**JAK inhibitors prevent migration of rheumatoid arthritis neutrophils towards interleukin-8, but do not inhibit priming of the respiratory burst or ROS production**

Short title: Effect of JAK inhibitors on RA neutrophil priming and chemotaxis

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Key words: Neutrophils, rheumatoid arthritis, JAK inhibitors, tofacitinib, baricitinib, ROS, apoptosis

Abbreviations: ACPA, anti-citrullinated peptide antibodies; DMARD, disease-modifying anti-rheumatic drugs; ECL, Enhanced chemiluminescence; fMLP, f-Met-Leu-Phe; GM-CSF, granulocyte/macrophage-colony stimulating factor; IFN, interferon; IL, interleukin; JAKi, janus kinase inhibitors; MMP, matrix metalloproteinases; NET, neutrophil extracellular traps; PAD4, protein arginine deiminase 4; PMA, phorbol-12-myristate-12-acetate; RA, rheumatoid arthritis; ROS, reactive oxygen species; STAT, signal transducer and activator of transcription; TNFi, TNF inhibitors.

**SUMMARY**

Neutrophils play a crucial role in the pathophysiology of rheumatoid arthritis (RA), via the release of reactive oxygen species (ROS), proteases and cytokines. Orally-active JAK inhibitors (JAKi), e.g. baricitinib and tofacitinib, have high clinical efficacy in RA but are linked with neutropenia and increased infections. Our aim was to determine the effect of JAK inhibition with baricitinib and tofacitinib on healthy control and RA neutrophil lifespan and function. RA (n=7) and healthy control (n=7) neutrophils were treated with baricitinib or tofacitinib for 30 min, prior to incubation in the absence or presence of GM-CSF or IFNγ. JAKi prevented GM-CSF- and IFNγ-induced apoptosis delay in RA and healthy control neutrophils in a dose-dependent manner. Baricitinib decreased the rate of chemotaxis towards IL-8, but not fMLP, in RA neutrophils. Whilst healthy control neutrophils incubated with GM-CSF became primed to produce ROS in response to stimulation with fMLP and PMA, RA neutrophils produced increased levels of ROS without the need for priming. JAKi prevented ROS release from primed healthy control neutrophils in response to fMLP, but had no effect on ROS production by RA neutrophils. Baricitinib reversed GM-CSF priming of ROS production in response to fMLP in healthy control, but not RA, neutrophils. We conclude that incubation with JAKi prevents chemotaxis of RA neutrophils towards IL-8, but does not prevent the production of ROS or increase the level of apoptosis. This may be due to the *in vivo* exposure of RA neutrophils to priming agents other than those that activate JAK/STAT signalling*.*

**INTRODUCTION**

Orally-active JAK inhibitors (JAKi), including tofacitinib (JAK3/JAK1 inhibitor) and baricitinib (JAK1/JAK2 inhibitor), show efficacy in treating rheumatoid arthritis (RA) patients who are refractory to disease-modifying anti-rheumatic drugs (DMARDs) and biologic therapies, including tumour necrosis factor inhibitors (TNFi)1-5. The molecular mechanisms of JAK inhibitors in decreasing disease activity in RA are still emerging. For example, tofacitinib decreases IL-17/IFN-γ production and CD4+ T cell proliferation6, and suppresses synovial production of matrix metalloproteinases (MMPs) and chemokines via decreased STAT1 and STAT3 activation7. However, whilst baricitinib is clinically effective in the treatment of RA4, to date there are no published studies on the molecular effects of baricitinib at the cellular level in RA or any other disease.

