




SHORT REPORT

JAK inhibitors disrupt T cell-induced proinflammatory macrophage activation

Mukanthu H Nyirenda ^{1,2}, Jagtar Singh Nijjar,¹ Marina Frleta-Gilchrist,¹ Derek S Gilchrist,¹ Duncan Porter,^{1,3} Stefan Siebert,¹ Carl S Goodyear ^{1,2},
Iain B McInnes ^{2,4}

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¹School of Infection and Immunity, University of Glasgow

College of Medical Veterinary and Life Sciences, Glasgow, UK

²The Research into Inflammatory Arthritis Centre of Excellence - Versus Arthritis (RACE-VA) Universities of Glasgow, Birmingham, Newcastle, Oxford, and Newcastle, UK

³Gartnavel General Hospital, Glasgow, UK

⁴College of Medical Veterinary and Life Sciences, University of Glasgow, Glasgow, UK

Correspondence to

Dr Mukanthu H Nyirenda;
mukanthu.nyirenda@glasgow.ac.uk

ABSTRACT

Objectives Macrophage subsets, activated by T cells, are increasingly recognised to play a central role in rheumatoid arthritis (RA) pathogenesis. Janus kinase (JAK) inhibitors have proven beneficial clinical effects in RA. In this study, we investigated the effect of JAK inhibitors on the generation of cytokine-activated T (Tck) cells and the production of cytokines and chemokines induced by Tck cell/macrophage interactions.

Methods CD14⁺ monocytes and CD4⁺ T cells were purified from peripheral blood mononuclear cells from buffy coats of healthy donors. As representative JAK inhibitors, tofacitinib or ruxolitinib were added during Tck cell differentiation. Previously validated protocols were used to generate macrophages and Tck cells from monocytes and CD4⁺ T cells, respectively. Cytokine and chemokine including TNF, IL-6, IL-15, IL-1RA, IL-10, MIP1 α , MIP1 β and IP10 were measured by ELISA.

Results JAK inhibitors prevented cytokine-induced maturation of Tck cells and decreased the production of proinflammatory cytokines TNF, IL-6, IL-15, IL-1RA and the chemokines IL-10, MIP1 α , MIP1 β , IP10 by Tck cell-activated macrophages in vitro ($p < 0.05$).

Conclusions Our findings show that JAK inhibition disrupts T cell-induced macrophage activation and reduces downstream proinflammatory cytokine and chemokine responses, suggesting that suppressing the T cell-macrophage interaction contributes to the therapeutic effect of JAK inhibitors.

INTRODUCTION

The hallmarks of synovitis in rheumatoid arthritis (RA) include the infiltration of inflammatory cells, predominantly blood-derived lymphoid and myeloid cells.¹ Infiltrating T-cells and monocytes/macrophages reside collaboratively within the inflamed RA synovium; hence, the established heterogeneity of inflammatory synovial macrophages² can drive synovitis via cytokine production by activated T-cells.³ Conversely, T-cells activate macrophages, leading to a proinflammatory cytokine forward feedback loop.⁴

Perpetuation of inflammation by monocytes/macrophages stimulated by

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Janus kinase (JAK) inhibitors modulate the activity of proinflammatory cytokines implicated in rheumatoid arthritis (RA) progression.

WHAT THIS STUDY ADDS

⇒ JAK inhibition disrupts cytokine-activated T cell-induced macrophage activation, an in vitro model for RA synovial T-cell-mediated stimulation of monocytes/macrophages, and reduces proinflammatory cytokine and chemokine responses.
⇒ Suppression of the T cell: macrophage interaction may contribute to the therapeutic efficacy of JAK inhibitors.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Although this study may not directly change policy or practice, it highlights that JAK inhibition may affect multiple steps involved in mediating inflammation by inhibiting cytokine and chemokine production and disrupting the function of innate and acquired immune cells.

