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Rheumatoid arthritis

Augmentation of immunothrombosis as a key mechanism underlying JAK inhibition associated hypercoagulability in rheumatoid arthritis

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ABSTRACT

Objectives: Venous thromboembolism (VTE), following Janus kinase inhibitors treatment (JAKi), is poorly understood in rheumatoid arthritis (RA). We investigated whether JAKi augmented immune cell-driven clotting or immunothrombosis in RA.

Methods: Peripheral blood leukocytes (PBLs) isolated from patients with RA and healthy controls were treated with various JAKi classes, before stimulation with Toll-like receptor (TLR)-4 (lipopolysaccharide [LPS]) or TLR3 (polyinosinic-polycytidylic acid—poly (I:C)) agonists. Conditioned supernatants were used in plasma turbidity assays to evaluate clot formation and lysis dynamics, while bulk RNA sequencing, enzyme-linked immunosorbent assay, and bead-based immunoassays were used to explore immunothrombosis mechanisms.

Results: Turbidity analyses showed that conditioned media from PBLs treated with LPS and tofacitinib significantly accelerated clot formation when compared to LPS alone, and this effect was tissue factor pathway dependent and accompanied by elevations in immunothrombotic cytokines, including tumour necrosis factor α, interleukin (IL)-1β, and IL-6. PBLs from patients with active RA exhibited significantly greater immunothrombotic potential compared to those with low disease activity, despite comparable baseline cytokine levels. RNA sequencing analysis revealed significant pathway enrichment in tofacitinib/LPS-treated PBLs, including activation of Nuclear Factor (NF)-κB pathways, increased tissue factor expression, and reduced levels of anticoagulant factors such as protein S. Pharmacological inhibition assays with 5 JAK therapies suggested that JAK1/tyrosine kinase 2-dependent effect underscored increased thrombosis but selective JAK3 inhibition did not reproduce the prothrombotic effects. Finally, patients with RA with JAK-associated pulmonary embolism showed interstitial changes compatible with immunothrombosis in 4/6 (67%).

Conclusions: Immunothrombosis offers a novel explanation for JAKi-associated VTE in RA.

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WHAT IS ALREADY KNOWN ON THIS TOPIC

- Janus kinase inhibitors (JAKis), particularly tofacitinib, are associated with increased risk of venous thromboembolism (VTE) in rheumatoid arthritis.
- The mechanisms underlying JAKi-related VTE remain unclear and are not explained by classical thrombophilia risk factors.
- Immunothrombosis, driven by innate immune activation, is increasingly recognised as a contributor to thrombosis in inflammatory diseases and under some conditions JAKi may actually increase innate immune activation.

WHAT THIS STUDY ADDS

- JAKis amplified toll-like receptor 4-induced cytokine production and accelerated clot formation in rheumatoid arthritis leukocyte assays and more pronounced in patients with active RA.
- The prothrombotic effect was tissue factor-dependent and associated with increased F3 (tissue factor) expression and reduced Protein S.
- Selective JAK3 inhibition did not reproduce these effects, suggesting pathway-specific risk.
- Pulmonary emboli under JAKi frequently showed imaging features compatible with in situ pulmonary immunothrombosis rather than DVT with PE development.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- This work provides mechanistic evidence that JAKi-associated VTE is linked to immunothrombosis rather than inherited clotting disorders.
- This study also highlights that the JAK and VTE association may be more likely in active RA.
- It also suggests that preserving IL-10 signalling or combining with protective strategies could mitigate thrombotic risk in future JAKi development.

INTRODUCTION

The risk factors for deep vein thrombosis (DVT) and pulmonary embolism (PE), collectively known as venous thromboembolism (VTE), include female sex, surgery, immobilisation, older age, heart failure, and cancer, among others [1–3]. Recent studies have also shown higher rates of VTE in patients with rheumatoid arthritis (RA) [4–7], which can be potentially further exacerbated upon treatment with Janus kinase inhibitors (JAKi), particularly tofacitinib [8–10]. Increased incidence of atherosclerotic disease upon treatment of patients with RA with JAKi has also been documented, although findings across cohorts remain inconsistent [11–15]. The pathogenesis of both VTE and major adverse cardiac events in JAKi-treated cases remains poorly understood.

PE is classically viewed as a consequence of DVT, where thrombi originating in the peripheral venous system embolise to the pulmonary arteries, leading to vascular occlusion and hypoxia. However, a notable subset of PE cases—estimated to account for nearly one-third—occur without detectable DVT, even after thorough imaging evaluation [16,17]. This phenomenon, referred to as isolated PE (iPE) or *in situ* pulmonary thrombosis (iPT), is increasingly recognised as a distinct clinical entity and may be particularly relevant in the setting of JAKi-associated thrombosis in RA [8,18,19]. Epidemiologic studies suggest that iPE occurs more frequently in women and older individuals and is less often associated with cancer or peripheral symptoms such as leg swelling or pain [20]. Furthermore, individuals with iPE appear to have a higher prevalence of atherosclerotic comorbidities—including coronary artery disease, atrial fibrillation, stroke, and peripheral artery disease—compared to patients with other VTE phenotypes [21]. The field is further complicated by the fact that both active RA and active inflammatory disease, in general, are also associated with DVT and PE [4,8], making it challenging to disentangle disease-specific and treatment-related thrombotic mechanisms.

Thus far, genetic risk factors for hypercoagulability have not been incriminated in JAKi-associated thrombosis [22]. The term ‘immunothrombosis’ describes activation of blood clotting mechanisms through innate immune defensive mechanisms triggered in several different pathologies [23]. Activated monocyte production of tissue factor, a crucial coagulation initiator, plays an important role in immunothrombosis. Other mechanisms, including dysregulation of platelets and key inflammatory cytokines dysregulation, including interleukin (IL)-1, tumour necrosis factor α (TNF-α), and IL-6, have been proposed [24]. Interestingly, pulmonary immunothrombosis is a key pathogenic mechanism in COVID-19 [25–29]. Of note, such thrombosis occurs without evident DVT, which has also been reported in RA under JAKi [8]. Thus, both conditions are linked to iPT, where PE without DVT may be evident [30].

In rheumatology and other medical specialties, JAK pathway antagonism—comprising JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK2) inhibition—has been used increasingly for inflammatory disease therapeutics [31–35]. Although generally overlooked by rheumatologists, it has been reported that JAKi elevates murine proinflammatory cytokine production in response to bacterial lipopolysaccharide (LPS) challenge, likely due in part to antagonism of IL-10 signalling, an anti-inflammatory cytokine [36,37]. Indeed, we recently confirmed a dissociation between JAKi on innate and adaptive immune compartments with seemingly paradoxical augmentation of myeloid inflammation despite robust suppression of adaptive T cell responses in an *in vitro* enthesitis model [38]. Given the present enigmatic basis for JAKi-associated VTE in RA, we tested the hypothesis that JAKi could promote immunothrombosis *in vitro*, depending on RA disease activity. Accordingly, we report that immunothrombosis represents a simple explanation for VTE associated with JAK inhibition, which may also be associated with JAK pathway selectively.

METHODS

Ethical approval and patients

Ethical approval for the study was obtained from the Leeds Teaching Hospitals NHS Trust (Rheumatoid Arthritis DiseAse Research ethics, protocol number RR09/9134, version 12.0). Patients already under JAKi were excluded, but disease-modifying antirheumatic drugs (DMARDs) or biological therapy was permitted. A volume of 8 mL of blood obtained in EDTA tubes was collected from patients with RA in remission/low disease activity (LDA) (Disease Activity Score [DAS]-28-C-reactive protein [CRP] score $\leq$ 3.2, n = 12), with moderate-high disease activity (DAS-28-CRP > 3.2, n = 14) and from healthy controls (n = 6). For clotting studies, home-made normal pooled plasma (NPP) was prepared by collecting free-flowing blood from the antecubital vein of at least 25 healthy donors using 19-gauge butterfly needles. Blood was drawn into 0.109 M trisodium citrate tubes and processed within 1 hour. Samples were centrifuged at 3000 relative centrifugal force for 20 to 30 minutes to

obtain platelet-poor plasma, which was subsequently pooled. The pooled NPP was divided into 0.8 to 1 mL aliquots, snap-frozen in liquid nitrogen, and stored at −80°C until use.

Peripheral blood leukocyte isolation and stimulation

Peripheral blood leukocytes (PBLs) were isolated from peripheral blood using centrifugation, following ammonium chloride-mediated lysis of red blood cells (RBCs). Blood was diluted 10-fold in RBC lysis buffer (Cytek Biosciences) and incubated at room temperature for 10 minutes. After incubation, the samples were centrifuged at 400 *g* for 10 minutes, and the supernatants were discarded. The PBLs were washed 3 times in phosphate-buffered saline, each time centrifuging at 300 *g* for 10 minutes. Cells were resuspended in Roswell Park Memorial Insistitute (RPMI) 1640 media (Gibco) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 U/mL penicillin (Sigma-Aldrich) and 100 µg/mL streptomycin (Sigma-Aldrich) and plated at a density of 5×10^5 cells per well in cell culture-treated 96-well plates (Thermofisher).

Cell stimulations

Cells were treated with either vehicle control (0.01% Dimethyl Sulfoxide -DMSO; Sigma-Aldrich) or JAK inhibitors (tofacitinib, upadacitinib, deucravacitinib, baricitinib, ruxolitinib, or ritlecitinib; MedChemExpress), which were dissolved in 0.01% DMSO. To account for any effect of the solvent, 0.01% DMSO was included in all experimental conditions, including LPS-only and poly(I:C)-only samples. A second control condition consisting of unstimulated cells treated with DMSO alone was also included to assess baseline cytokine expression. Treatments were applied using clinically relevant concentrations (1 μM, 100 nM, and 10 nM) for 1 hour before stimulation with either LPS (100 ng/mL; Sigma-Aldrich) or polyinosinic-polycytidylic acid (poly(I:C), 1 μg/mL; Invivogen). To assess the role of steroid therapy on JAKi-induced coagulation, cells were treated with 1 µM tofacitinib 1 hr before LPS stimulation in combination with incremental doses of methylprednisolone (1 µM, 100 nM, and 10 nM; Cayman Chemical). For transcriptomics analysis, cells were collected 24 hours post-stimulation for RNA extraction. Supernatants were collected 48 hours post-stimulation for analysis of clotting dynamics and cytokine quantification and stored at -80°C.

