The JAK inhibitor baricitinib inhibits Oncostatin M induction of proinflammatory mediators in ex-vivo synovial derived cells

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Abstract

Objectives: To investigate the ex vivo effect of the JAK1/2 inhibitor baricitinib on expression of pro-inflammatory mediators in Rheumatoid Arthritis (RA) fibroblast like synoviocytes (FLS) stimulated with TNFα, IL-1β, and Oncostatin M (OSM), and in RA synovial membrane cells (SMCs).

Methods: RA and OA SMCs, were isolated from arthroplasty specimens of RA (n-=8) and OA (n=8) patients respectively using enzymatic digestion followed by cell propagation to obtain RA (n=5) and OA (n=3) FLS. Normal FLS and normal human foreskin fibroblasts (HSF) were purchased from commercial sources. Fibroblasts were stimulated with cytokines with or without baricitinib. RA SMCs were cultured in the presence of baricitinib without stimulation. JAK/STAT activation and levels of mRNA and proteins of the various inflammatory cytokines (IL-6, IL-8, MCP-1, RANTES and IP-10) were determined by qPCR, ELISA and MSD.

Results: Baricitinib inhibited OSM-induced JAK signaling in RA synovial fibroblasts and effectively suppressed subsequent expression of the proinflammatory mediators IL-6, MCP-1 and IP-10. However, baricitinib was not effective in altering levels of spontaneously released TNFα, IL-6 and IL-8 in RA SMC. Although both TNFα and IL-1β signal independently of the JAK/STAT pathway, in HSF, but not in RA FLS, baricitinib significantly inhibited TNFα and IL-1β-induced MCP-1 and IP-10 protein levels in a dose dependent manner. Furthermore, baricitinib did not inhibit TNFα– and IL-1β– induced expression of IL-6, IL-8 and MCP-1 in RA FLS.

Conclusions: These findings are consistent with known signaling pathways employed by OSM, TNFα and IL-1β, but our data suggest that in HSF, baricitinib may have anti-

inflammatory effects via downstream modulation of cytokines and chemokines produced in response to TNFα or IL-1β.

Introduction

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory syndrome in which the predominant disease manifestation is peripheral joint synovitis. Although the importance of various molecules and cells involved in RA pathogenesis is now better understood and validated through the availability of targeted therapies, the heterogeneity of clinical responses observed in clinical practice within subjects meeting classification criteria for RA suggest that the dominant pathways driving inflammation may vary widely [1]. Furthermore, the mechanisms underlying perpetuation of joint pain, swelling and inflammation are not completely dissected. It is well established that in most patients, the pro-inflammatory cytokines tumor necrosis factorα (TNFα) and interleukin-6 (IL-6) play dominant pathological roles. While several TNFα inhibitors and other biological therapies using a different mode of action, including biologics targeting IL-6 receptor, B cell depletion or co-stimulation are in routine clinical use in RA, the fact that not all patients respond adequately, coupled with the increased risk of immunosuppression and corresponding infectious complications, suggest that better understanding of the biology of the disease and improvements to current treatments are still necessary with the ideal goal of achieving immune homeostasis while avoiding the risks associated with immune suppression [1, 2].

A number of pro-inflammatory cytokines including IL-6, GM-CSF, interferons and OSM are involved in the pathogenesis of inflammatory arthritis and signal through the Janus kinase (JAK) enzyme family. These cytokines, together with growth factors, play an important role both in the continuous stimulation of fibroblast like synoviocytes (FLS) towards an aggressive behavior as well as in the crosstalk between FLS and other cell types in the synovium [3].

The JAK family consists of four members: JAK1, JAK2, JAK3, and TYK2. JAKs signal through binding of the intracytoplasmic tail of a specific group of so-called Type I and Type II cytokine receptors, which are structurally distinct from other cytokine receptors such as those that bind TNFα and IL-1. Type I receptors bind several interleukins, colony stimulating factors (CSF) and hormones whereas Type II receptors bind interferons and IL-10 related cytokines. Cytokine receptor engagement leads to receptor oligomerization and the recruitment and activation of cytoplasmic JAKs. Because JAKs are phosphotransferases, they catalyse phosphorylation of the receptor, which allows recruitment and tyrosine phosphorylation of members of the signal transducer and activator of transcription (STAT) family of DNA binding proteins. Finally, phosphorylated STAT dimers translocate to the nucleus and regulate the transcription of target genes [4, 5].

Baricitinib, a novel, potent and selective oral inhibitor of JAK1/JAK2 enzymes with IC50 values in the nM range, has recently been approved for the treatment of RA and trials are currently underway for other inflammatory disorders [6]. Baricitinib is well tolerated and significantly improves symptoms and signs of RA across a range of patient populations with active disease, with a particularly impressive set of outcomes in phase III studies. One such phase III study was the first to formally demonstrate superiority of a small molecule plus methotrexate over adalimumab plus methotrexate in a range of clinically relevant outcomes [7]. However, there is a pressing need to investigate the mechanisms of action of baricitinib on synovial tissue in order to better understand the clinical correlates.

In this study, we aimed to investigate the effect of baricitinib ex vivo, in cells derived from inflamed RA synovium including disaggregated synovial membrane cells and FLS.

Methods

Cell culture

Cell culture experiments were carried out using established in house protocols as previously described [8-10]. FLS were obtained from synovial tissue from five RA and three osteoarthritis (OA) patients undergoing joint replacement surgery with the OA synovial tissue serving as a control. All RA patients met American College of Rheumatology/ European League Against Rheumatism 2010 classification criteria for RA.[11] Informed written consent was obtained prior to obtaining the tissue samples as described in the protocol that has obtained approval of the regional ethics committee (REC 07/H0706/81), in accordance with the Declaration of Helsinki.