cytokine-activated T (Tck) cells may be modelled in vitro by the macrophage: T-cell coculture assay.^{5,6} Signalling pathways elicited by Tck cells in responding monocytes were identical to those induced by T-cells isolated from RA synovial tissue.^{6,7} Thus, Tck cells have been proposed as a model for RA synovial T-cell-mediated stimulation of monocytes/macrophages via cell-cell contact-dependent mechanisms.^{5,7}

Janus kinases (JAKs) are tyrosine kinases that recruit and activate signal transducers and activators of transcription, which drive proinflammatory cellular responses. There are four JAKs in humans, JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2)⁸ that subserve the biology of a range of cytokines critical in the pathogenesis of RA.⁹ The JAK inhibitor tofacitinib interacts with multiple JAKs, although preferentially inhibiting JAK1-dependent and JAK3-dependent cytokines.¹⁰

At the pathophysiological level, tofacitinib and now other JAK inhibitors, modulate the activity of proinflammatory cytokines implicated in RA progression.^{10 11} Though the biological roles of JAK inhibitors in lymphocytes are well known, their function in the context of macrophage: T-cell interactions has not been defined. Here, we investigated the effect of tofacitinib, ruxolitinib and Tyrphostin AG-490 on the generation of Tck cells and assessed whether JAK inhibition in Tck cells impacts cytokine and chemokine production by macrophages in the vitro coculture system.

MATERIALS AND METHODS

Isolation of peripheral blood mononuclear cells from buffy coats

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats using Histopaque 1077 (Sigma) density centrifugation. The buffy coats were obtained from healthy volunteers from the Scottish National Blood Transfusion Service UK (SNBTS). All subjects provided informed consent with the appropriate ethical approvals in place.

Reagents

Tofacitinib, ruxolitinib and Tyrphostin AG-490 were obtained from LC-Labs and dissolved in dimethyl sulfoxide (DMSO) (Sigma). Serial dilutions were employed to ensure that DMSO concentration did not exceed 0.001% in cell culture. Recombinant TNF, IL-6, IL-2 and MCSF (Peprotech); IFN- γ , GM-CSF and IL-4 (R&D Systems); Lipopolysaccharide (Salmonella typhimurium; Sigma).

Purification of CD14⁺ monocytes and differentiation into macrophages

CD14⁺ monocytes were purified by positive magnetic separation using CD14 immunomagnetic microbeads (Miltenyi Biotec). Purities of >98% were routinely confirmed by flow cytometry (data not shown). CD14⁺ monocytes were cultured in 24 well-plates with MCSF (50 ng/mL) for 3 days. Cells were detached using a non-enzymatic dissociation solution (Sigma), washed in Dulbecco's phosphate-buffered saline and then replated in 96 well plates at a density of 5×10^5 cells/mL with MCSF (50 ng/mL) for the additional 3 days before harvesting and used in subsequent experiments.

Purification and differentiation of CD4⁺ Tck cells and the effect of JAK inhibition on Tck cell maturation

CD4⁺ T-cells were isolated from PBMC using CD4 microbeads (Miltenyi Biotec). To generate Tck cells, CD4⁺ T-cells were cultured at 1×10^6 cells/mL with IL-2 (25 ng/mL), IL-6 (100 ng/mL) and TNF (25 ng/mL) (Peprotech) for 6 days.¹² In separate experiments, the JAK inhibitor tofacitinib was added to the culture medium during the 6-day maturation period to assess the effect of tofacitinib on Tck cell maturation. The cells were washed and cultured with mature macrophages at a 4:1

Tck: macrophage ratio. The supernatants were collected and assessed for TNF using ELISA.