Neutrophils are implicated in the pathophysiology of RA via secretion of degradative enzymes such as elastase and collagenase, and reactive oxygen species (ROS)8-10. Neutrophils also contribute to the cytokine/chemokine cascades that accompany inflammation and regulate immune responses11. Production of neutrophil extracellular traps (NETs), containing citrullinated peptide residues, are implicated in the production of anti-citrullinated peptide antibodies (ACPA) and development of RA12-15, and neutrophils are one of the few cells expressing high levels of active protein arginine deiminase 4 (PAD4), the enzyme principally responsible for the citrullination of peptides8, 15, 16. However, neutrophils play a key role in immunity and host protection against micro-organisms via phagocytosis and production of ROS10, and therefore drug-induced neutropenia and/or impairment of normal neutrophil function has serious implications for host-defence in immuno-suppressed patients. Clinical trials of JAKi have reported transient drops in neutrophil counts during therapy1, 4, 5, 17 and increased rates of infection in patients receiving JAKi (compared to TNFi or placebo). More specifically, clinical trials have reported increased cases of bronchitis, herpes zoster, influenza, and urinary- and upper respiratory-tract infections in patients receiving JAKi2, 3, 5, 18, suggesting drug-induced neutropenia and/or decreased neutrophil function.

The aim of this study was to investigate the effect of JAK inhibition with baricitinib (JAK1/2 inhibitor) and tofacitinib (JAK3 inhibitor) on the normal neutrophil response to *in vitro* cytokine priming in healthy controls, and on the *ex vivo* activated phenotype of neutrophils isolated from the peripheral blood of patients with RA. We present data showing the effect of baricitinib and tofacitinib on three key aspects of neutrophil function which are important in the response to infection, but also implicated in unwanted activation during inflammatory disease: apoptosis, chemotaxis and ROS production.

**METHODS**

**Materials**

Ficoll-Paque, Horse Radish Peroxidase-linked anti-rabbit IgG antibody, photographic film (GE Healthcare, Chalfont St Giles, UK); RPMI 1640 media, Annexin V-FITC (Life Technologies, Paisley, UK); 3μm porous membrane hanging cell inserts, phosphatase inhibitor cocktail II, PVDF membrane ECL reagent (Merck, Watford, UK); Propidium Iodide, luminol, phorbol 12-myristate 13-acetate (PMA), human AB serum, f-Met-Leu-Phe (fMLP), interleukin-8 (IL-8), HRP-linked anti-mouse IgG antibody, poly-hema (Sigma, Gillingham, UK); Actin antibody (Abcam, Cambridge, UK); phosphorylated-STAT1, phosphorylated-STAT3 antibodies (New England Biosciences, Hitchin, UK); tofacitinib, baricitinib (Stratech, Newmarket, UK); GM-CSF, interferon-γ (Roche Applied Sciences, Burgess Hill, UK).

**Patients and Controls**

This study was approved by the University of Liverpool Committee on Research Ethics for healthy controls, and NRES Committee North West (Greater Manchester West, UK) for RA patients. All participants gave written, informed consent. Patients with active RA were recruited from University Hospital Aintree. Clinical demographics are shown in Table 1. All patients were receiving DMARD therapy with methotrexate (n=7), along with concominant hydroxychloriquine (n=4), sulfasalazine (n=3) and/or leflunamide (n=1).

**Isolation of neutrophils**

Neutrophils (purity typically >97%) were isolated from heparinised whole blood using Ficoll-Paque as previously described9, 19 and resuspended in RPMI 1640 media plus 25mM Hepes. Neutrophils were incubated with therapeutically and experimentally relevant concentrations6, 20 of tofacitinib (200ng/mL) and baricitinib (200ng/mL) for 30 min prior to assay. DMSO was used as a vehicle control in all incubations at the same concentration as JAKi (v/v).

**Measurement of apoptosis**

Neutrophils (106/mL) were incubated at 37°C in 5% CO2 in RPMI 1640 (+Hepes, +L-glutamine, +10% AB serum) for up to 20h in the absence/presence of GM-CSF (5ng/mL) or interferon (IFN)-γ (10ng/mL). Apoptosis was measured using Annexin V-FITC/propidium-iodide (PI, 1μg/mL) staining19 before analysis by flow cytometry (>5,000 events analysed) using a Dako CyAn flow cytometer. Apoptotic cells were defined as those being positive for Annexin V.

