophagy by repressing the mechanistic target of rapamycin (mTOR) [35]. This was confirmed in PsA FLS, since the treatment with rapamycin increased the LC3-II levels and suppresses phosphorylated mTOR and its substrate P70S6K, and tofacitinib did not interfere with them (Fig. 4A).

We then investigated if the autophagy-inducer rapamycin exhibited an effect that was comparable to that of tofacitinib, slowing down FLS migration attitude. Our data confirm that rapamycin, similarly to tofacitinib, reduced FLS migration at 24 h with respect to DMSO (p = 0.0156). In addition, co-exposure to rapamycin and tofacitinib did not show synergistic or additive effects (p = 0.0078), suggesting that both compounds act on the same pathway (Fig. 4B).

We finally explored if the decrease in ROS production could have a positive effect on pro-invasive properties of FLS, as well. First, we selected the proper dosage of the NAC antioxidant agent. As shown in Figure S4C-D, a concentration of 5 mM of NAC for 48 h reduced intracellular ROS production by FLS. This concentration of NAC did not affect cell viability (Fig. S4E). Next, we performed migration analysis in presence of vehicle control, tofacitinib or NAC 5 mM for 48h. As a result, both tofacitinib and NAC reduced FLS migration (p = 0.0156 and 0.0313, respectively) (Fig. 4C).

4. Discussion

This *in vitro* study demonstrates that the JAK inhibitor tofacitinib increases autophagy in FLS obtained from active PsA patients, as shown