Macrophage activation by Tck cells in coculture and the effect of JAK inhibition

Fixed numbers of monocyte-derived macrophages (5.0×10^5 cells) were cocultured with autologous Tck cells, achieving macrophage: Tck ratios of 1:8, 1:4, 1:2 and 1:1. Macrophages and Tck cells were also cultured alone. A 1:4 macrophage/Tck cell ratio was optimal and was used to assess the effect of JAK inhibitors. DMSO (0.001%) was used as a control. The cocultured cells were incubated for 24 hours, and supernatants were collected for analysis using ELISA or Luminex. Trans-well inserts (0.4 μ m pore size; Corning) were used to assess cell-cell contact independent mechanisms.

Measurement of cytokines in culture supernatants

IL-6, IL-15, IL-1RA, IL-10, MIP1 α , MIP1 β , IP10 and MIG were assessed using Luminex (Invitrogen), and TNF was determined using ELISA (Invitrogen); all following the manufacturer's protocols.

Statistical analysis

Results were analysed using GraphPad Prism V.9 Software, (San Diego, California, USA). Statistical differences between groups were analysed using the Wilcoxon rank test, Student's t-test or one-way analysis of variance with Bonferroni post hoc corrections as appropriate.

RESULTS

Tck induce contact-dependent and concentration-dependent TNF production by human macrophages

To delineate the effect of JAK inhibition on macrophages, we revisited the generation of Tck cells and their ability to activate monocyte-derived macrophages. In coculture, we established a 1:4 macrophage/Tck cell ratio as the optimal ratio of macrophages to Tck cells. In agreement with previous reports,^{12 13} Tck cells activated macrophages contact-dependently, secreting TNF (online supplemental figure 1A,B).

JAK inhibitors prevent cytokine-induced maturation of Tck cells and inhibit the production of IL-6, IL-15 and IL-1RA by Tck-activated macrophages

The effect of tofacitinib or ruxolitinib was evaluated on Tck cell maturation. JAK inhibitors diminished the capacity to induce TNF production by macrophages in coculture, suggesting that Tck cells failed to mature sufficiently to be able to activate macrophages (figure 1A). The reduced TNF production was not due to cell death caused by the toxicity of DMSO (figure 1B). Tofacitinib was selected as it is in clinical use, while ruxolitinib and AG-490 typhostin extended the generability of the findings. We chose a range of concentrations (online supplemental figure 2A,B) corresponding to approximate blood levels reflected by peak and trough dosage of tofacitinib by mouth.¹⁴ We also explored the effect of JAK inhibitors