**Chemotaxis assay**

The chemotaxis assay was carried out in 24-well tissue culture plates (coated with 12mg/mL poly-hema to prevent cell adhesion) using hanging chamber inserts with a 3μM porous membrane to separate media in the top and bottom chamber, as previously described21. fMLP (10-8M) or IL-8 (100ng/mL) was added to RPMI media in the bottom chamber. Neutrophils (106/mL) were added to the top chamber and incubated for 90 min at 37°C and 5% CO2. The number of migrated cells after 90 min incubation was measured using a Coulter Counter Multisizer-3 (Beckman Coulter).

**Measurement of the respiratory burst**

Neutrophils (5x106/mL) were incubated with JAK inhibitors for 30 mins prior to GM-CSF (5ng/mL) priming for 45 min. ROS production was stimulated with fMLP (1μM) or phorbol-12-myristate-13-acetate (PMA, 100ng/mL). Luminol-enhanced chemiluminescence (luminol, 10μM) was measured continuously for 30 min using a Tecan plate reader at 37°C. For reverse-priming experiments, neutrophils were incubated with GM-CSF (5ng/mL) prior to the addition of JAKi. ROS production was measured in response to fMLP and PMA at 30 min intervals for up to 120 min after addition of JAKi.

**Protein analysis**

Neutrophils were incubated as detailed in Results, with or without the addition of JAKi (0-200ng/mL), GM-CSF (5ng/mL), IFN-γ (10ng/mL), fMLP (10-8M) or IL-8 (100ng/mL) for up to 60 min. Neutrophils were lysed in boiling, reducing Laemmli buffer containing phosphatase inhibitor cocktail II, and proteins separated by 10% SDS-PAGE prior to transfer onto PVDF membrane. Primary antibodies were: anti-phosphorylated STAT1 (1:1,000), anti-phosphorylated STAT3 (1:1,000) and actin (1:10,000). Secondary antibodies were HRP-linked anti-rabbit IgG (1:20,000) and HRP-linked anti-mouse IgG (1:10,000). Bound antibodies were detected using the ECL system on carefully-exposed film to avoid saturation. Protein expression was measured as relative abundance to Actin loading control.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism (v5).

**RESULTS**

**Effect of JAK inhibition on neutrophil apoptosis**

Delayed neutrophil apoptosis is an important response to infection, but an unwanted phenomenon in inflammatory diseases such as RA22. We determined the dose-dependent effect of JAKi on neutrophil apoptosis using healthy control neutrophils incubated with baricitinib and tofacitinib for 20h over a range of clinically relevant concentrations, with and without the addition of GM-CSF or IFNγ, cytokines known to signal via the JAK/STAT pathway. GM-CSF and IFNγ significantly delayed neutrophil apoptosis compared to unstimulated (constitutive) levels (Figure 1A, n=4, §p<0.05). Neither JAKi exerted any effect on the constitutive rate of neutrophil apoptosis. GM-CSF-delayed apoptosis was abrogated by both baricitinib and tofacitinib over a range of concentrations (10-200ng/mL) (Figure 1A, n=4, \*p<0.05). Whilst baricitinib inhibited IFNγ-delayed apoptosis at higher concentrations (100-200ng/mL), tofacitinib only had a significant effect at the highest concentration (200ng/mL). The specificity of the inhibition of GM-CSF- and IFNγ-induced JAK signalling was confirmed by Western blotting for STAT proteins know to be unphosphorylated in healthy control neutrophils23, 24 but activated by both cytokines (STAT3 by GM-CSF; STAT1 by IFNγ). Neutrophils were incubated with increasing concentrations of baricitinib or tofacitinib (0-200ng/mL) for 30 min prior to the addition of GM-CSF (5ng/mL) or IFNγ (10ng/mL). Baricitinib and tofacitinib both blocked the phosphorylation of STAT3 by GM-CSF at concentrations >50ng/mL (Figure 1B, n=3, \*p<0.05). However, significant inhibition of STAT1 phosphorylation by IFNγ was only obtained when incubating with baricitinib at 100 and 200ng/mL and tofacitinib at the highest concentration (200ng/mL, Figure 1B, n=3, \*p<0.05). A concentration of 200ng/mL was therefore used for both drugs for further experiments.