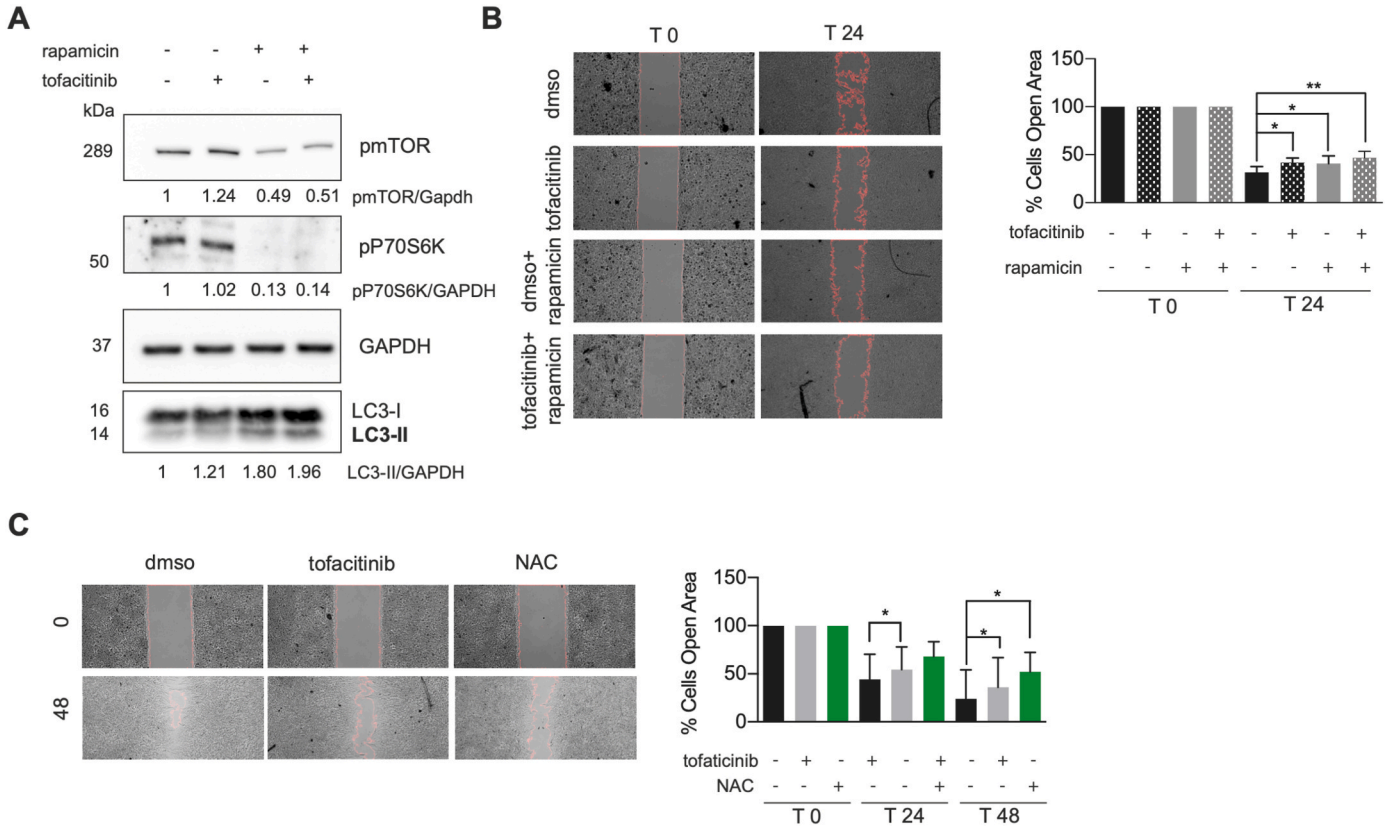

Fig. 4. *Modulation of autophagy or ROS production reduces FLS pro-migratory properties.*
(A) Immunoblot of rapamycin efficacy on FLS cell lysates. GAPDH was used as loading control. pMTOR and p70S6K decrease as readout of rapamycin treatment, as widely published elsewhere. LC3-II increases following tofacitinib or rapamycin *in vitro* administration. This is representative of 3 biological replicates. (B–C) Analysis of FLS migration by *in vitro* wound healing assay. Representative photomicrographs showing FLS migration following treatment with rapamycin (B) or NAC (C) and tofacitinib for 24 and 48 h *: $p < 0.05$; **: $p < 0.01$. B: n = 8; C: n = 7. Abbreviations: pmTOR, phosphorylated mechanistic target of rapamycin; GAPDH, Glyceraldehyde 3-Phosphate Dehydrogenase; LC3, Microtubule-associated protein; DMSO, dimethyl sulfoxide; NAC, N-acetylcysteine; ROS, reactive oxygen species; AFU, arbitrary fluorescence units.

by enhanced LC3-II and ATG7 levels without autophagy flux blockade, improves mitochondrial function and quality control, and shows anti-migratory and anti-inflammatory properties (Fig. 5). To the best of our knowledge, this is the first study evaluating *in vitro* autophagy and mitochondrial turnover modulation in PsA FLS, and the first assessing the synovial effect of an approved DMARD focusing on cells obtained with an ultrasound-guided synovial biopsy procedure in the context of PsA [6].

Since the effects of JAK inhibitors on autophagy have been scarcely characterized in the context of chronic inflammatory arthritis, an effect of tofacitinib on spontaneous autophagy activity of PsA FLS has not been reported yet. A recent work explored the *in vitro* effect of tofacitinib on FLS from RA, showing a reduction in spontaneous autophagy activity [17]. Differing from our conditions, FLS were obtained from a total knee/hip replacement surgery, with no information regarding disease activity or treatment status. Additionally, experiments involving the administration of rapamycin were performed before the administration of tofacitinib, diverging from the technique adopted in our experiments. Another JAK inhibitor, baricitinib, displayed a pro-autophagic role inducing autophagy, reducing ROS and increasing intracellular ATP in progeria fibroblasts with different experimental conditions [36]. A similar finding was reported by Pandey et al. in the experimental model of a lipodystrophic autoimmune disease [37], in which tofacitinib administered *ex vivo* to knock-out mice induced an increase in autophagy, coupled with a clinical amelioration of the disease phenotype. Likewise, tofacitinib restored autophagy levels in chondrocytes of an osteoarthritis model [38]. Here, we demonstrated that tofacitinib reduces pro-invasive and pro-inflammatory properties of PsA FLS when administered *in vitro*, in line with the literature [10]. Moreover, the autophagy-inducer rapamycin exhibited a similar effect to tofacitinib, as it effectively attenuated FLS migration attitude. Importantly, the pro-autophagic effect we observed was selective on FLS, as we did not observe any influence on either synovial cells or PBMC cultures. The complicated physiology of heterogeneous cellular populations might explain, at least partially, this evidence [39]. On the other hand, our experiments lack the analysis of single-cells technologies which could have informed on the properties of other cells relevant to disease pathogenesis [18].