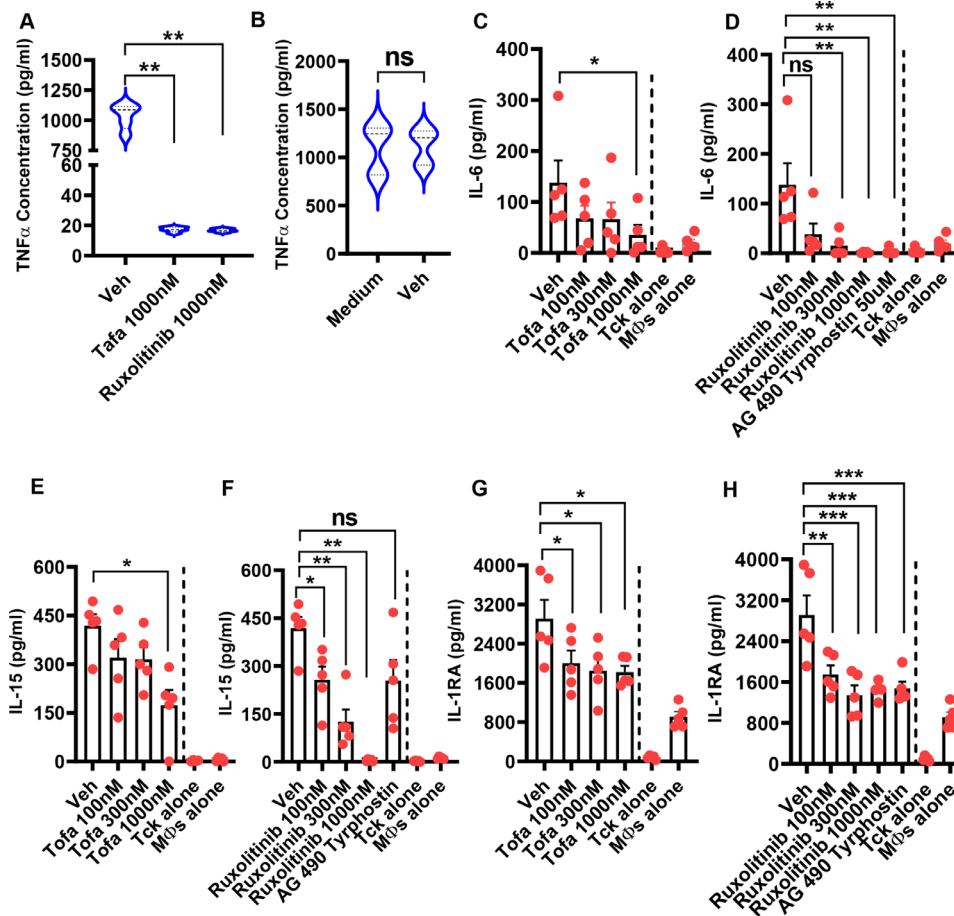


Figure 1 Janus kinase (JAK) inhibitors prevent cytokine-induced maturation of cytokine-activated T (Tck) cells and inhibit the production of IL-6, IL-15 and IL-1RA by activated macrophages. CD14⁺ monocytes (5×10^5 cells/mL) were differentiated into macrophages by culturing them in the presence of macrophage-colony stimulating factor (MCSF; 50 ng/mL) for 6 days in a complete medium in a 96-well plate. Purified CD4⁺ T cells (1×10^6 cells/mL) were stimulated with a cocktail of IL-2, IL-6 and TNF in the absence or presence of 0.001% DMSO (used as Vehicle), tofacitinib (100 nM) or ruxolitinib (1000 nM). The cells were incubated for 6 days to generate Tck. The Tck populations were washed and then cocultured with monocyte-derived macrophages at a concentration of 4:1 Tck-macrophage ratio. The supernatants were harvested, and the level of TNF was measured by ELISA. (A) The comparison of TNF production between DMSO and Tofacitinib (Tofa)-treated or ruxolitinib-treated Tck. (B) The comparison of TNF production between medium and DMSO-treated Tck. In separate experiments, cytokine-generated Tck were cocultured with monocyte-derived macrophages (MΦs) at 4:1 ratio in the absence or presence of 0.001% DMSO (Veh) or tofacitinib (100 nM, 300 nM and 1000 nM) or ruxolitinib (100 nM, 300 nM, 1000 nM, 50 μM). Culture supernatants were harvested, and the levels of (C, D) IL-6, (E, F) IL-15 and (G, H) IL-1RA were measured using Luminex. Data represent a mean of triplicate cultures \pm SE of the mean of at least five experiments. Statistically significant differences are indicated (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). DMSO, dimethyl sulfoxide; ns, not significant; Veh, vehicle.

on IL-6, IL-15 and IL-1RA synthesis, relevant cytokines in inflammatory processes in RA. Tofacitinib, ruxolitinib and AG-490 tyrophostin reduced IL-6, IL-15 and IL-1RA (figure 1C–H).

JAK inhibitors reduce MIP1 α , MIP1 β , MIG and IP10 production by Tck-activated macrophages

We then evaluated the effect of JAK inhibitors on chemokines, including macrophage inflammatory protein MIP1 α and MIP1 β , monokine induced by interferon- γ (MIG)/CXCL9) and interferon- γ -inducible protein-10 (IP10)/CXCL10. These chemokines induce the synthesis and release of other proinflammatory cytokines that play pathological roles. At rest, macrophages or Tck cells did not produce MIP1 α , MIP1 β , MIG and IP10 (data

not shown). Activation of macrophages with Tck cells induced increased production of MIP1 α , MIP1 β and MIG. The output of IP10 was generated by the treatment of macrophages with LPS. Treatment with tofacitinib, ruxolitinib or AG 490 tyrophostin reduced the release of MIP1 α , MIP1 β , MIG and IP10 (figure 2A–H).