Tissue was dissociated mechanically and digested enzymatically with Liberase (Roche, Basel, Switzerland). The disaggregated cells were filtered through a sterile nylon cell strainer to obtain a single cell suspension of synovial membrane cells (SMC). Cells were incubated for 24 hours in the presence of Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/l D-Glucose, L-Glutamine, Pyruvate free, (Gibco, ThermoFisher Scientific, Massachusetts, USA) supplemented with 10% fetal calf serum and 1 % Penicillin/Streptomycin (both obtained from Sigma-Aldrich, Missouri, USA), or DMEM containing baricitinib (1 μM) with an equivalent volume of dimethyl sulphoxide (DMSO) used as a vehicle control.

In parallel experiments, untreated SMCs were cultured overnight and non-adherent cells were removed by changing the medium to allow overgrowth of FLS. Cells were cultured for approximately 3 weeks before use. Cells used for the experiments were at early passages (3-5), to ensure a minimal change in phenotype. Normal synovial

fibroblasts (Normal FLS), and human foreskin fibroblasts (HSF) were purchased from Asterand bioscience, Detroit, USA, and from the American Type Culture Collection (ATCC), Manassas, USA respectively. Both cell types were used as additional healthy controls. Experiments were carried out in 6-well and 24-well plates at a concentration 1 x 10^4 cells per cm^2. Cells were pretreated for 2 hours with baricitinib (0.01 μM, 0.1 μM, and 1 μM), and an equivalent volume of DMSO (used as a vehicle control) and subsequently treated for 24 hours with TNFα (10 ng/ml), IL-1β (10 ng/ml) and OSM (20 ng/ml) following protocols described by [12, 13]. Supernatants from all cell cultures were aspirated and frozen at -20°C for future enzyme-linked immunosorbent assay (ELISA). Cells were then washed with ice-cold 1 x Phosphate Buffered Saline (PBS) and lysed with a lysis buffer as described by [16] to obtain protein and RNA.

Western blotting

Protein concentration was determined using the Bio-Rad protein assay kit. An identical amount of protein (50 μg) for each lysate was subjected to 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. Western blot analysis was conducted using a Western Blotting Application Solutions Kit #12957 and the following anti-rabbit antibodies: Phospho-Jak1(Tyr1034/1035), Phospho-Jak2 (Tyr1008), Phospho-Stat3 (Ser727) and Phospho-Stat5 (Tyr694) from Cell Signaling Technology following the manufacturers western blotting protocol (Cell Signaling Technology Massachusetts, USA).

RNA extraction qPCR

RNA isolation and complementary DNA (cDNA) synthesis was performed as

described previously [14]. 1 µl of complementary DNA was used in a 10 µl volume with Fast SYBR Green Master Mix (Applied Biosystems, Massachusetts, USA) and diluted with validated primers (Invitrogen) for IP-10 (fwd: TCCACGTGTTGAGATCATTGC, rev: TCTTGATGGCCTTCGATTCTG) MCP-1 (fwd: CCATTGTGGCCAAGGAGATC, rev: TGTCCAGGTGGTCCATGGA), RANTES (fwd: CCAGCAGTCGTCTTTGTCAC rev: CTCTGGGTTGGCACACACTT) and β-actin (fwd: CCTGGCACCCAGCACAAT rev: GCCGATCCACACGGAGTACT). The $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expression normalized to the expression of β-actin rRNA.

ELISA

Supernatants were then analyzed for measurement of IL-6, IL-8, MCP-1 (PharMingen, Oxford, UK) and IP-10 levels (R&D systems, Bio-Techne Ltd, Oxford) as described previously [8, 9]. Absorbance was examined on a spectrophotometric ELISA plate reader (Multiscan Biochromic; Labsystems, Helsinki, Finland) and analyzed using a DeltaSoft II.4 software program (DeltaSoft, Princeton, NJ).

MSD STAT pathway analysis

pSTAT3 and pSTAT5 samples were assayed in duplicates using the MSD Phospho-STAT3 (Tyr705) and Phospho (Tyr694)/Total STAT5a,b Assay Whole Cell Lysate Kit according to the manufacturer's instructions (MesoScale Discovery, Gaithersburg, MD, USA).

Statistical analysis

Graph Pad Prism 7.03 software package (Graph Pad Software, La Jolla, CA, USA) was employed to analyse the data using one-way ANOVA analysis of variance with Bonferroni adjustment for multiple comparisons.

Results:

We first investigated the effect of baricitinib in regulating JAK/STAT signaling in RA FLS by Western blotting using cell lysates. OSM induced phosphorylation of Jak1(Tyr1034/1035), Jak2 (Tyr1008), Stat3 (Ser727), and Stat5 (Tyr694) proteins which were significantly inhibited by baricitinib (Figure 1). Moreover, by implementing MSD assays in cell lysates we also demonstrated that baricitinib significantly inhibited phosphorylation of both STAT isoforms in RA, OA and Normal FLS (Figure 2A & 2B). Next, we sought to investigate whether baricitinib could suppress OSM induced cytokine production. OSM induced significant levels of IL-6 and MCP in RA FLS, but only enhanced production in OA FLS in one out of three donors tested. Baricitinib inhibited OSM-induced IL-6 and MCP-1 production in RA FLS in a dose dependent manner (Figure 3A). In comparison, in OA FLS, inhibition of OSM-induced IL-6 and MCP-1 was achieved by baricitinib at the highest 1 μM concentration in the one responsive donor (Figure 3B). Additionally, we investigated the effect of baricitinib on transcription and found that mRNA levels of both MCP-1 and IP-10 were inhibited in RA and OA FLS in a dose-dependent manner. Transcription of RANTES occurred only in OA FLS in response to OSM and was also inhibited by baricitinib (Figure 4A & 4B).