Cytokine quantification

The LEGENDplex Human Essential Immune Response Panel (13-plex; BioLegend) multiplex assay panel (IL-4, IL-2, C-X-C motif chemokine ligand (CXCL)-10 [interferon gamma inducible protein 10, IP-10], IL-1β, TNF-α, C-C-motif ligand (CCL2) [Monocyte Chemotatic Protein 1, MCP-1], IL-17A, IL-6, IL-10, interferon [IFN]-γ, IL-12p70, CXCL8 [IL-8], and Free Active transforming growth factor -TGF-β1) was used for supernatant cytokine quantification, measured on a Beckman Coulter Cytoflex S flow cytometer. Due to high concentrations of IL-6 and IL-1β, repeated measurements were performed on diluted samples using enzyme-linked immunosorbent assay kits (Thermo Fisher Scientific).

Turbidity and lysis

JAK inhibition has been shown to increase myeloid inflammatory activity in response to some pathogen-associated molecular pattern stimuli, including LPS [36,37], which could theoretically augment immunothrombosis. Therefore, we examined whether conditioned media from tofacitinib-treated PBLs impacted clotting using plasma turbidity.

Conditioned media were generated from isolated RA PBLs by pretreatment with either tofacitinib or DMSO vehicle control 1 hour before stimulation with LPS or poly(I:C) for 48 hours, and subsequent collection of cell-free supernatant. The conditioned supernatants were added to NPP along with $CaCl_2$ and thrombin to trigger clotting, and clotting metrics were analysed by measuring plasma turbidity.

For turbidity assays, stimulated cell supernatants (20 μL) were added to NPP (20 μL) obtained from healthy individuals. Clotting was triggered by the addition of clotting mix (20 μL) containing $CaCl_2$, human thrombin (Sigma)—at final concentrations 10 mM/mL and 0.1U/mL, respectively—in Hanks' balanced salt solution. For lysis assays, tissue plasminogen activator (tPA, 0.03 ng/mL; Technoclone) was also added to the clotting mix [39]. Absorbency was read on a Powerwave plate reader (Biotek) at 340 nm, every 12 seconds for 3.5 hours at 37°C [39]. Clot formation and lysis parameters, including lag time, maximum optical density, maximum turbidity velocity, and time to 50% lysis, were obtained as previously described [40]. To evaluate the impact on contact and tissue factor coagulation pathways, experiments were replicated using plasma sourced from patients deficient in Factor XI (FXI; George King Bio-Medical) and Factor VII (FVII; George King Bio-Medical), respectively. Experiments were performed in duplicate, for 10 RA samples (5 from the LDA group; 5 from the moderate-high disease activity group).

Bulk RNA sequencing and analysis

RNA extraction was performed using the Norgen Total RNA Purification Kit (Norgen Biotek, Canada) following manufacturer's instructions. Libraries were prepared and sequenced (PE150, 9 Gb per sample) on the Illumina NovaSeq X plus at Novogene. Following removal of low-quality reads and reads containing adapters and poly-N, reads were aligned to the reference genome using Hisat 2 v2.0.5. FeatureCounts v1.5.0-p3 was used to count reads and calculate Fragments Per Kilobase of transcript per Million base pairs sequenced. Differential expression analysis was performed with DESeq2 (DESeq2RPackage 1.20.0) [41], and adjusted *P* values (*P*adj) were calculated using Benjamin and Hochberg's approach [42]. Genes with a *P*adj $\leq$ .05 were assigned as differentially expressed. Pathway enrichment analysis was performed using clusterProfiler package in R.

Computed tomography pulmonary angiography imaging

Computed tomography pulmonary angiography (CTPA) studies of 6 patients with confirmed PE under JAK inhibition therapy were also reviewed for evidence of lung parenchymal changes supporting immunothrombosis with imaging analysis by an experienced radiologist (AS). Demographic, clinical, and serological relevant data were analysed.

Statistical analysis

Results were analysed with GraphPad Prism software. Data are presented as mean ± SEM and were compared by one-way analysis of variance when normal distribution was found. When not normally distributed, data were presented as median + IQR and compared by the Kruskal-Wallis test. Differences were

considered statistically significant for $P < .05$. Multiple comparisons were corrected by Tukey's post hoc method.

RESULTS

Patient demographics

A total of 32 patients with RA (4 males, 28 females) and 6 healthy individuals (3 males, 3 females) participated in this study. Blood was collected from 26 patients with RA (3 males, 23 females) who were separated into a moderate-high disease activity group (DAS-28-CRP > 3.2; n = 12) and an LDA group (DAS-28-CRP ≤ 3.2; n = 14). Additionally, 6 patients with RA who were diagnosed with PE while on JAK inhibition therapy were included for analysis of imaging features on CTPA. The overall median age of the patients was 61 (IQR 50.5-66.5), and the median age of the healthy individuals was 23 (IQR 16-29). Details of the patients and stage of RA are shown in Table 1. The samples and experimental conditions for each experiment are presented in Supplementary Figure S1.

Tofacitinib treatment of PBLs increases the rate of clotting

Compared to media from unstimulated cells, LPS-stimulated PBL conditioned media (LPS) induced a significant reduction in the time taken for clotting to start (lag time) (median 494 seconds vs 810 seconds, $P = .0229$) and showed a nonsignificant trend towards increased rate of clot formation (V_{max}) (0.0325 ΔOD/min vs 0.0263 ΔOD/min, $P = .057$) (Fig 1A,B). The addition of tofacitinib-treated LPS-stimulated PBL conditioned media (tofacitinib/LPS) further augmented this procoagulant effect, reducing the lag phase to a median of 349 seconds compared to 494 seconds in LPS alone ($P = .0454$), and significantly increasing V_{max} to 0.055 Δ Optical Density (OD)/min compared to 0.0335 ΔOD/min in LPS alone ($P = .0432$) (Fig 1A,B), suggesting that tofacitinib enhances PBL-driven coagulation in a TLR4-dependent manner. To determine whether JAK inhibitors alone could trigger prothrombotic responses, PBLs were also treated with tofacitinib (1 μM) without LPS. Tofactinib alone did not impact clotting metrics (Supplementary Fig S2A,B).

In contrast to LPS, poly(I:C) stimulated PBL conditioned media, with or without the addition of tofacitinib, did not significantly alter plasma turbidity compared to unstimulated PBL conditioned media (Fig 1C,D), indicating no role for TLR3-induced activation of leukocytes in promoting clotting. Furthermore, analysis of each RA subgroup showed that clotting rates and lag time were not significantly different between moderate-high disease activity and LDA RA groups, suggesting that tofacitinib-promoted coagulation is independent of disease activity (Supplementary Fig S3).

To elucidate which pathway of the coagulation cascade was responsible for the alteration in clotting observed above, we repeated these experiments using coagulation FVII ($FVII^{-/-}$) or XI ($XI^{-/-}$) deficient plasma, lacking activation of the tissue factor and contact pathways, respectively. As with complete plasma, in $FXI^{-/-}$ plasma, tofacitinib/LPS-conditioned media significantly reduced lag time (396 seconds vs 620 seconds for LPS alone, $P = .0094$) and increased V_{max} (0.040 ΔOD/min vs 0.0275 ΔOD/min, $P = .0053$) (Fig 1E,F). However, in $FVII^{-/-}$ plasma, neither LPS nor tofacitinib/LPS altered lag time compared to unstimulated media (LPS: 1178 seconds vs 798 seconds, $P = .481$; tofacitinib/LPS: 900 seconds vs 798 seconds, $P = .4895$). Interestingly, LPS decreased V_{max} in $FVII^{-/-}$ plasma (median 0.005 δOD/min vs 0.035 δOD/min in unstimulated, $P = .0123$), yet the observed V_{max} for tofacitinib/LPS was not significantly different from untreated levels (median 0.0212 δOD/min vs 0.035 δOD/min in unstimulated, $P = .7684$) (Fig 1G,H). Therefore, the loss of tofacitinib plus LPS-induced augmentation of clotting in $FVII^{-/-}$ plasma suggests the clotting potential of PBL supernatant is driven by the tissue factor pathway.

Clot thrombolysis kinetics were also assessed by turbidity by addition of the tPA in the assays. We found no overall significant effect of TLR stimulation ± tofacitinib on plasma clot time to half-lysis when compared to untreated conditioned media suggesting that JAK inhibition impacts on clot formation but not lysis dynamics (Fig 1I).

Tofacitinib increases LPS-induced inflammatory cytokine production in PBLs

We and others have previously reported that JAK inhibition can counterintuitively increase the inflammatory activity of myeloid cells, increasing the production of proinflammatory cytokines. Given the importance of inflammatory cytokines in promoting immunothrombosis, we measured the concentration of cytokines, known to play a role in immunothrombosis, in the conditioned media of stimulated RA PBLs (IL-1β, TNFα, IL-6, IL-10, IL-12, and TGFβ). As expected, all cytokines measured were upregulated following LPS stimulation, compared to controls, while poly(I:C) had no effect (Fig 2A). Importantly, addition of tofacitinib before LPS stimulation further elevated secretion of each cytokine in a dose-dependent fashion, and while the effect was statistically significant for IL-6 and IL-1β, the trend was maintained for all cytokines (Fig 2A). To determine whether JAK inhibitors alone could trigger cytokine, PBLs were also treated with tofacitinib (1 μM) in the absence of LPS. Tofacitinib alone did not induce secretion of measured cytokines (Supplementary Fig S2C-E).