We next measured the effect of JAK inhibition by baricitinib and tofacitinib at the highest concentration (200ng/mL) on apoptosis in neutrophils from patients with RA, with and without stimulation with GM-CSF and IFNγ. After 2h incubation the JAK inhibitors had no significant effect on constitutive or cytokine-delayed neutrophil apoptosis (data not shown). By 20h, both baricitinib and tofacitinib inhibited the anti-apoptotic effect of GM-CSF (Figure 1C, n=7; \*p<0.05, \*\*p<0.01). IFNγ did not significantly delay RA neutrophil apoptosis.

**Effect of JAK inhibitors on neutrophil chemotaxis**

Neutrophil migration from the blood stream to sites of inflammation is an essential process in the innate immune response to infection. However, in inflammatory diseases such as RA, migration of neutrophils into synovial joints, and their subsequent activation within the joint, contributes to joint damage and persistent inflammation8. In order to determine the effect of baricitinib and tofacitinib on neutrophil chemotaxis we measured the effect of both drugs on migration towards fMLP (a bacterial peptide) and IL-8 (a chemokine found at high levels in RA joints)25. Increased numbers of neutrophils from healthy controls and patients with RA migrated towards fMLP and IL-8. Whilst this was significant in healthy controls (Figure 2A, n=6, \*p<0.05, \*\*p<0.01), this did not reach significance across the entire population of RA patients (n=7, p=0.06). Similarly, comparison of the number of neutrophils migrating towards fMLP between healthy controls and patients with RA was not statistically significant (p=0.06). Both inhibitors significantly decreased the level of random migration in RA neutrophils (Figure 2B, n=7, \*p<0.05), and baricitinib significantly decreased the number of RA neutrophils that migrated towards IL-8 (Figure 2C, n=7, \*p<0.05). Neither inhibitor affected the rate of chemotaxis towards fMLP in healthy control or RA patients. Western blotting of neutrophils incubated for up to 60 min with fMLP or IL-8 showed that neither STAT1 nor STAT3 was phosphorylated by either chemoattractant at the concentrations used in the migration experiments (Figure 2E, n=3).

**Effect of JAK inhibitors on ROS production**

Neutrophils produce ROS as part of the respiratory burst, a vital process in bacterial killing10. However, ROS production within RA synovial joints contributes to inflammation and is associated with the release of proteases that can damage the cartilage and joint tissues8, 9. We measured production of ROS stimulated by fMLP (receptor-dependent) and PMA (receptor-independent) in neutrophils incubated with baricitinib and tofacitinib for 30 min, and in JAKi-treated neutrophils incubated with and without GM-CSF priming for 45 min. Neither inhibitor affected the level of ROS production in unprimed control or RA neutrophils stimulated with fMLP (Figure 3A, n=7). In healthy controls, the level of ROS production in response to fMLP stimulation was significantly increased by GM-CSF priming, and this effect was completely blocked by baricitinib and tofacitinib (Figure 3B, n=7, p<0.05). The level of ROS produced by unprimed RA neutrophils was higher than in healthy controls, and was not increased following incubation with GM-CSF (Figure 3B). Incubation with baricitinib and tofacitinib did not inhibit the production of ROS by RA neutrophils in response to fMLP (Figure 3B). We also measured ROS production in response to PMA in inhibitor treated neutrophils, before and after priming with GM-CSF. Tofacitinib significantly increased the amount of ROS produced by unprimed RA neutrophils in response to PMA (Figure 3C, n=7 p<0.05). Following priming with GM-CSF, the amount of ROS produced by control neutrophils treated with baricitinib and tofacitinib was significantly increased (Figure 3D, n=7, p<0.05).