We then investigated the effect of baricitinib in HSF as well as in RA FLS stimulated with either TNFα or IL-1β to observe any differences in the expression of pro inflammatory cytokines and chemokines between healthy and pathological cells in response to baricitinib. As depicted in Figure 5A, baricitinib did not directly affect IL-6 and IL-8 protein levels in HSF. Interestingly, we found that baricitinib significantly inhibited TNFα and IL-1β-induced MCP-1 and IP-10 protein levels in a dose dependent

manner in HSF (Figure 5B). In RA FLS stimulated with either TNFα or IL-1β, we were not able to observe any inhibitory effect of baricitinib (Figure 6). Similar data was obtained from experiments using FLS derived from normal or OA synovial samples (not shown).

In parallel experiments, we examined the effect of baricitinib on the spontaneous production of IL-6 and IL-8, by freshly isolated RA and OA synovial membrane cells (RA SMCs and OA SMCs). As depicted in Figure 7A and 7B, baricitinib at 1μM concentration was not effective in inhibiting IL-6, IL-8 and MCP-1 expression in these cells. However, baricitinib inhibited basal mRNA levels of the cytokines IP-10, MCP-1 and RANTES in RA synovial mixed cell cultures with no effect in OA SMC levels (Figure 8).

Discussion

In this study we aimed to investigate the effect of baricitinib, a JAK1/2 inhibitor, in FLS obtained from surgical synovial tissue of patients with RA, as these cells are considered a major contributor to the pathogenesis of RA. In our experimental design we included OA FLS and fibroblasts derived from normal synovium as control tissue to allow us to better understand differences between cells derived from an inflammatory and non-inflammatory milieu. As a stimulus we used OSM as a previous study by Migita et al has shown that OSM activates the JAK/STAT pathway in RA FLS [15]. In their study, they compared the effects of PF-956980 (JAK3 inhibitor), tofacitinib (JAK1/3 inhibitor) and baricitinib, and they reported that complete inhibition of OSM-induced JAK (1,2,3) activation and downstream STAT (1,3,5) phosphorylation in RA and OA FLS was achieved by baricitinib at a concentration of 0.5-1 μM [15]. We were

also able to confirm inhibition of OSM induced JAK1/2 and STAT 3/5 phosphorylation in RA FLS by baricitinib. Moreover, we were able to repeat the findings of Migita et al on the effect of baricitinib on JAK/STAT activation in RA, OA and HC FLS, using a novel and less time-consuming approach utilizing MSD kits. In another study investigating the effect of the pan-JAK inhibitor peficitinib in activated RA synovial fibroblasts, tofacitnib and baricitinib were used as a comparators and baricitinib was shown to inhibit OSM induced IL-6 release by RA FLS at a concentration between 1-5 μM. However, baricitinib failed to inhibit IL-1β induced IL-6 expression by these cells [16]. By repeating their experiments, we confirmed that baricitinib was not effective in inhibiting the production of TNFα- and IL-1β-induced cytokines or chemokines in RA FLS. However, in contrast to the findings in RA FLS, in our study baricitinib was found to inhibit the TNFα- and IL-1β-induced MCP-1 and IP-10, but not IL-6 or IL-8, from HSF, another cell line often used as a control. Our findings suggest that in HSF, baricitinib has an indirect anti-inflammatory effect that acts “downstream” of cytokines which do not signal through JAKs, presumably by inhibition of induced JAK-dependent cytokines.

Further investigation will be necessary to understand the intermediary steps involved. Rosengren et al reported that in RA FLS, the JAK1,3 selective inhibitor tofacitinib inhibited TNF-induced expression of several chemokines, including IP-10, RANTES and MCP, both at the messenger RNA and protein levels [13]. The effect of tofacitinib on IP-10 was particularly remarkable with inhibition in excess of 80–90%, with a lesser although still significant inhibition of MCP-1 and RANTES expression [13]. It was also shown that TNFα indirectly induced IP-10 and RANTES production via the release of type I IFN-β, which in an autocrine fashion was able to stimulate JAK/STAT activation that was inhibited by tofacitinib. Since the use of control cells was missing in these

studies [13, 16], in our experiments we included OA FLS as a control. Whereas we observed similar findings in RA FLS, demonstrating inhibition of OSM induced IL-6 and MCP-1 release, in OA FLS we found that OSM induced cytokine and chemokine release at similar concentrations to those of RA FLS and that baricitinib was also effective in inhibiting their release.

In our study we have also included RA SMCs. This cell population represents a complex but pathophysiologically relevant mixture mainly comprising T cells, as well as monocyte/macrophages and FLS, which spontaneously secrete a variety of pro and anti-inflammatory cytokines in quantities readily detectable by ELISA [17, 18].[17, 18] However, we did not observe inhibition of spontaneous production of IL-6. IL-8 or MCP-1 by baricitinib at the concentrations tested. Moreover JAK inhibition by peficitinib has been shown to decrease cell proliferation and migration of RA FLS [16]. Further studies will be required to investigate the pharmacological effects of baricitib in RA, OA and HC FLS with regard to cell proliferation and migration.

Several JAK inhibitors with variable degrees of selectivity for the JAK enzymes have been investigated in inflammatory arthritis. Although the JAK inhibitors have proven to be efficacious in the clinical trial programs and real-world data to date, concerns remain regarding long-term safety and rarer target related toxicities that might be associated with JAK inhibition. Thus, more data are needed to better understand the relationship between mechanism of action of JAK inhibition and the benefit: risk profile at an individual level [19-22].

Synovial samples obtained at the time of synovial joint surgery need to be carefully examined both macroscopically and microscopically for features of RA such as hyperplasia of the intimal lining layer and the accumulation of inflammatory cells [23]. While we are confident that synovial tissue obtained from RA patients comprised

inflammatory cells, we cannot exclude the possibility that patients also had features of secondary osteoarthritis. The possible heterogeneity of disease features in subjects with established RA represents a potential limitation of our study.

In summary, our data shows that baricitinib does not alter spontaneously released pro-inflammatory mediators from RA synovial membrane cultures. However, our data has shown that baricitinib has a direct effect on proinflammatory cytokine production in FLS induced by cytokines such as OSM which signal through JAK1,2. Moreover, our data is in agreement with recent studies which show that baricitinib does not affect TNFα– and IL-1β-induced IL-6, IL-8 and MCP-1 protein levels in RA FLS, confirming that both stimuli signal through JAK-independent pathways in these cells.