DISCUSSION

We show that tofacitinib and ruxolitinib, two structurally unrelated small molecule inhibitors, inhibit the production of TNF, IL-6, IL-15, IL-1RA and chemokines MIP1 α , MIP1 β , MIG and IP10 by macrophages interacting with cytokine activated memory T-cells. This was due to tofacitinib and ruxolitinib preventing cytokine-induced

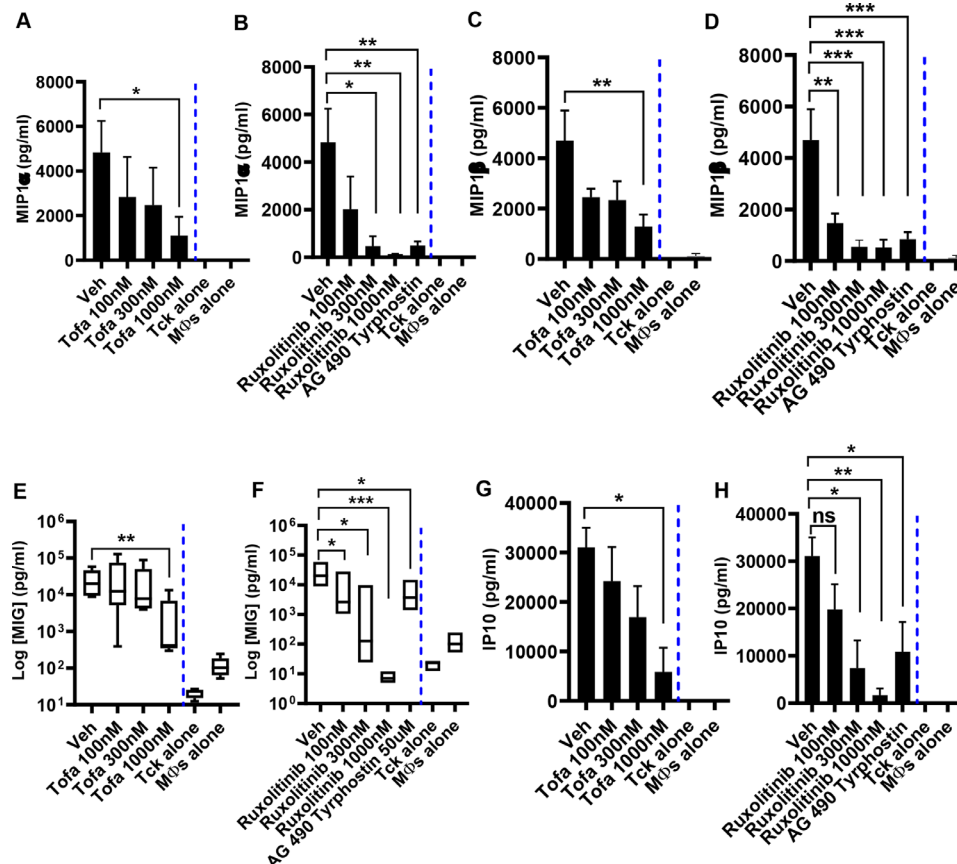


Figure 2 Janus kinase (JAK) inhibitors reduce MIP1 α , MIP1 β , MIG and IP10 production by activated macrophages. Cytokine-generated cytokine-activated T (Tck) cells were washed and then cocultured with monocyte-derived macrophages (M Φ s) at a 4:1 ratio. The cells were treated with 0.001% dimethyl sulfoxide (DMSO) (Veh) or tofacitinib (100 nM, 300 nM and 1000 nM) or ruxolitinib (100 nM, 300 nM, 1000 nM, 50 μ M). In some experiments, M Φ s were also cultured alone. Culture supernatants were harvested, and the levels of (A, B) MIP1 α ; (C, D) MIP1 β ; (E, F) MIG; and (G, H) IP10 were measured using Luminex. Data represent a mean of triplicate cultures \pm SE of the mean of at least five experiments. Statistically significant differences are indicated (* p <0.05; ** p <0.01; *** p <0.001; ns=not significant). Veh, vehicle.