Moreover, when comparing patients with RA with moderate-high disease activity to those with LDA, the effects of LPS and tofacitinib on IL-1β and TNF-α were significantly enhanced, while IL-6 showed a strong trend towards increase, despite similar baseline cytokine levels (Fig 2B).

Table 1
Patient demographics and details

Variables	Moderate-high disease activity RA (n = 12)	Low disease activity RA (n = 14)	Healthy individuals (n = 6)	Patients with RA who were diagnosed with PE when treated with JAKi (n = 6)
Age, median (IQR)	59 (50-66)	61 (50-62)	23 (16-29)	66 (60-70)
Female, n (%)	11 (92%)	12 (86%)	3 (50%)	5 (83%)
DAS-28-CRP score, mean (SD)	4.83 (1.35)	2.35 (0.46)	NR	NR

CRP, C-reactive protein; DAS, disease activity score; JAKi, Janus kinase inhibition; NR, not recorded; PE, pulmonary embolism; RA, rheumatoid arthritis.

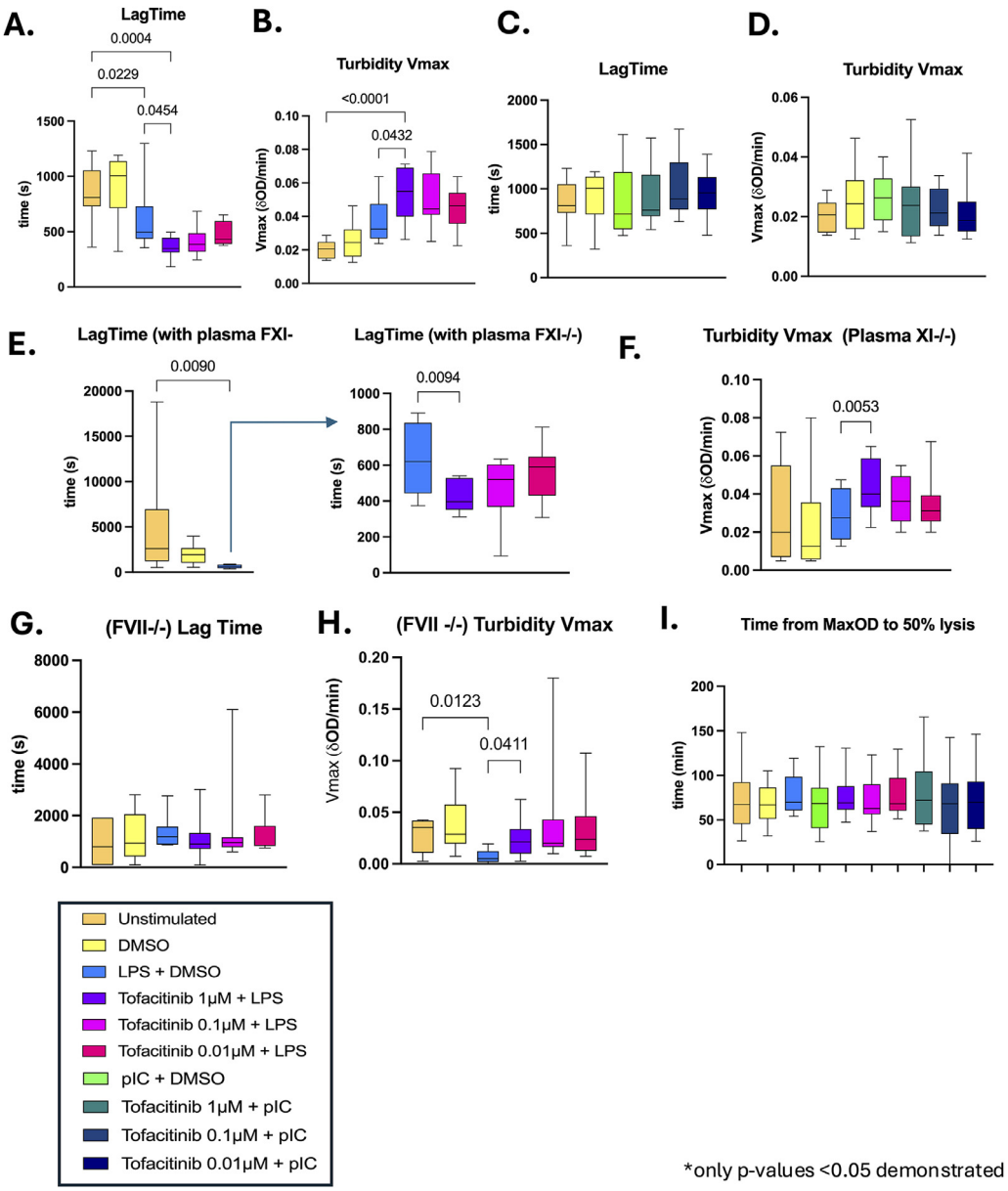

Figure 1. Effect of tofacitinib-treated leukocyte conditioned media on clotting. (A-I) Turbidity assays were conducted using conditioned media generated by PBLs isolated from patients with RA (n = 10). Conditioned media generated from stimulated RA PBLs were added to normal pool plasma (A-D, I), FXI-deficient plasma (E, F), and FVII-deficient plasma (G, H). Turbidity was measured to assess clot formation lag time (A, C, E, G) and rate of clot formation (V_{max}; B, D, F, H). (I) Time from maximum optical density to 50% lysis when the normal pool was supplemented with RA PBL conditioned media. FVII, Factor VII; FXI, Factor XI; LPS, lipopolysaccharide; PBL, peripheral blood leukocyte; RA, rheumatoid arthritis. DMSO, dimethyl sulfoxide; pIC, poly (I:C)

Transcriptional analysis shows increased innate immune activity and dysregulated coagulation factors after tofacitinib treatment

To better understand the immunological effects induced by tofacitinib pretreatment that may contribute to accelerated and increased clotting, transcriptional analysis of stimulated PBLs was performed. As the changes in clotting dynamics and cytokine secretion were most strongly observed in the moderate-high disease activity RA group, PBLs from this group were used for analysis. PBLs were treated with tofacitinib 1 hour before 24-hour stimulation with LPS. Principal component analysis showed that treatment condition accounted for 82% of variance, while participant variability accounted for 7% of variance (Fig 3A). Differential gene expression analysis comparing tofacitinib-pretreated LPS-stimulated PBLs with LPS only stimulated cells identified 1805 upregulated genes and 1990 downregulated genes (Supplementary Fig S4), with significant upregulation of inflammatory genes including IFN signalling components, antimicrobial peptides, colony-stimulating factors, costimulatory receptors, and ILs (Supplementary Fig 3B,C). Pathway enrichment analysis shows tofacitinib-pretreated LPS-stimulated PBLs were enriched for pathways of innate immune responses, leukocyte activation, regulation of T cell activity and cell migration, suggesting tofacitinib pretreatment has a significant impact on PBLs (Fig 3D). Increases in inflammatory markers from tofacitinib-pretreated PBLs also support our previous findings of increased innate cell inflammatory activity.

In addition to assessing whole transcriptome changes, we analysed expression of a custom set of genes associated with coagulation and further highlighted altered gene expression in tofacitinib-pretreated PBLs (Fig 3E). Differential gene expression identified tissue factor pathway inhibitor 2 (TFPI-2) and F3