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Author conflicts of interest

PCT has received research grants from Lilly, and has received consultation fees from Lilly.

Figures

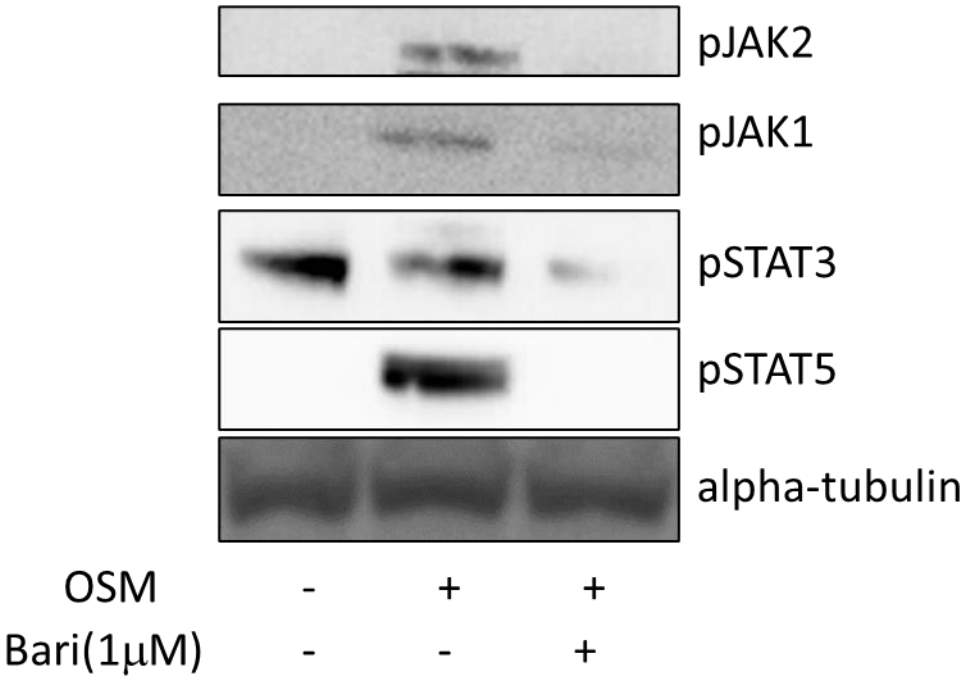

Figure 1: Baricitinib inhibits phospho JAK1 and 2 and phospho STAT 5 and 3 in RA FLS. FLS were pre-incubated for 2 hours with baricitinib before stimulating with 20 ng/ml of OSM for 30 mins. JAK1 /2 and STAT3/5 phosphorylation was determined in cell lysates using a Western Blotting Application Solutions Kit #12957 and anti-rabbit antibodies for Phospho-Jak1(Tyr1034/1035), Phospho-Jak2 (Tyr1008), Phospho-Stat3 (Ser727) and Phospho-Stat5 (Tyr694) from Cell Signaling Technology following the manufactures western blotting protocol (Cell Signaling Technology Massachusetts, USA).