maturation of Tck cells. Thus, tofacitinib and ruxolitinib impact the production of proinflammatory cytokines and chemokines crucial in the pathogenesis of immune-mediated inflammatory diseases. Although the effects of JAK inhibition on macrophages and T-cells have been studied,^{15–18} no study has thus far demonstrated the impact of JAK inhibition on Tck cell-activated macrophages.

Our observations concur with studies showing that JAK inhibitors mediate the suppression of TNF and IL-6 release, in keeping with the suggestion that JAK inhibitors work, in part, by suppressing the TNF-IFN β -JAK-STAT1 autocrine loop.¹⁷ Tofacitinib, ruxolitinib and AG490 Tyrphostin also led to the inhibition of Tck cell maturation. Likely, the inhibition of IL-6 and IL-15 by tofacitinib and ruxolitinib contributed to the failure to generate mature Tck cells since these cytokines are crucial in forming mature Tck cells, as previously suggested.^{12–13} Indeed, we previously demonstrated that IL-15 (which signals via JAK1 and JAK3) is sufficient to form Tck cells and alters the balance between effector and memory T cells.¹³ These observations suggest that JAK inhibition may effectively disrupt cell contact-mediated inflammation at

the synovial site and prevent a priori development of Tck cells.

Since Tck cells in our setting were not fixed, it is likely that a small proportion of the cytokines came from Tck cells and not all from macrophages. However, using fixed Tck cells, a previous study demonstrated that Tck cell-activated macrophages induced TNF¹⁹; therefore, it is reasonable to suggest that the TNF synthesis seen in our study is of macrophage origin.

The limitation of this study is that the investigation did not include RA patient samples, which would provide direct clinical relevance. However, the data suggest that JAK inhibition may effectively treat established inflammation by disrupting the crosstalk between macrophages and T-cells (online supplemental figure 3). Overall, JAK inhibition may affect multiple steps in mediating inflammation by targeting cytokine and chemokine production and affecting the function of innate and acquired immune cells, contributing to the beneficial effect of JAK inhibitors in inflammatory diseases like RA.

Contributors MHN analysed the data and wrote the manuscript. JSN performed the assays. MF-G, DSG, SS, DP, CSG and IBM supervised the study and edited the manuscript. All authors approved the final version of the manuscript.

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Competing interests DP has received consultancy and sponsorship from Abbvie and Eli Lilly to attend scientific meetings. SS has received institutional research grants from Amgen (previously Celgene), Boehringer Ingelheim, Bristol Myers Squibb, Eli Lilly, GlaxoSmithKline (GSK), Janssen, and UCB; and honoraria/speaker fees from AbbVie, Amgen, Eli Lilly, GSK, Janssen, and UCB. IBM has received consultancy and research support from Bristol Myers Squibb, Pfizer, Abbvie, Eli Lilly and Gilead, all of whom manufacture inhibitors of JAKs.

Patient consent for publication Not applicable.

Ethics approval This study complied with the World Medical Association Declaration of Helsinki and was approved by the Glasgow East ethics committee. Participants gave informed consent to participate in the study before taking part.

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ORCID iDs

Mukanthu H Nyirenda <http://orcid.org/0000-0001-5290-4990>

Carl S Goodyear <http://orcid.org/0000-0001-5926-5941>

Iain B McInnes <http://orcid.org/0000-0002-6462-4280>

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