Our data also demonstrated that tofacitinib improves mitochondrial function in PsA FLS, and mitochondria from tofacitinib-treated FLS appeared to display a more quiescent phenotype, with a change in the bioenergetic profile. This confirms that the regulation of the metabolic pathways in FLS is strongly linked with the resolution of inflammation in the inflamed joint, and our work contributes to this growing knowledge, arising not only in RA but even in PsA synovitis [11,12,26,40]. Furthermore, as cells maintain a functional and healthy mitochondrial population through mitochondrial quality control, ensuring the replacement of defective mitochondria with newer and more functional ones, we conducted experiments specifically focusing on mitophagy. Mitophagy is a selective form of autophagy, which ensures the preservation of healthy mitochondria through the removal of damaged or unnecessary ones, by mediating lysosomal degradation [41,42]. Specifically, mitophagy neutralizes the excess of mitochondrial ROS and damaged mitochondrial DNA relevant for inflammation [43]. Our experiments proved that tofacitinib increased the co-localization of mitochondria and lysosomes, indicating an active process of internalization of defective mitochondria inside autophagolysosomes. Even if the number of mitochondria was not altered following tofacitinib treatment, they resulted more fragmented, suggesting that tofacitinib renews mitochondria population. The analysis with the fluorescent probe MitoTimer confirmed the hypothesis that an improved mitochondrial turnover takes place in PsA FLS in the presence of tofacitinib, rescuing intracellular ROS accumulation and reducing mitochondrial stress [44,45]. We could speculate that a balance between newly synthesized and removed mitochondria could justify the absence of a net effect on the total volume of mitochondria, which are more fragmented and healthier [46].

Our study has limitations that need to be mentioned. First, FLS is a heterogeneous population [47,48], and dissecting the effect of tofacitinib on relevant FLS sub-populations using high throughput technologies was out of the scope of our work. Second, patients enrolled were not all treatment-naïve, and this could have generated a heterogeneous cellular

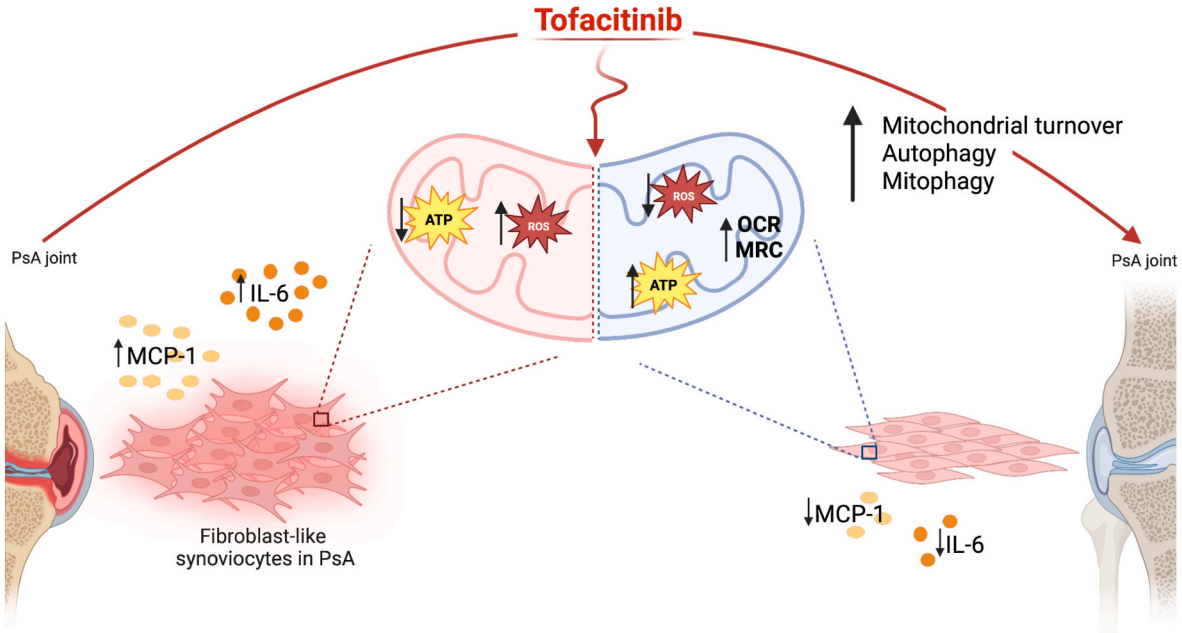

Fig. 5. *Schematic representation of the effects of tofacitinib on PsA FLS.*
Tofacitinib enhances autophagy activity and improves mitochondrial function in PsA FLS. Specifically, it reduces ROS production, stimulates oxidative phosphorylation through increasing OCR, ATP production and MRC, favours mitophagy and mitochondrial turnover, and reduces pro-migratory and pro-inflammatory properties of FLS. Abbreviations: PsA, psoriatic arthritis; FLS, fibroblast-like synoviocytes, ROS, reactive oxygen species; OCR, oxygen consumption rate; ATP, Adenosine triphosphate; MRC, maximal respiratory capacity. This image was created with © BioRender 2021.