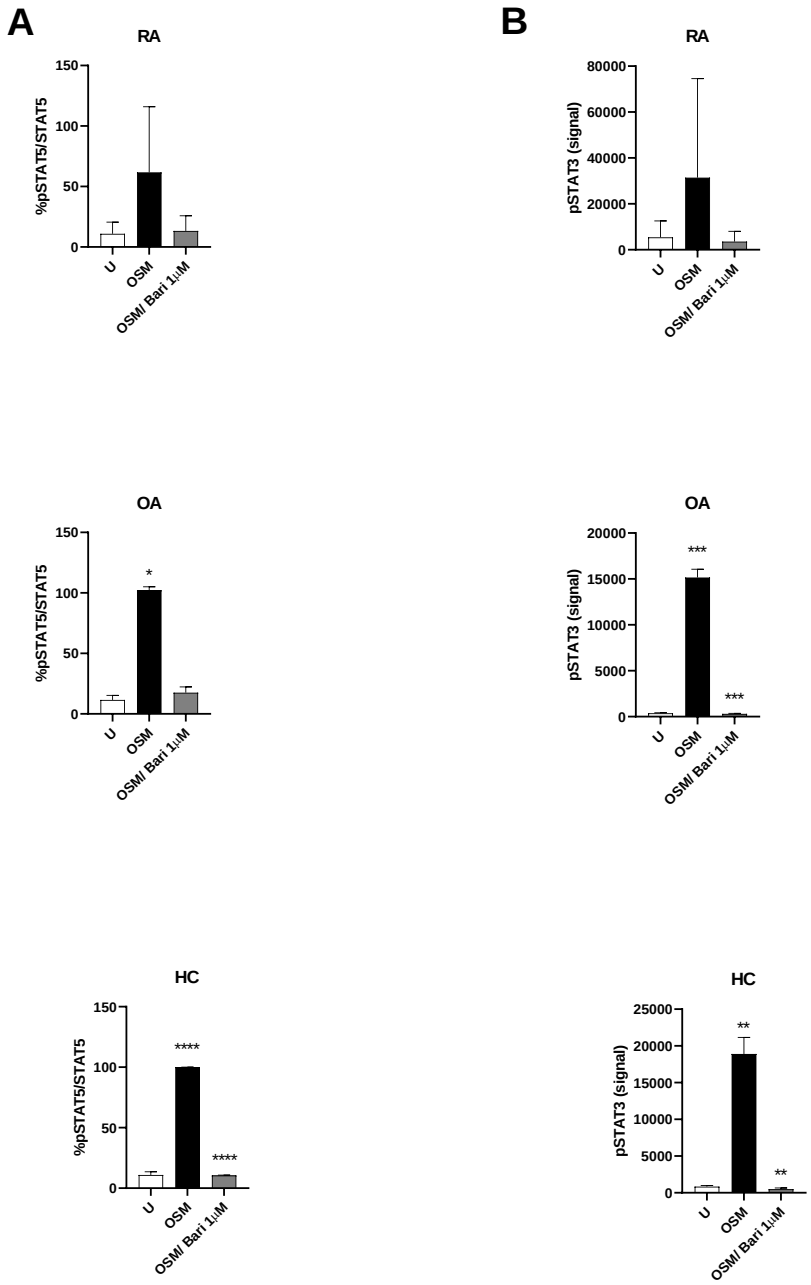

Figure 2: Baricitinib inhibits phospho STAT 5 and 3 in RA, OA and Healthy control (HC) FLS. FLS were pre-incubated for 2 hours with baricitinib before stimulating with 20 ng/ml of OSM for 30 mins. Phospho STAT3, phospho STAT5 and total STAT5 was determined in cell lysates using MSD assays. The mean ± s.d. of doublets from 3 experiments of each cell type is shown. ∗ = P < 0.05 unstimulated versus OSM: ∗∗ = P < 0.01; ∗∗∗ = P < 0.001; ∗∗∗∗ = P < 0.0001 unstimulated versus OSM and OSM versus OSM/ baricitinib.

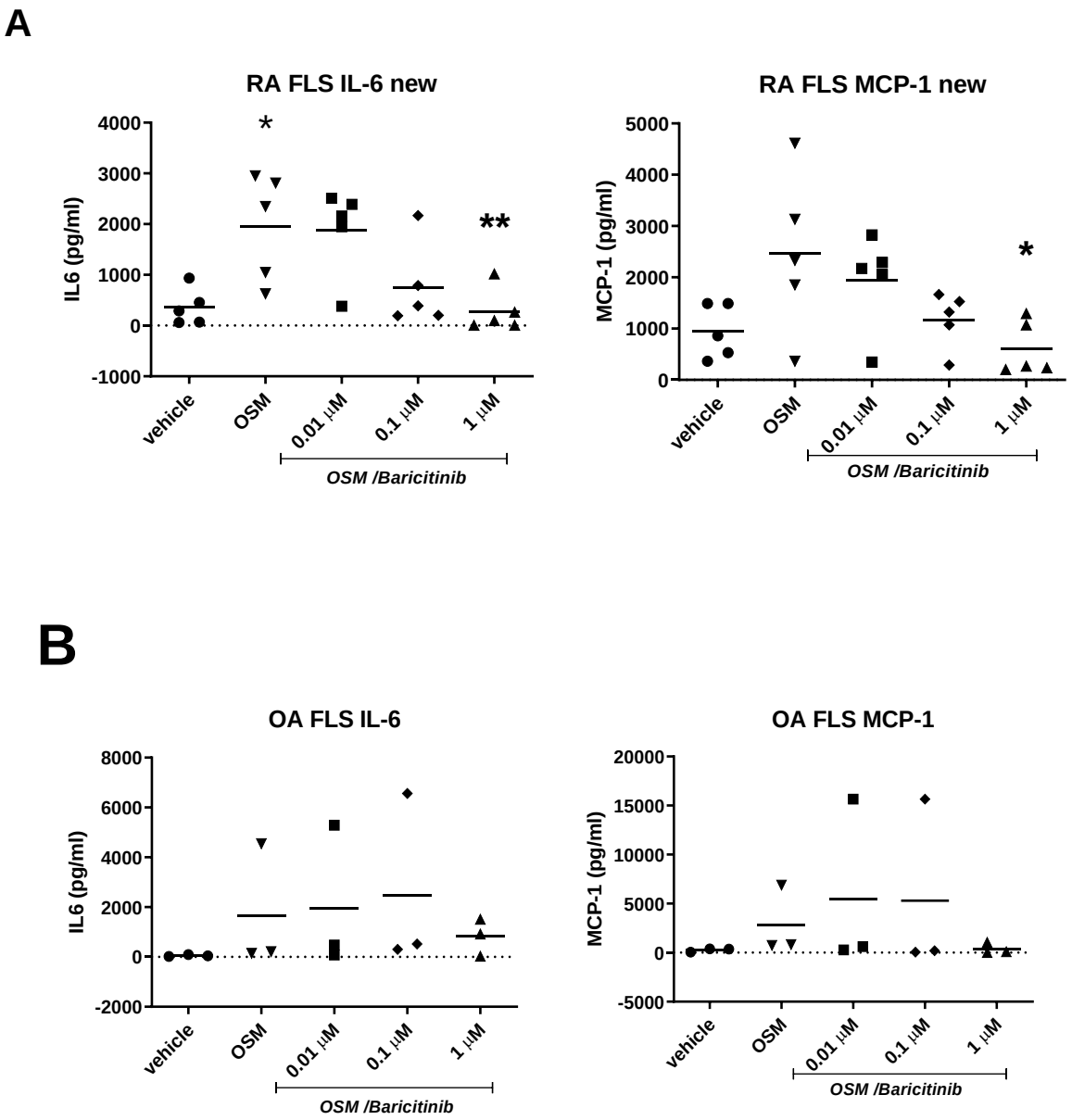

Figure 3: Barcitinib inhibits OSM-induced IL-6 and MCP-1 production by (A) RA FLS and (B) OA FLS. FLS were pre-incubated for 2 hours with baricitinib (0.01, 0.1 and 1 μM) before stimulating with 20 ng/ml of OSM for 24 hours. Cell supernatants (n=4 for RA FLS and n=3 for OA FLS) were collected and levels of IL-6 and MCP-1 were measured by ELISA. Each point represents the mean ± s.d. of triplicates from one individual experiment. ∗= P < 0.1; (versus unstimulated); ∗= P < 0.1; ∗∗ = P < 0.01 (versus cells stimulated with OSM).