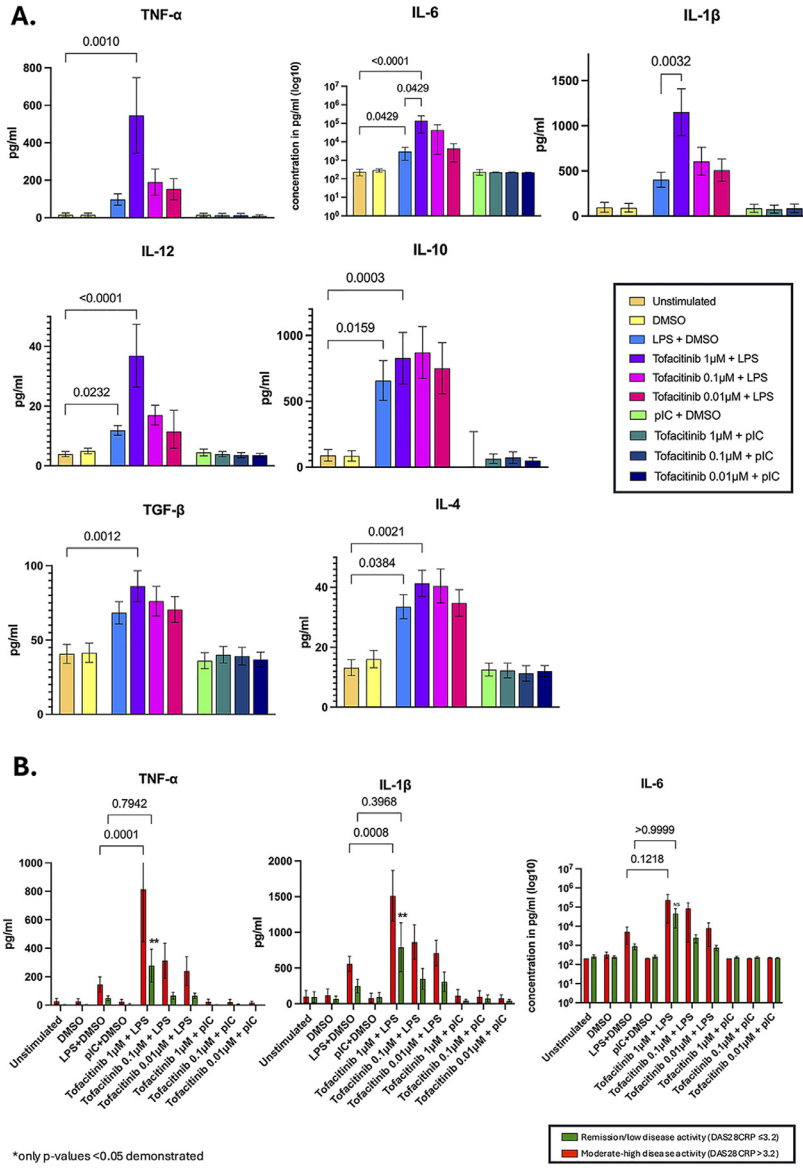

Figure 2. Effect of tofacitinib and Toll-Like receptor (TLR) stimulation on cytokine production. PBLs isolated from 12 patients with RA (6 with moderate-high Disease Activity Score [DAS > 3.2]; 6 with low disease activity or in remission [DAS ≤ 3.2]) were pretreated with vehicle control (0.01% DMSO) or tofacitinib (1 μM, 100 nM, 10 nM) before stimulation with LPS (100 ng/mL) or poly (I:C) (1 μg/mL). (A) Following 48 hours of stimulation, supernatants were measured for secreted cytokines (TNFα, IL-6, IL-1β, IL-12, IL-10, TGF-β, IL-4). (B) Secretion of TNFα, IL-1β, and IL-6 was compared between the PBLs from moderate-high disease activity and low disease activity patients with RA. Data are presented as mean ± SD compared by one-way ANOVA. ANOVA, analysis of variance; IL, interleukin; LPS, lipopolysaccharide; PBL, peripheral blood leukocyte; RA, rheumatoid arthritis; TNFα, tumour necrosis factor α. DMSO, dimethyl sulfoxide; TGF-β, transformig growth factor β

(encoding for tissue factor) as more than 2-fold upregulated, while F2RL1 (encoding for factor II - or thrombin -receptor like 1), protein S gene (PROS1), and versican gene (VCAN) showed a more than 2-fold downregulation in the tofacitinib group (Fig 3E). Of these genes, TFPI-2 and VCAN were significantly regulated (*P*adj > .05). TFPI-2 and PROS1 are important regulators of the coagulation cascade, while F3 encodes tissue factor which is a critical activator of coagulation via FVII. Tofacitinib-induced changes in transcription of these factors and regulators may therefore impact clotting kinetics, with increased tissue factor expression.

JAK3 inhibition does not promote LPS-induced immunothrombosis

Previous work has shown that JAK inhibition-dependent increases in inflammatory cytokines are in part due to inhibition of anti-inflammatory IL-10 signalling, which signals through JAK1/TYK2 pathways, preventing effective negative feedback [36,38]. Several JAKi with differing specificities are available to target different JAK signalling pathways. Next, we compared the effect of those inhibitors on LPS-induced cytokine production to understand the importance of each pathway in cytokine regulation. We therefore compared IL-1β and IL-6 production from PBLs isolated from 6 healthy volunteers, after treatment with various JAKi. We found that inhibitors of JAK1, JAK2, and TYK2 (tofacitinib, upadacitinib, baricitinib, deucravacitinib, and ruxolitinib) led to a trend towards increased expression of IL-1β and IL-6, while ritlecitinib, the JAK3 selective inhibitor, did not impact LPS-induced production of either cytokine (Fig 4A). Conditioned media of JAKi-pretreated LPS-stimulated PBLs were also analysed by clotting turbidity assays in NPP. Conditioned media from LPS-stimulated PBLs significantly reduced clotting lag time (and increased V_{max}, data not shown) compared to unstimulated controls (mean 394 seconds vs 566 seconds, P = .0179). Tofacitinib-pretreated, LPS-stimulated PBLs exhibited a slightly decreased mean lag time of 370 seconds, which was not significantly different from LPS alone (P = .9982), but remained significantly shorter than unstimulated controls (P = .0065) (Fig 4B). Similar reductions in lag time compared to unstimulated supernatants were observed with other JAK1, JAK2, and TYK2 inhibitors: upadacitinib (mean 325 seconds, P = .0008), baricitinib (358 seconds, P = .0047), deucravacitinib (365 seconds, P = .0059), and ruxolitinib (410 seconds, P = .0294) (Fig 4B). In contrast, ritlecitinib (selective JAK3i) pretreated LPS-stimulated conditioned

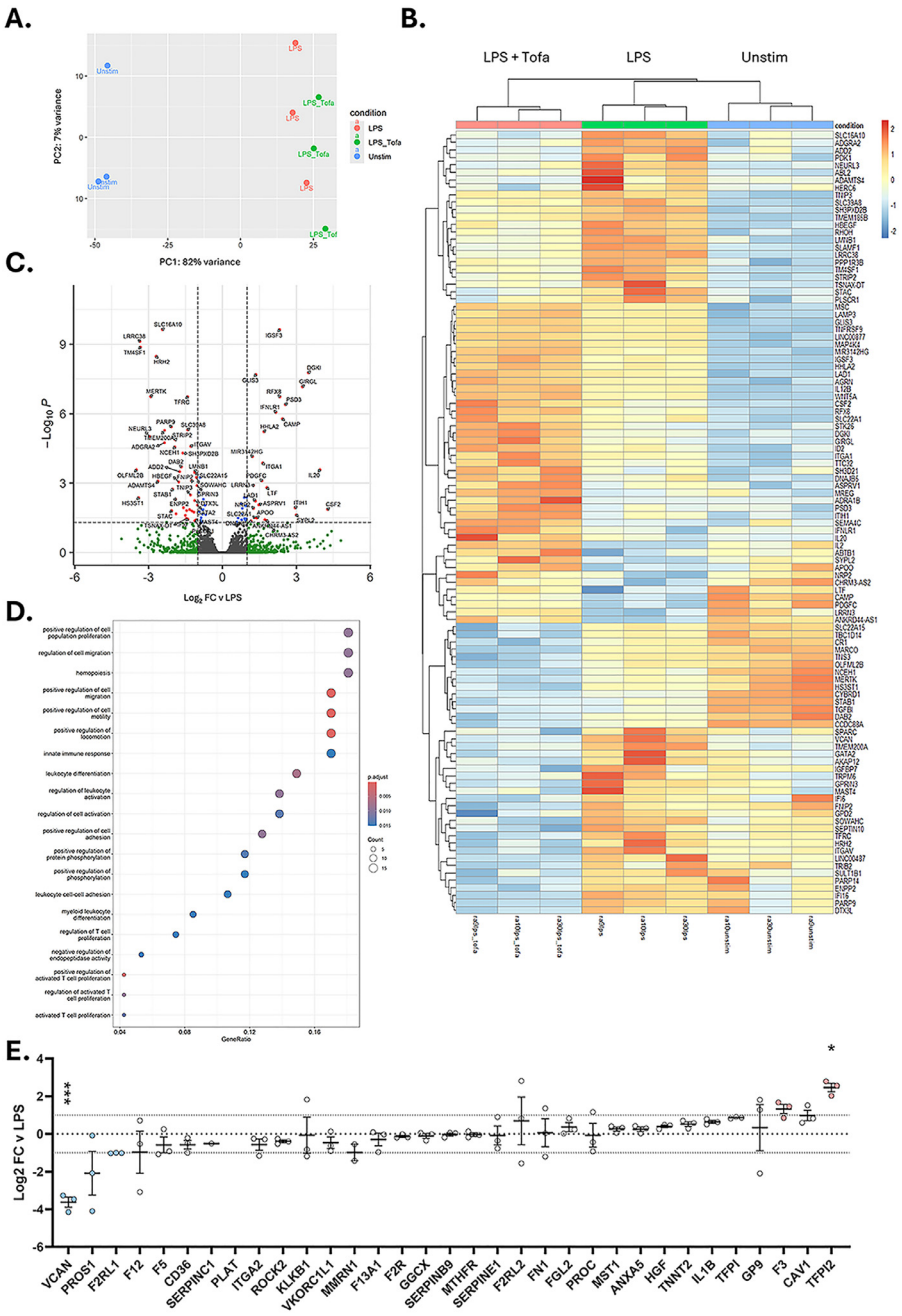

Figure 3. Bulk RNA sequencing of PBLs shows tofacitinib-induced increase in PBL inflammation and dysregulated coagulation-related genes. (A) Principal components analysis of samples analysed by bulk RNA sequencing of PBLs isolated from moderate-high disease activity patients with RA (n = 3, DAS > 3.2) showing that treatment condition is the primary contributor of variance in the data. (B) Heatmap showing differential gene expression of the top 100 significantly regulated genes in unstimulated (blue), LPS-stimulated (green), and tofacitinib-pretreated LPS-stimulated (LPS + tofa; red) PBLs. (C) Volcano plot showing differentially expressed genes of tofacitinib-pretreated LPS-stimulated (LPS + tofa) vs LPS-stimulated PBLs. Green dots indicate > 2-fold change in expression. Red dots indicate significant (*P*adj < .05) > 2-fold change in expression. (D) Pathway enrichment dot plot of the 20 most significantly enriched pathways in tofacitinib-pretreated LPS-stimulated PBLs. Dot size indicates counts of genes enriched in the pathway. Dot colour indicates significance. (E) Log_2 fold changes in gene expression of clotting factors and genes related to regulation and control of coagulation in tofacitinib-pretreated LPS-stimulated PBLs vs LPS-stimulated PBLs. Coloured dots indicate > 2-fold change in expression. Data are presented as mean ± SD compared by one-way ANOVA for (E). **P* < .05, ****P* < .001. ANOVA, analysis of variance; LPS, lipopolysaccharide; PBL, peripheral blood leukocyte; RA, rheumatoid arthritis.

media did not significantly alter lag time compared to unstimulated media (mean 502 seconds vs 566 seconds, $P = .4842$), and showed a nonsignificant trend towards prolongation relative to LPS alone (Fig 4B).