We next measured whether JAK inhibitors could “reverse prime” neutrophils that had been primed with GM-CSF. Neutrophils from healthy controls and RA patients were primed with GM-CSF (5ng/mL) for 45 min prior to the addition of baricitinib and tofacitinib (200ng/mL). ROS production was measured every 30 min for a further 120 min. Baricitinib significantly decreased ROS production in response to fMLP, but not PMA, in GM-CSF-primed control neutrophils after 30 and 60 min (Figure 4A,B \*p<0.05, \*\*p<0.01, n=3). This effect was not observed in RA neutrophils (Figure 4C,D n=3). Tofacitinib had no reverse-priming effect in either control or RA neutrophils.

**Effect of JAK inhibitors on STAT activation in RA neutrophils**

We have shown previously that STAT1 and STAT3 are activated in neutrophils freshly isolated from the peripheral blood of RA patients23. In order to investigate the effect of JAK inhibitors on STAT activation in RA neutrophils, we incubated freshly isolated neutrophils for up to 60 min in the absence and presence of baricitinib and tofacitinib (200ng/mL). Western blotting for phosphorylated STAT1 and STAT3 showed that in untreated RA neutrophils, STAT1 and STAT3 activation decreased by around 50% over 60 min incubation (Figure 5, n=4). The addition of baricitinib and tofacitinib increased the loss of phosphorylated STAT1 and STAT3 to around 50% by 30 min, although this did not reach statistical significance.

**DISCUSSION**

In this study we determined the effect of JAK inhibition by baricitinib (JAK1/2 inhibitor) and tofacitinib (JAK3 inhibitor) on three key aspects of neutrophil function which are important in the response to infection, but implicated in unwanted activation during inflammatory disease: apoptosis, chemotaxis and ROS production. In line with other published work, we found that GM-CSF decreased the level of apoptosis in healthy control and RA neutrophils22. IFNγ delayed apoptosis in healthy control, but not RA, neutrophils. Neutrophils from RA patients have a gene and protein expression profile indicating activation by interferons *in vivo*23. Therefore, one explanation of our observation is that RA neutrophils are un-responsive to further stimulation by IFNγ *in vitro* because this signalling pathway has already been activated *in vivo*.23Baricitinib and tofacitinib significantly abrogated the anti-apoptotic effect of GM-CSF and IFNγ on healthy control neutrophils in a dose-dependent manner, although the effect of tofacitinib was less evident. The GM-CSF receptor signals via a JAK2 homodimer, whilst the IFNγ receptor signals via a JAK1/JAK2 heterodimer1, therefore both cytokine:receptor signalling complexes should be direct targets for baricitinib (JAK1/2 inhibitor). The main inhibitory target of tofacitinib is JAK3. However, data from cell-free kinase assays demonstrate that tofacitinib also inhibits JAK2 and JAK1, albeit with 20- and 112-fold less potency26. Whilst tofacitinib had a less potent effect on GM-CSF-delayed neutrophil apoptosis *in vitro* than baricitinib, we did not investigate higher concentrations of tofacitinib, as this would not have reflected serum concentrations of the drug6, 20. Clinical studies have also shown that tofacitinib inhibits signalling via JAK1 and prevents phosphorylation of STAT1 in RA synovial tissue7. Indeed, our experiments show that incubation of RA neutrophils with tofacitinib (and baricitinib) enhanced the loss of phosphorylated STAT1. These published data, in concert with our own findings, would indicate that tofacitinib has a greater effect on the JAK1/JAK2 heterodimer than previously thought.

Both healthy control and RA neutrophils migrated towards fMLP and IL-8. The number of migrated cells was lower in RA patients, in line with other studies21, and may be due to prior exposure to DMARDs *in vivo.* All patients in the study were receiving methotrexate, which has previously been shown to decrease neutrophil migration both *in vivo* and *in vitro*27. Baricitinib consistently and significantly decreased RA neutrophil migration towards IL-8. IL-8 has previously been described as activating JAK2 in hepatocellular carcinoma cell lines28, which could explain our observation. However, we did not see phosphorylation of STAT1 or STAT3 in neutrophils in response to IL-8 in this study, and therefore this phenomenon remains unexplained and warrants further investigation. The effect of tofacitinib on migration towards IL-8 was not significant in our study.