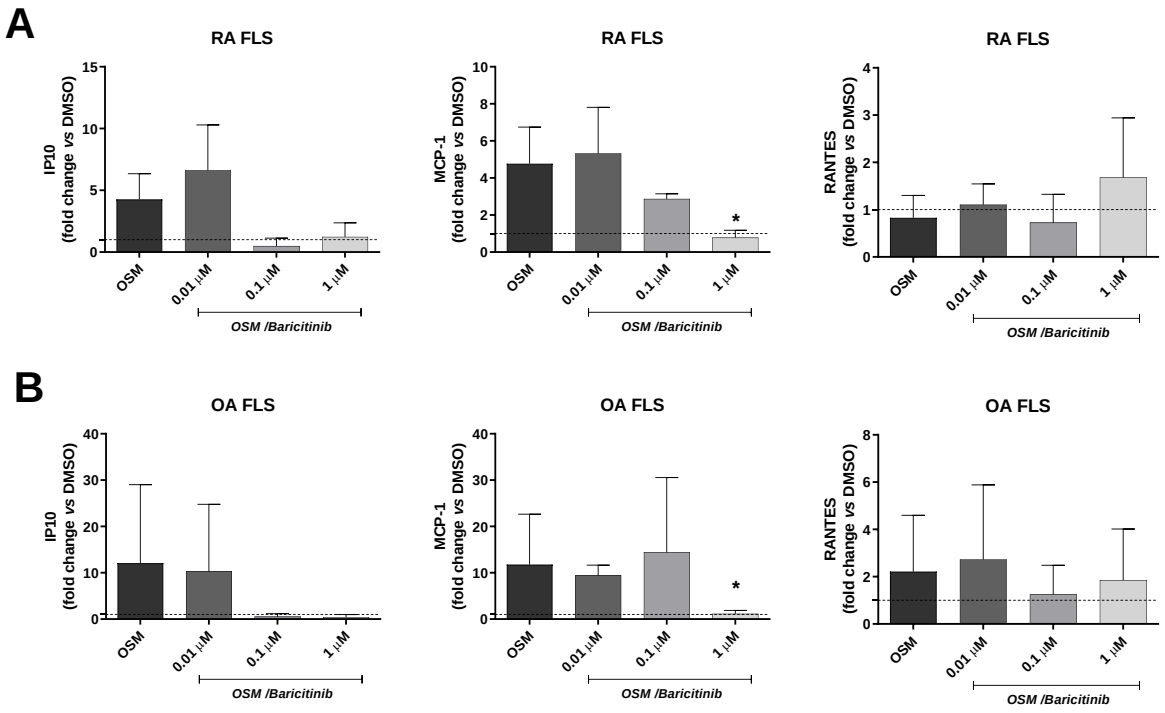

Figure 4: Barcitinib inhibits OSM-induced IP-10 and MCP-1 mRNA expression in (A) RA FLS and (B) OA FLS. FLS were pre-incubated for 2 hours with baricitinib (0.01, 0.1 and 1 μM) before stimulating with 20 ng/ml of OSM for 24 hours. Results are expressed as fold change mRNA expression (compared to β-actin). The mean ± s.d. of 4 experiments for RA and 3 for OA is shown ∗ = P < 0.1 (versus cells stimulated with OSM).