Corticosteroids do not mitigate tofacitinib-induced clotting despite inflammatory cytokine reduction

Corticosteroids are commonly used alongside DMARDs in treating RA. Given their significant ability to reduce inflammation and production of inflammatory cytokines, we tested whether concomitant corticosteroid therapy mitigated against or blocked JAKi-induced *in vitro* thrombotic potential. PBLs from patients with RA were pretreated with vehicle control or tofacitinib for 1 hour before stimulation with LPS with or without methylprednisolone for 48 hours. Following stimulation, supernatants were collected for use as conditioned media in turbidity assays and measured for cytokine concentrations. In LPS-stimulated cells pretreated with tofacitinib, the addition of methylprednisolone significantly reduced secretion of IL-1β, TNFα, and IL-12 (Fig 5A-C). However, the addition of methylprednisolone to tofacitinib/LPS-treated PBLs did not mitigate the increased clotting potential observed by turbidity assays. While, as before, the addition of tofacitinib/LPS to plasma reduced clotting lag time and increased V_{max} compared to LPS, the addition of methylprednisolone did not significantly change lag time or V_{max} compared to tofa/LPS at any concentration (Fig 5D,E), suggesting that corticosteroids did not block the immunothrombotic tendency linked to JAKi and LPS stimulation.

CTPA evaluation in RA cases with PE for immunothrombosis

In 6 patients with RA who developed PE under JAK inhibitor therapy (median age 66 years, 5 females), 4 were receiving baricitinib, 1 tofacitinib, and 1 upadacitinib. The median

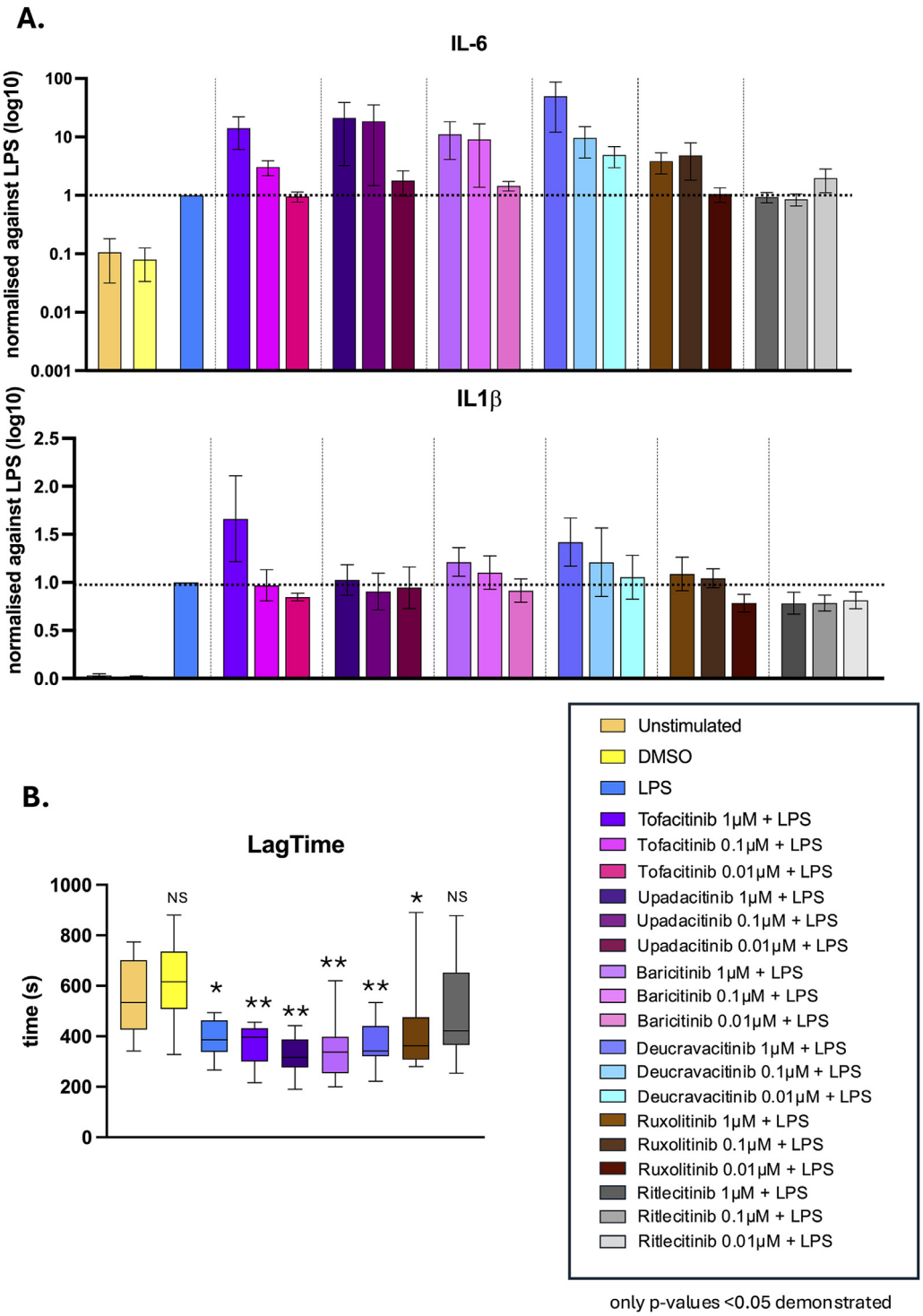

Figure 4. JAK3 inhibition may avoid JAKi-induced increase in inflammatory cytokine production and clot acceleration. (A) PBLs isolated from healthy participants (n = 6) were pretreated with either vehicle control (0.01% DMSO) or JAK inhibitor (1 μM, 100 nM, 10 nM) before LPS stimulation. Secreted IL-6 and IL-1β were measured by ELISA 48 hours post-stimulation. Data are normalised to the LPS-induced cytokine level for each donor. (B) Turbidity assays (n = 6) were performed using normal pool plasma supplemented with the conditioned media collected from the stimulation assay in (A), lag time to clot formation was calculated for conditioned media of LPS plus 1 μM of each JAK inhibitor. Data are presented as mean ± SD compared by one-way ANOVA for (A) and as median + IQR compared by Kruskal-Wallis test against the unstimulated condition for (B). *$P < .05$, **$P < .01$. ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; JAKi, Janus kinase inhibitors; LPS, lipopolysaccharide; PBL, peripheral blood leukocyte.

duration of JAKi exposure before the thrombotic event was 7.5 months (range: 1.0-18.0), and none of the patients were receiving glucocorticoids at the time of the event. CTPA revealed segmental-level thrombi in 5 patients, and involvement of a branch of the main pulmonary artery in 1 (Fig 6A). Figure 6B shows wedge-shaped peripheral opacification representing pulmonary infarction as a result of the thrombus. Ground-glass opacities were present in 3 cases, showing bilateral and focal or dependent patterns (Fig 6C). Concomitant DVT was confirmed in only 1 patient, suggesting a predominantly *in situ* pulmonary thrombotic process (Table 2).