Priming of healthy control neutrophils with GM-CSF prior to fMLP activation resulted in a significantly greater ROS production compared to unstimulated neutrophils, in line with previous publications24. Priming was inhibited by pre-treatment with both baricitinib and tofacitinib in healthy control neutrophils. Unprimed RA neutrophils demonstrated higher levels of ROS production than healthy controls after being activated by fMLP. This was prior to the addition of the priming agent GM-CSF, suggesting that RA neutrophils were already primed for ROS production *in vivo*29. GM-CSF priming did not increase ROS production by RA neutrophils, suggesting that once RA neutrophils have been primed *in vivo* they don’t have the ability to “re-prime”. Healthy control and RA neutrophils lost the GM-CSF-induced priming response over time *in vitro*, so that after 165 min in culture the levels of ROS produced in response to fMLP were only ~40% of the levels produced after 45 min priming. This is in line with observations of de-priming following exposure of neutrophils to other agents, such as platelet activating factor30. Addition of baricitinib to healthy neutrophils primed with GM-CSF successfully reversed the priming response (i.e. enhanced de-priming) and generation of ROS in response to fMLP after 30 min. However, neither JAK inhibitor reversed the priming effect in RA neutrophils either before or after the addition of GM-CSF, suggesting that RA neutrophils had been primed *in vivo* by an inflammatory agonist that did not activate the JAK/STAT pathway. These data are further supported by our observation that JAK inhibitors were able to increase the rate of STAT de-phosphorylation in RA neutrophils.

All RA patients in our study were receiving DMARDs, including methotrexate. The effect of existing medications such as methotrexate and TNFis on neutrophils has been described, extensively in the case of methotrexate, which has been shown to abrogate delayed apoptosis of neutrophils, and decrease both chemotaxis and ROS production27, 31. To reduce the potential effect of this confounder, further studies analysing the effect of JAK inhibitors should recruit newly diagnosed RA patients prior to commencing DMARD therapy, as this may give a clearer picture as to how JAK inhibitors affect RA neutrophil function. Further work should now focus on the ability of JAK inhibitors to modulate other key aspects of neutrophil activation in RA (e.g. NET production)8, 9, with particular attention to the risk of impaired host defence and the ability of JAK inhibitor-treated neutrophils to carry out key protective functions such as bacterial killing.

In conclusion, incubation of healthy control and RA neutrophils with baricitinib and tofacitinib inhibited the anti-apoptotic effect of GM-CSF and IFNγ in a dose-dependent manner. JAKi increased the turnover of active, phosphorylated STAT1 and STAT3 in RA neutrophils, and prevented *ex vivo* chemotaxis of RA neutrophils towards IL-8. However, JAKi did not prevent the production of ROS or increase the level of constitutive apoptosis in RA neutrophils. This may be due to the prior exposure of RA neutrophils to priming agents other than those which activate JAK/STAT signalling *in vivo.*

**ACKNOWLEDGEMENTS**

TSM was funded by the University of Liverpool MRes Clinical Sciences Research Support Fund. HLW was funded by the University of Liverpool Faculty of Health and Life Sciences.We would like to thank the rheumatology nurses and consultants at University Hospital Aintree for their assistance in recruiting patients.

**CONFLICTS OF INTEREST**

None

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**TABLES**

**Table 1. Clinical demographics of RA patients included in the study.** 1Mean; 2Range.

|  |  |
| --- | --- |
| N | 7 |
| Age (y)1,2 | 58 (42-84) |
| Sex: male, female | 4,3 |
| Disease duration (y)1,2 | 5 (0-11) |
| RF+ | 5 |
| ACPA+ | 3 |
| CRP (g/L)1,2 | 11 (2-28) |
| ESR (mm/h)1,2 | 23 (12-40) |
| DAS281,2 | 4.67 (3.1-6.6) |
| Current therapy:  - Methotrexate  - Hydroxychloriquine  - Sulfasalazine  - Leflunamide | 7  4  3  1 |