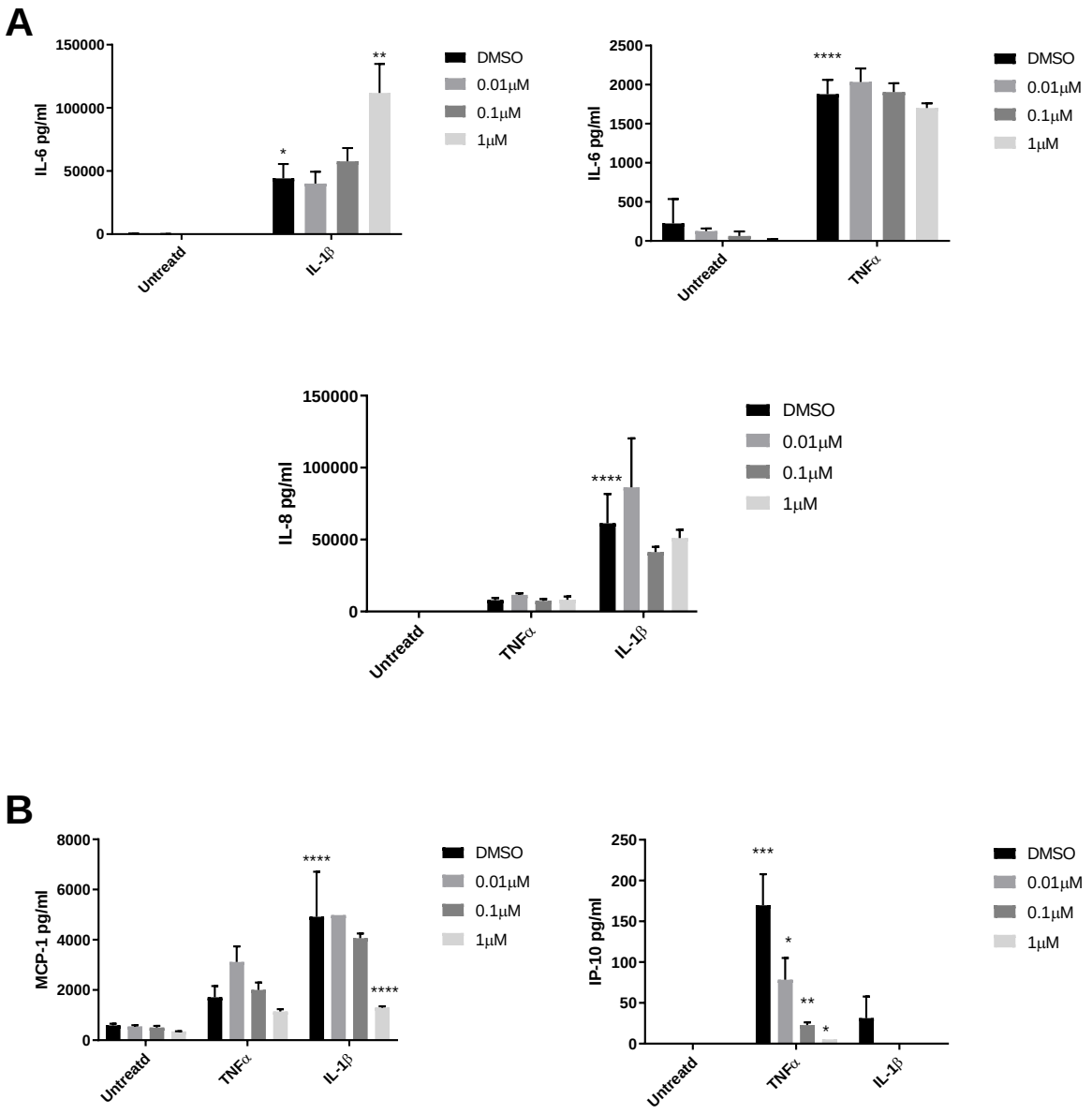

Figure 5: Baricitinib does not inhibit TNFα or IL-1β induced cytokines (IL-6 and IL-8) but does inhibit chemokines (MCP-1 and IP-10) in HSF. HSF were pre-incubated for 2 hours with baricitinib (0.01, 0.1 and 1 μM) before stimulating with 10 ng/ml TNFα or 10 ng/ml IL-1β for 24 hours. Cell supernatants were collected and levels of cytokines and chemokines were measured by ELISA. The mean ± s.d. of triplicates from one representative experiment of a total of 2 is shown. (A) IL-6 and IL-8 levels are not affected by baricitinib * = P < 0.1, **** = P < 0.0001 (versus untreated), ** = P < 0.01 (versus IL-1β). (B) TNFα- and IL-1β-induced MCP-1 and IP-10 are both inhibited by baricitinib. *** = P < 0.001; **** = P < 0.0001 (versus unstimulated); * = P < 0.01; ** = P < 0.01 (versus stimulated with TNF α); **** = P < 0.0001 (versus cells stimulated with IL-1β).

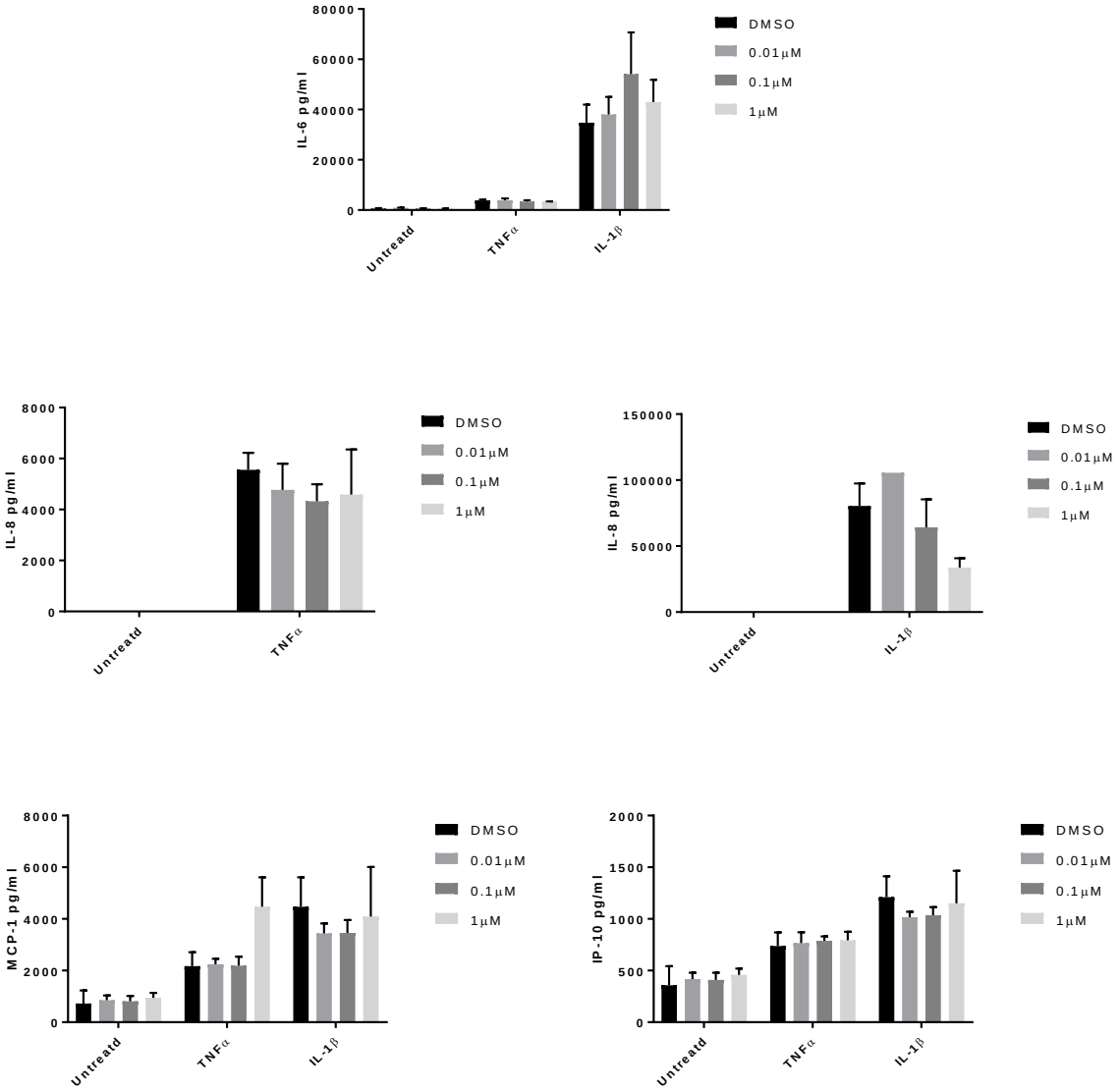

Figure 6: Baricitinib does not inhibit TNFα or IL-1β induced cytokines and chemokines in RA FLS. RA FLS were pre-incubated for 2 hours with the baricitinib (0.01, 0.1 and 1 μM) before stimulating with 10 ng/ml TNFα or 10 ng/ml IL-1β for 24 hours. Cell supernatants were collected, and levels of cytokines and chemokines were measured by ELISA. The mean ± s.d. of triplicates from one representative experiment of a total of three is shown.