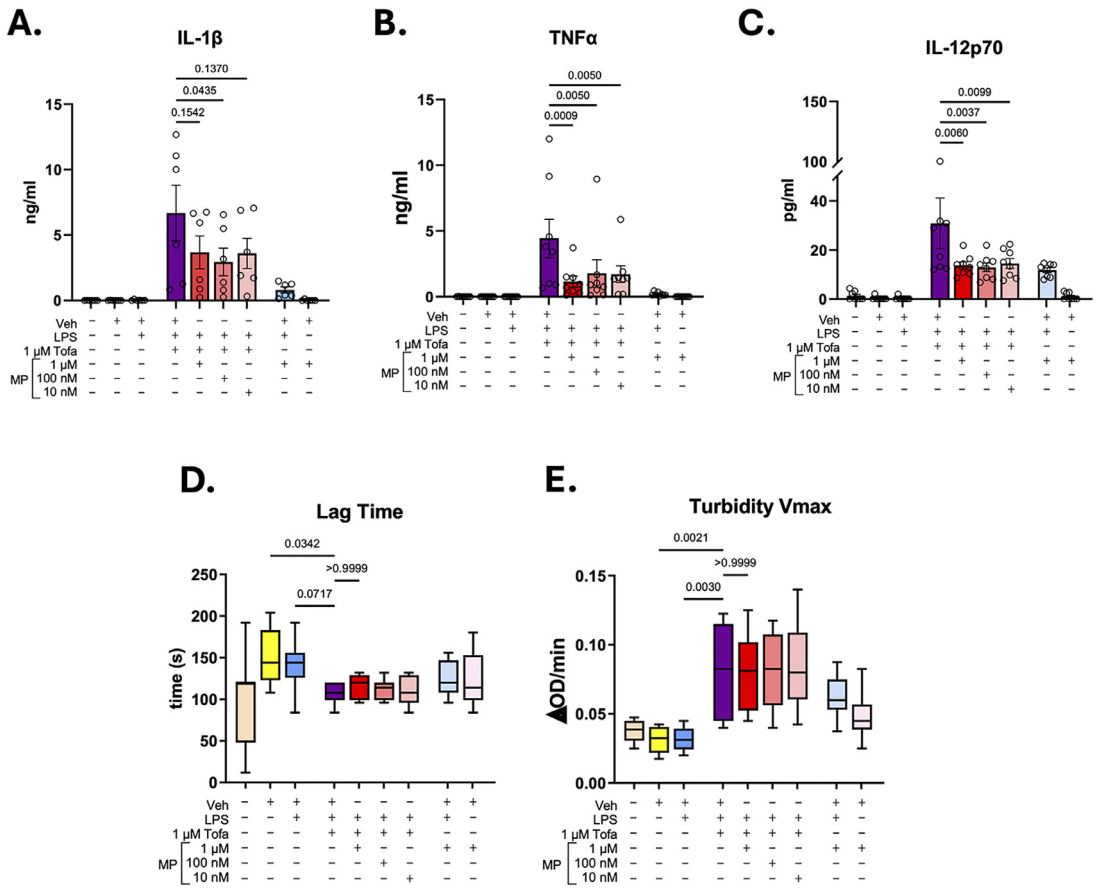

Figure 5. Methylprednisolone reduces tofacitinib-induced increase in proinflammatory cytokines but does not reduce accelerated clotting. PBLs isolated from patients with RA (n = 6) were pretreated with either vehicle control (0.01% DMSO) or 1 μM tofacitinib (Tofa) before LPS stimulation. Cells were also treated with methylprednisolone (MP; 1 μM, 100 nM, 10 nM) at the time of LPS stimulation as indicated. Secreted IL-1β (A), TNFα (B), and IL-12p70 (C) were measured by bead-based immunoassay 48 hours post-stimulation. Turbidity assays were performed using normal pool plasma supplemented with the conditioned media collected from the stimulation assay in (A-C). Lag time to clot formation (D) and clotting velocity (E; V_{max}) were calculated for the conditioned media. Data are presented as mean ± SD compared by one-way ANOVA for (A-C) and as median + IQR compared by Kruskal-Wallis test for (D-E). ANOVA, analysis of variance; IL, interleukin; LPS, lipopolysaccharide; PBL, peripheral blood leukocyte; RA, rheumatoid arthritis; TNFα, tumour necrosis factor α.

DISCUSSION

A link between JAKi and both DVT and VTE has been reported, but the underlying mechanism(s) remain elusive [8,9,19]. Increased incidence of VTE in patients with RA treated with tofacitinib was reported particularly in those with active disease, and receiving higher doses of the medication [8,10]. Herein, we report a TLR4, but not TLR3, JAKi-dependent mechanism associated with accelerated *in vitro* immunothrombosis that was more pronounced in RA samples from moderate-high disease, in keeping with clinical observations linking active RA to clotting [5]. In addition to upregulation of mononuclear cell-derived clotting factors, JAKi also upregulated cytokines, including IL-6, IL-1β, IL-12, and TNF-α, that have previously been associated with immunothrombosis [43–46]. Supernatants from TLR4-activated PBLs, in conjunction with tofacitinib and other JAKi, accelerated clot formation *in vitro*. The exception was JAK3 inhibition which was not linked to the acceleration of *in vitro* thrombosis. Specifically, ritlecitinib, a selective JAK3 inhibitor, unlike upadacitinib (JAK1) and deucravacitinib (Tyk2), does not perturb regulatory IL-10-mediated negative feedback [38]. These findings offer novel insights into immunothrombosis with JAK inhibition in rheumatology and potential strategies for therapy development to mitigate against VTE.

The observation of accelerated clotting exhibited by tofacitinib-treated PBLs in normal and $FXI^{-/-}$ plasma, but not $FVII^{-/-}$ plasma, suggests the effect primarily influences the tissue factor pathway. This aligns with our bulk RNA expression analysis, where we examined specific coagulation-related genes and found a significant overexpression of F3 (tissue factor). Tissue factor plays a crucial role in the crosstalk between the immune system and thrombosis, serving as a key initiator of physiological and pathological coagulation. When complexed with FVII, tissue factor activates factor X, leading to thrombin formation [47,48]. While the tissue factor pathway is typically associated with tissue damage, many immune-related molecules, such as TNFα, IL-1β, and IL-6, have been shown to induce the expression and secretion of tissue factor by monocytes [49,50]. Notably, secretion of TNFα and IL-1β, whose signalling pathways do not involve the JAK family, was increased under tofacitinib treatment. Thus, the tofacitinib-induced increases of TNFα and IL-1β, among others, may directly stimulate tissue factor expression by PBLs, thereby enhancing coagulation and contributing to a prothrombotic environment.

Our transcriptional analysis of LPS-stimulated PBLs with or without tofacitinib pretreatment also revealed dysregulated coagulation pathway-related genes that may further impact clotting dynamics. TFPI-2 was significantly overexpressed in PBLs

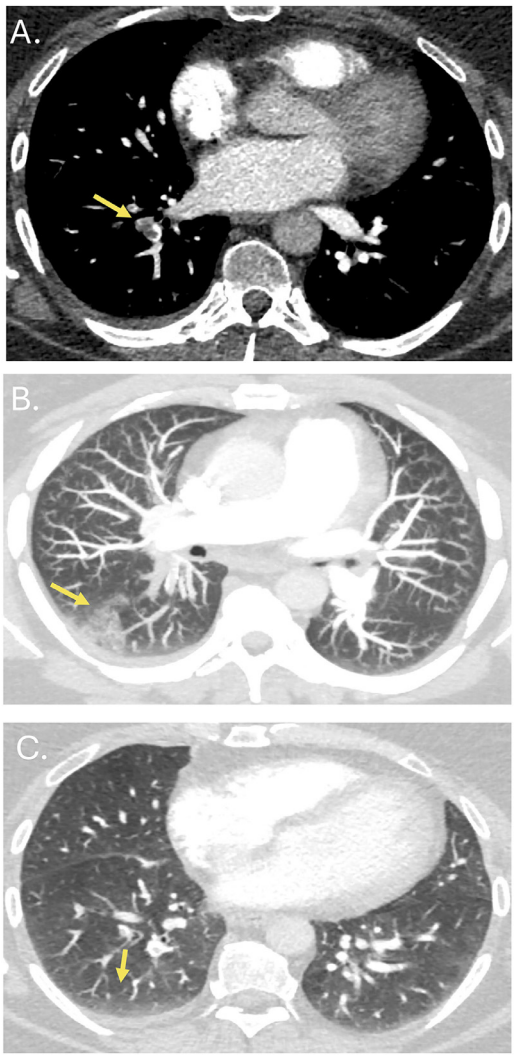

Figure 6. CTPA findings in a patient with pulmonary embolism under JAK inhibitor therapy. (A) CTPA showing right lower lobar pulmonary emboli. (B) Maximum intensity projection from CTPA showing wedge-shaped peripheral opacification within the apical segment of the right lower lobe, which may reflect pulmonary infarction, marked by arrow. (C) CTPA revealing patchy bibasal subpleural ground-glass opacification, marked by arrow. CTPA, computed tomography pulmonary angiography; JAK, Janus kinase.

treated with tofacitinib in conjunction with LPS. Despite its nomenclature, TFPI-2 does not inhibit the tissue factor pathway; instead, it belongs to the Kunitz-type family akin to tissue factor pathway inhibitor 1 (TFPI-1), sharing a closely aligned domain organisation and amino acid sequence [51]. TFPI-2 exerts inhibitory effects on serine proteases [51], including plasmin, thereby impeding fibrinolysis more profoundly than FXIa activity, fostering a hypercoagulative state [52]. Indeed, this molecule was associated with a higher risk of thrombosis in some patients with cancer [53,54]. Protein S expression was significantly reduced in PBLs treated with LPS and tofacitinib compared to LPS alone. Protein S serves as a cofactor for protein C, which, when activated, inhibits FVa and FVIIIa [55], as well as for TFPI-1, which in turn inhibits FXa [56]. Reduced protein S levels would therefore diminish its direct inhibitory effect on FXa, further enhancing coagulation and contributing to a prothrombotic state. Intriguingly, TFPI-1 also inhibits the tissue factor-FVIIa complex, and inhibition of protein S has been shown to reduce the efficacy of TFPI-1 in regulating tissue factor-FVIIa activity [57]. Given the dependence of tofacitinib-induced increase in coagulation on FVII in our turbidity assays, a decrease in protein S expression may contribute to loss of TFPI-1-mediated control of tissue factor-FVIIa and downstream FXa.

Our sequencing data also showed a reduction in expression of *F5* and *F12* in tofacitinib-pretreated LPS-stimulated PBLs. While this did not reach statistical significance, it is noteworthy that both are prothrombotic factors, as they belong to the common and contact coagulation pathway. This could initially seem contradictory, given that contact pathway factors are associated with thrombosis. However, the procoagulant activity of the tissue factor-FVIIa complex is significantly stronger than the amplification role of the contact pathway, specifically in the context of immunothrombosis, where the tissue factor pathway—primarily driven by tissue factor and FVII—plays a more potent role in initiating thrombosis, while the contact pathway mainly serves to amplify coagulation [48,58].

As previously suggested by Pattison et al [36] and recently shown by our group, the overexpression of inflammatory cytokines is likely due to predominant inhibition of IL-10 signalling, thereby preventing adequate negative feedback and further driving inflammation [38]. Given that IL-10 signals through a JAK1/TYK2 complex [59], JAK inhibition that spares IL-10 signalling may theoretically circumvent their immunothrombotic effects. To test this, we performed a comparison with healthy control samples (n = 6) to assess the effects of other JAK inhibitors on LPS-induced cytokine expression and clotting dynamics. Interestingly, every JAK inhibitor that targets either JAK1 or TYK2 exhibited comparable effects on IL-1β and IL-6 production, increasing their secretion when compared to LPS alone. In contrast, ritlecitinib, a selective JAK3 inhibitor, did not augment the TLR4 cytokine response following drug treatment. Intriguingly, in healthy donor PBLs, supernatants from LPS-stimulated cells accelerated clot formation in turbidity assays, but the addition of JAK inhibitors did not significantly further enhance this procoagulant effect. This contrasts with RA-derived PBLs, where JAK inhibition appeared to further promote clotting metrics. Yet, while JAK1 and TYK2 inhibitors had no impact on clotting metrics, JAK3 inhibition normalised LPS-induced lag time to that of untreated PBLs. These results suggest that additional activation of the myeloid compartment by JAKi may not be as impactful on clotting in people without background risk (as in RA), and that blockade of JAK3 signalling may circumvent JAKi-associated immunothrombosis by sparing JAK1/TYK2-dependent negative feedback loops.