**FIGURE LEGENDS**

**Figure 1. Effect of JAK inhibition on neutrophil apoptosis**. Neutrophils from healthy controls (A,B) and (C) RA patients were incubated with baricitinib or tofacitinib over a range of concentrations (50-200ng/mL) for 30 min prior to addition of GM-CSF (5ng/mL) or interferon-γ (IFNG, 10ng/mL) for 20h. DMSO was used as a vehicle control. (A) GM-CSF- and IFNG-delayed apoptosis (§p<0.01) was abrogated by baricitinib and tofacitinib in a dose-dependent manner (\*p<0.05, \*\*p<0.01). (B) Phosphorylation of STAT3 by GM-CSF and STAT1 by IFNG occurred after 15 min incubation, and was inhibited by high concentrations of baricitinib and tofacitinib (n=3, \*p<0.05). (C) GM-CSF-delayed apoptosis was abrogated by baricitinib and tofacitinib (200ng/mL) in RA neutrophils (\*p<0.05, \*\*p<0.01).

**Figure 2. Effect of JAK inhibition on neutrophil chemotaxis.** (A) Neutrophil migration towards fMLP (10-8M) and IL-8 (100ng/mL) over 90 min was increased compared to random migration (\*p<0.05, \*\*p<0.01). (B) Pre-incubated with baricitinib and tofacitinib (200ng/mL) for 30 min decreased the rate of random migration in RA neutrophils (\*p<0.05, \*\*p<0.01). Chemotaxis towards IL-8 (C) but not fMLP (D) was inhibited by baricitinib in RA neutrophils (\*p<0.05). (E) fMLP and IL-8 did not phosphorylate STAT1 or STAT3 in healthy neutrophils over a period of 15-60 min (representative Western blot of n=3 experiments).

**Figure 3. Effect of JAK inhibition on neutrophil ROS production.** (A) Pre-incubation with baricitinib and tofacitinib for 30 min did not affect the production of ROS by unprimed neutrophils in response to fMLP (1μM), but did inhibit ROS production in healthy control neutrophils primed with GM-CSF for 45 min (B, \*p<0.05). GM-CSF priming did not increase ROS production in RA neutrophils, and JAK inhibitors had no effect on ROS production. (C) ROS production was increased by tofacitinib in response to PMA (100ng/mL) in RA neutrophils (\*p<0.05). (D) Both baricitinib and tofacitinib increased ROS production in GM-CSF primed neutrophils from healthy controls (\*p<0.05).

**Figure 4. Effect of JAK inhibition on reverse priming of ROS production**. The priming effect of GM-CSF (5ng/mL) on neutrophils from healthy controls (A) and RA patients (C) stimulated with fMLP (10-8M) decreased to ~40% of the levels observed at 45min, following a further 120 min in culture. Baricitinib (200ng/mL) significantly enhanced the depriming of healthy control neutrophils after 30 and 60 min incubation (A, \*p<0.05, \*\*p<0.01, n=3), but was not able to reverse GM-CSF priming of RA neutrophils (C). Tofacitinib was not able to reverse prime GM-CSF-treated neutrophils from controls (A,B) or RA patients (C,D). No significant reverse priming effect was seen with either JAK inhibitor in PMA-treated (100ng/mL) neutrophils from healthy controls (B) or RA patients (D).

**Figure 5. Effect of JAK inhibition on STAT phosphorylation in RA neutrophils.** Freshly isolated (0h) RA neutrophils exhibited elevated levels of phosphorylated STAT1 and STAT3, which decreased over 60 min incubation in untreated conditions. Addition of baricitinib and tofacitinib (200ng/mL) at 0h induced a loss of phosphorylated STAT1 and STAT3 compared to untreated cells at 30 and 60 min.



FIGURE 1



FIGURE 2



FIGURE 3



FIGURE 4



FIGURE 5