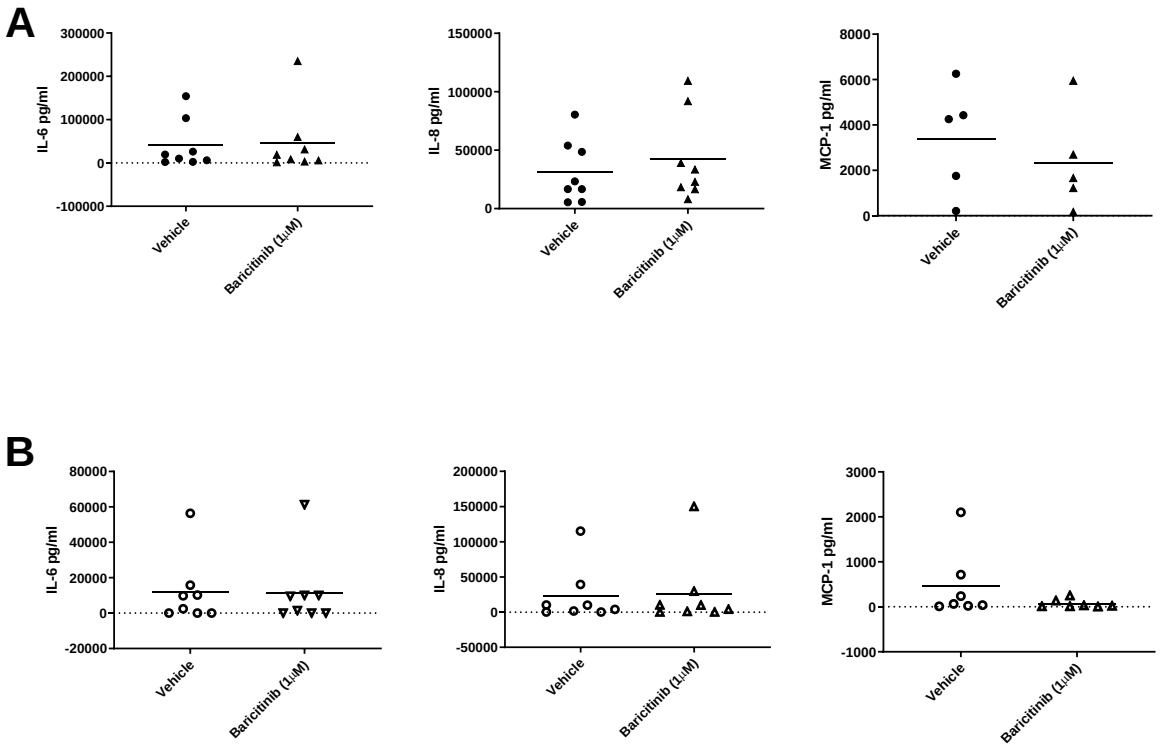

Figure 7: Baricitinib does not reduce spontaneous IL-6, IL-8 but does inhibit MCP-1 release in (A) RA synovial membrane cells (RA SMC, n=8), and (B) OA synovial membrane cells (OA SMC, n=8) derived from patients undergoing joint replacement surgery. Synovial membrane cells were cultured for 24 hours in the presence of baricitinib. Cell supernatants were collected, and levels of cytokines were measured by ELISA. Each point represents the mean ± s.d. of triplicates from one individual patient.

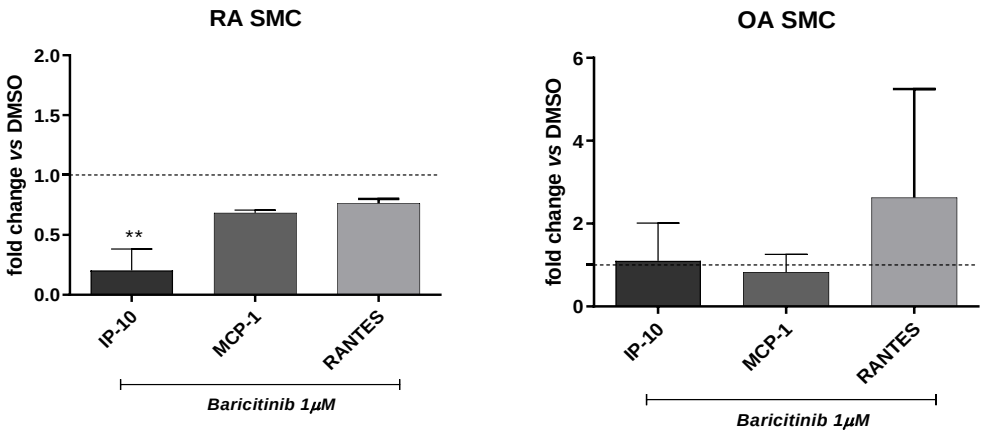

Figure 8: Barcitinib inhibits IP-10, MCP-1 and RANTES mRNA expression only in RA but not OA SMC derived from patients undergoing joint replacement surgery. Synovial membrane cells were cultured for 24 hours in the presence of baricitinib. Results are expressed as fold change mRNA expression (compared to β-actin). The mean ± s.d. of 2 experiments for RA and 4 for OA is shown. ** = $P < 0.01$ (versus DMSO).