While JAK inhibitors such as tofacitinib are clinically effective in reducing systemic inflammation in RA by suppressing multiple cytokine pathways, including IL-6, IFN-γ, and GM-CSF, emerging data suggest that their effects on innate immunity are more complex and context dependent. Several mechanistic studies have shown that JAK inhibition disrupts IL-10–mediated negative feedback in macrophages and dendritic cells, resulting in exaggerated TLR-induced secretion of proinflammatory cytokines such as TNF, IL-6, and IL-12 [36,38,60,61]. This paradox —where JAKi dampen systemic inflammation but enhance innate immune activation under specific stimuli—may have clinical relevance beyond traditional immunosuppression. IL-10 plays a central role in regulating thromboinflammatory pathways, including tissue factor expression and platelet activation [62–66]. By attenuating IL-10 signalling in myeloid cells, JAK inhibitors may inadvertently promote a prothrombotic phenotype, particularly in settings of heightened innate immune activation. These findings support a model in which JAKi-driven dysregulation of innate immune checkpoints—such as IL-10—may serve as a mechanistic link between inflammation and

Table 2

Demographic and disease-related characteristics of patients with pulmonary embolism under JAK inhibitor therapy

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age	72	68	71	62	71	33
Diagnosis age	53	56	42	50	50	23
Gender	Female	Female	Female	Female	Male	Female
Primary diagnosis	Seronegative RA/PsA	Seropositive RA	Seronegative RA	Seropositive RA	Seropositive RA	Seronegative RA
Comorbidities	Osteoporosis, osteoarthritis, hemochromatosis,	Osteoporosis, diabetes	Post-knee replacement-DVT, osteoarthritis, osteoporosis, hypertension	Diabetes, NSTEMI, COPD, osteoarthritis, COPD, TIA	ILD, chronic hepatitis	None
BMI	29.10	23.77	23.35	38.45	30.67	28.79
Smoking	No	No	No	Yes	No	No
Anti-CCP	Negative	Positive	Negative	Positive	Negative	Negative
RF	Negative	Positive	Negative	Positive	Positive	Negative
ANA	Negative	Negative	Negative	Negative	Positive	Positive
Antiphospholipid antibodies	N/A	N/A	Negative	Negative	Negative	Lupus anticoagulant (one-time positive)
Biological treatments before JAKi	Certolizumab, adalimumab, etanercept, tocilizumab, sarilumab	Abatacept, rituximab, etanercept, golimumab, sarilumab	Infliximab, etanercept, adalimumab golimumab, tocilizumab, abatacept	Enbrel, rituximab, tocilizumab, certolizumab, abatacept	Abatacept, tocilizumab, rituximab, infliximab, etanercept, certolizumab	Rituximab
Previously received JAKi	No	No	No	Yes (baricitinib stopped due to oral ulcers and mood disorder)	No	No
Which JAKi is used during the event	Baricitinib	Baricitinib	Baricitinib	Tofacitinib	Baricitinib	Upadacitinib
Duration of JAKi	14 mo	18 mo	1 mo	1 mo	13 mo	2 mo
Thrombosis age	69	65	65	57	66	32
Level of thrombosed vessel	Segmental	Segmental	Segmental	Branch of main pulmonary artery	Segmental	Segmental
Presence of ground-glass changes at CT	No	Yes, bilateral/dependent changes	Yes, bilateral/focal	No	Yes, bilateral/focal	No
CRP levels (mg/L) before the event	5	5.1	125	19.7	5.5	90
DVT concurrence	No (clinical)	No (clinical)	No (clinical)	No (US Venography)	No (clinical)	Yes
Any risk factor	Pancreatic cancer	No	Cholecystitis (no surgery was performed)	History of COVID infection within the last 2 wk	No	No
Receiving GCs during the event	No	No	No	No	No	No
Active arthritis during the event	No	No	2 wk ago	No	No	No
Continuing JAKi after the event	No	No	Yes	Yes	Yes	No
Current treatment	Abatacept	Tocilizumab	Filgotinib	Tofacitinib	Baricitinib	Tocilizumab

ANA, antinuclear antibody; BMI, body mass index; CCP, cyclic citrullinated peptide; COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; CT, computed tomography; DVT, deep vein thrombosis; GC, glucocorticoid; ILD, interstitial lung disease; JAKi, Janus kinase inhibitors; N/A, not applicable; NSTEMI, non-ST-elevation myocardial infarction; PsA, psoriatic arthritis; RA, rheumatoid arthritis; RF, rheumatoid factor; TIA, transient ischaemic attack; US, ultrasound.

thrombosis and highlight the need for individualised risk stratification in patients receiving these therapies.

In addition, CTPA appearance of patients with RA who developed PE during JAK inhibition treatment often demonstrated concomitant ground-glass opacities that fit with immunothrombosis taking place *in vivo* in these patients. Interestingly, during the COVID-19 pandemic, where iPT was commonly seen and often related to immunothrombosis [25], JAK inhibition with baricitinib was successfully used as a therapy [67]. In COVID-19, JAKi was used with corticosteroids as standard therapy. We thus used corticosteroids in our *in vitro* clotting model to check if it would neutralise JAK inhibition prothrombotic effect. Despite reductions in inflammatory cytokines, no significant changes were observed in *in vitro* clot dynamics, thus making it uncertain what impact concomitant corticosteroids might have in active RA cases starting JAKi. Corticosteroids have previously been shown to increase the levels of plasminogen activator inhibitor-1, thrombin, and von Willebrand factor in the blood of healthy individuals [68]. Indeed, clinical observations show that corticosteroid use is associated with an increased risk of rethrombosis [69], indicating a broader impact on coagulation regulation. The procoagulant mechanisms of corticosteroids could explain why, despite their ability to reduce cytokine levels, their effects on turbidity remained evident and like JAK inhibition alone [70]. It is also of interest that the beneficial effects of JAKi with baricitinib in COVID-19 pneumonia was only evident in subjects with high body mass index (BMI), that is a known risk factor for thrombosis.

This study was designed as a mechanistic *in vitro* investigation and, as such, has several important limitations. First, although our experimental model focused on PBL responses under inflammatory stimulation, it does not fully recapitulate the complex physiological environment involved in coagulation and fibrinolysis. Key contributors to immunothrombosis—such as endothelial cells, platelets, plasmatic coagulation factors, and complement pathways—were not included in our assays. Therefore, while our findings provide mechanistic insight into leukocyte behaviour during JAK inhibition, they cannot capture the full complexity of thromboinflammatory responses *in vivo*.

In addition, we did not assess haematologic or genetic predispositions to thrombosis, such as JAK2 V617F mutations or age-related clonal haematopoiesis, which could influence individual susceptibility to thrombotic events during JAK inhibitor therapy. Furthermore, we lacked longitudinal clinical data such as plasma cytokine levels, CRP, or body temperature during treatment, and were therefore unable to directly correlate *in vitro* findings with *in vivo* inflammatory activity or thrombotic outcomes. Future studies incorporating these elements will be important for validating and extending our observations in clinically relevant settings. Although PE cases in patients with RA treated with JAK inhibitors were included for clinical context, no peripheral blood samples from these individuals were used in the experimental analyses. We also recognise that certain imaging findings, such as ground-glass opacities observed on CTPA scans, may be confounded by unrelated processes including subclinical interstitial lung disease. As such, these features should not be overinterpreted as definitive evidence of *in situ* immunothrombosis.

While our transcriptomic and clotting assays revealed JAKi-related alterations in key coagulation-related genes (eg, F3, TFPI-2, PROS1), these findings were based solely on tofacitinib exposure. However, previous work conducted by our lab [38], exploring upadacitinib in entheseal bone marrow leukocytes, has shown similar LPS-induced transcriptional changes after upadacitinib treatment. Indeed, interrogation of this dataset for the coagulation genes of interest outlined in Figure 3 showed F3 and TFPI-2 to be strongly upregulated in the Giryes dataset as well (Supplementary Fig S5). Given the genes implicated in both datasets and the reliance on FVII demonstrated in Figure 1, future studies should explore the relationship between JAK inhibition and F3/TFPI-2-regulated coagulation in inflammation. Nonetheless, caution is warranted when extrapolating our findings to other JAK inhibitors, given the variability in JAK specificity and mechanism of inhibition across this drug class.

Finally, while our transcriptomic and turbidity assays revealed JAKi-associated alterations in coagulation-related genes, we did not perform proteomic validation or direct functional assays of the affected pathways. These findings, therefore, remain at the transcript level and require further investigation to confirm their biological relevance.

Our study provides proof of concept that JAK inhibition, especially JAK1 or TYK2, promotes immunothrombosis by increasing inflammatory cytokines, clotting factors, and their regulators triggered by TLR4 stimulation in PBLs from patients with RA, in an *in vitro* setting. These findings invite the notion that JAKi, targeting JAK1 and TYK2 in particular, may reduce the threshold required for an immune insult, such as a respiratory tract infection, to initiate *in situ* clotting. This effect is particularly notable in cases of active disease, potentially elucidating the heightened risk of VTE observed in active patients with RA [4]. Interestingly, a study published at the time of writing described a parallel platelet-dependent mechanism by which JAKi can promote thrombosis via thromboxane production in systemic lupus and axial spondyloarthritis, complementing our results in support of prothrombotic JAKi effects in inflammatory conditions [71]. Overall, our findings suggest that VTE under JAK inhibition is distinct from clotting pathway mutations and autoantibodies associated with thrombosis in rheumatology, thus offering insights into distinct clotting mechanisms that are part of the innate immune response, which has broad implications for understanding the clinical pharmacology of JAKi.