population for *in vitro* studies, since previous treatments could have influenced the features of synovial inflammation. However, our results were consistent across different treatment lines and disease activity phases, thus providing a picture applicable to the whole PsA disease spectrum. We did not provide information about the synovial pathotype (i.e., myeloid, lymphoid, pauci-immune) [49–51]. However, pathotypes definition in PsA is less validated than in RA, and we performed IHC staining in line with international consensus [52]. The relatively small sample size was in line with similar reports in the field [10–12], while the ethnic origin of the patients limits the validity of our results to Caucasian patients. Finally, our study does not include investigations using specimens obtained from non-PsA individuals, since collecting synovial tissues from healthy adults was not feasible due to ethical considerations.

Notwithstanding these limitations, the strengths of this study refer to the well-characterized clinical population at study entry, the focus on clinically-active disease, the adoption of a minimally-invasive ultrasound-guided synovial biopsy procedure, the adherence to the OMERACT (Outcome Measures in Rheumatology) consensus statement regarding synovial tissue handling and reporting, and the EULAR minimal reporting requirements in synovial tissue research [52,53], as well as to the innovation of the matter, with data regarding the actually unsolved correlation between JAK inhibition at synovial level and mitochondrial function improvement. Furthermore, we decided to focus on unstimulated cellular populations for experiments. Despite boosting autophagy far more was out of the scope, diverging from the observations of other researchers (e.g. adopting serum starvation) [17], and whereas *in vitro* stimulation with cytokines or other mediators could have amplified the synovial effects of tofacitinib [12,54], these additions remain an exogenous manipulation that could limit the interpretation of results [6]. Moreover, we confirmed that FLS derived from synovial joints isolated by outgrowth from synovial tissue fragments are able to constitutively release cytokines and chemokines into supernatants [24], providing a mechanistic rationale for *in vitro* administration of JAK inhibitors [10]. Regarding synovial cells cultures, we adopted an approach by digesting synovial tissue and culturing it immediately after processing, to minimize the progressive time-dependent reduction in mediators release; this was in line with several similar approaches [27,55].

To summarize, the possibility to assess the intimate effect of approved DMARDs might expand the knowledge of their properties at synovial level, going beyond a mere anti-inflammatory action [4,6], and this achievement could expand the possibility to test a specific drug in future synovial biomarkers retrieval studies, applicable also to the context of *in vitro* experiments [27,55,56], in the search of precision medicine.

4.1. Conclusions

In conclusion, tofacitinib enhances autophagy activity and improves mitochondrial function in PsA FLS, reducing their pro-invasive and pro-inflammatory properties. Induction of autophagy and modulation of mitochondrial quality control by tofacitinib might permit the removal of damaged mitochondria and a better functioning of the new organelles. This reinforces the significance of the beneficial effects of JAK inhibitors at synovial level, expanding the rationale for the use of these drugs in the systemic management of PsA patients.

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Author contributions

All authors have made substantial contributions to all of the following: (i) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (ii) drafting the article or revising it critically for important intellectual content, (iii) final approval of the version to be submitted.

Data availability statement

All data are available upon appropriate request. Data pertaining to this manuscript were presented at EULAR Congress 2022 (Poster Tour Session, June 4, 2022, POS0328, abstract presenter Dr. E. Silvagni).

Patients' involvement

Patients or the public were not involved in the design, or conduct, or reporting, or dissemination plans of our research. All subjects provided signed informed consent and were asked to assess the burden of the intervention.

Declaration of competing interest

ES has received research support from AbbVie and Lilly and consulting/speaker's fees from AbbVie, Galapagos, Lilly, Novartis, Astra-Zeneca and Amgen. MG has received research support from AbbVie, Pfizer and Lilly and consulting/speaker's fees from AbbVie, Galapagos, Lilly, Alfa-Sigma, Astra-Zeneca and Amgen. The other authors declare no conflicts of interest.

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Appendix A. Supplementary data

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