From a clinical standpoint, these results underscore the importance of identifying patient populations at increased risk of thrombotic complications during JAKi therapy, particularly those with active disease or concurrent inflammatory stressors. The data also suggest that temporary discontinuation of JAK inhibitors during episodes of acute infection or fever might be a reasonable precaution in selected cases, especially in patients with known thrombotic risk factors.

Moreover, by implicating the disruption of IL-10-mediated immunoregulatory feedback as a key mechanistic driver, our findings raise the possibility that next-generation JAK inhibitors could be optimised to preserve IL-10 signalling, retaining their anti-inflammatory benefits while reducing prothrombotic risk. Ultimately, this work contributes to a better understanding of the immunothrombotic landscape in inflammatory diseases and may help guide personalised treatment decisions and safer drug design in the era of targeted immunomodulation.

Competing interests

DM reports financial support was provided by NIHR Leeds Biomedical Research Centre. RA reports financial support was provided by NIHR Leeds Biomedical Research Centre. KM reports financial support was provided by NIHR Leeds Biomedical Research Centre. PE reports financial support was provided by NIHR Leeds Biomedical Research Centre. AS reports financial

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Contributors

PD and TM contributed equally to this work and share first authorship. They were responsible for conceptualisation, formal analysis, investigation, methodology, and writing—original draft. DM, CD, and RA contributed to conceptualisation, methodology, supervision, investigation, writing—original draft, and writing—review and editing. KA, SG, AA, MH, CW, and PG were involved in methodology, investigation, and writing—review and editing. GdMS contributed to investigation and writing—original draft. YS, PE, KM, BS, and AS contributed to writing—review and editing.

Patient consent for publication

Written informed consent was obtained from all participants.

Ethics approval

Ethical approval for the study was obtained from the Leeds Teaching Hospitals NHS Trust (Rheumatoid Arthritis DiseAse Research ethics, protocol number RR09/9134, version 12.0) and were in accordance with the Decleration of Helsinki-Ethical Principles for Medical Research Involving Human Subjects.

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REFERENCES

[1] Naess IA, Christiansen SC, Romundstad P, Cannegieter SC, Rosendaal FR, Hammerstrøm J. Incidence and mortality of venous thrombosis: a population-based study. J Thromb Haemost 2007 Apr;5(4):692–9.
[2] Heit JA, Silverstein MD, Mohr DN, Petterson TM, O'Fallon WM, Melton LJ. Predictors of survival after deep vein thrombosis and pulmonary embolism: a population-based, cohort study. Arch Intern Med 1999 Mar 8;159(5):445–53.
[3] Palareti G, Antonucci E, Dentali F, Mastroiacovo D, Mumoli N, Pengo V, et al. Patients with isolated pulmonary embolism in comparison to those with deep venous thrombosis. Differences in characteristics and clinical evolution. Eur J Intern Med 2019 Nov;69:64–70.
[4] Molander V, Bower H, Askling J. Validation and characterization of venous thromboembolism diagnoses in the Swedish National Patient Register among patients with rheumatoid arthritis. Scand J Rheumatol 2023 Mar;52(2):111–7.
[5] Molander V, Bower H, Frisell T, Askling J. Risk of venous thromboembolism in rheumatoid arthritis, and its association with disease activity: a nationwide cohort study from Sweden. Ann Rheum Dis 2021 Feb;80(2):169–75.
[6] Li L, Lu N, Avina-Galindo AM, Zheng Y, Lacaille D, Esdaile JM, et al. The risk and trend of pulmonary embolism and deep vein thrombosis in rheumatoid arthritis: a general population-based study. Rheumatology (Oxford) 2021 Jan 5;60(1):188–95.
[7] Hu LJ, Ji B, Fan HX. Venous thromboembolism risk in rheumatoid arthritis patients: a systematic review and updated meta-analysis. Eur Rev Med Pharmacol Sci 2021;25(22):7005–13.
[8] Molander V, Bower H, Frisell T, Delcoigne B, Di Giuseppe D, Askling J, et al. Venous thromboembolism with JAK inhibitors and other immune-modulatory drugs: a Swedish comparative safety study among patients with rheumatoid arthritis. Ann Rheum Dis 2023 Feb;82(2):189–97.
[9] Ytterberg SR, Bhatt DL, Mikuls TR, Koch GG, Fleischmann R, Rivas JL, et al. Cardiovascular and cancer risk with tofacitinib in rheumatoid arthritis. N Engl J Med 2022 Jan 27;386(4):316–26.
[10] Charles-Schoeman C, Fleischmann R, Mysler E, Greenwald M, Ytterberg SR, Koch GG, et al. Risk of venous thromboembolism with tofacitinib versus tumor necrosis factor inhibitors in cardiovascular risk-enriched rheumatoid arthritis patients. Arthritis Rheumatol 2024 Mar 13;76(8):1218–29.
[11] Dijkshoorn B, Raadsen R, Nurmohamed MT. Cardiovascular disease risk in rheumatoid arthritis anno 2022. J Clin Med 2022 May 11;11(10):2704.
[12] England BR, Thiele GM, Anderson DR, Mikuls TR. Increased cardiovascular risk in rheumatoid arthritis: mechanisms and implications. BMJ 2018 Apr 23;361:k1036.
[13] Semb AG, Ikdahl E, Wibetoe G, Crowson C, Rollefstad S. Atherosclerotic cardiovascular disease prevention in rheumatoid arthritis. Nat Rev Rheumatol 2020 Jul;16(7):361–79.
[14] Wu JJ, Strober BE, Hansen PR, Ahlehoff O, Egeberg A, Qureshi AA, et al. Effects of tofacitinib on cardiovascular risk factors and cardiovascular outcomes based on phase III and long-term extension data in patients with plaque psoriasis. J Am Acad Dermatol 2016 Nov;75(5):897–905.
[15] Charles-Schoeman C, DeMasi R, Valdez H, Soma K, Hwang LJ, Boy MG, et al. Risk factors for major adverse cardiovascular events in phase III and long-term extension studies of tofacitinib in patients with rheumatoid arthritis. Arthritis Rheumatol 2019 Sep;71(9):1450–9.
[16] Giuntini C, di Ricco G, Marini C, Melillo E, Palla A. Pulmonary embolism: epidemiology. Chest 1995 Jan;107(1 suppl):3S–9S.
[17] Hull RD, Hirsh J, Carter CJ, Jay RM, Dodd PE, Ockelford PA, et al. Pulmonary angiography, ventilation lung scanning, and venography for clinically suspected pulmonary embolism with abnormal perfusion lung scan. Ann Intern Med 1983 Jun;98(6):891–9.
[18] Dörner T. Lessons from tofacitinib in patients with cardiovascular risk factors: increased pulmonary embolism or isolated (thrombotic) pulmonary occlusion rates? Ann Rheum Dis 2020 Nov;79(11):1389–92.
[19] Mease P, Charles-Schoeman C, Cohen S, Fallon L, Woolcott J, Yun H, et al. Incidence of venous and arterial thromboembolic events reported in the tofacitinib rheumatoid arthritis, psoriasis and psoriatic arthritis development programmes and from real-world data. Ann Rheum Dis 2020 Nov;79(11):1400–13.
[20] Lee YH, Cha SI, Shin KM, Lim JK, Lee WK, Park JE, et al. Clinical characteristics and outcomes of patients with isolated pulmonary embolism. Blood Coagul Fibrinolysis 2021 Sep 1;32(6):387–93.
[21] Ten Cate V, Eggebrecht L, Schulz A, Panova-Noeva M, Lenz M, Koeck T, et al. Isolated pulmonary embolism is associated with a high risk of arterial thrombotic disease: results from the VTEval study. Chest 2020 Jul;158(1):341–9.
[22] Mori S, Ogata F, Tsunoda R. Risk of venous thromboembolism associated with Janus kinase inhibitors for rheumatoid arthritis: case presentation and literature review. Clin Rheumatol 2021 Nov 23;40(11):4457–71.
[23] Engelmann B, Massberg S. Thrombosis as an intravascular effector of innate immunity. Nat Rev Immunol 2013 Jan;13(1):34–45.
[24] von Brühl ML, Stark K, Steinhart A, Chandraratne S, Konrad I, Lorenz M, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. J Exp Med 2012 Apr 9;209(4):819–35.
[25] Portier I, Campbell RA, Denorme F. Mechanisms of immunothrombosis in COVID-19. Curr Opin Hematol 2021;28(6):445–53.
[26] Bonaventura A, Vecchié A, Dagna L, Martinod K, Dixon DL, Van Tassell BW, et al. Endothelial dysfunction and immunothrombosis as key pathogenic mechanisms in COVID-19. Nat Rev Immunol 2021;21(5):319–29.
[27] Nicolai L, Leunig A, Brambs S, Kaiser R, Joppich M, Hoffknecht ML, et al. Vascular neutrophilic inflammation and immunothrombosis distinguish severe COVID-19 from influenza pneumonia. J Thromb Haemost 2021 Feb 1;19(2):574–81.
[28] McGonagle D, Bridgewood C, Meaney JFM. A tricompartmental model of lung oxygenation disruption to explain pulmonary and systemic pathology in severe COVID-19. Lancet Respir Med 2021;9(6):665–72.
[29] McGonagle D, O'Donnell JS, Sharif K, Emery P, Bridgewood C. Immune mechanisms of pulmonary intravascular coagulopathy in COVID-19 pneumonia. Lancet Rheumatol 2020 Jul;2(7):e437–45.
[30] Baranga L, Khanuja S, Scott JA, Provancha I, Gosselin M, Walsh J, et al. In situ pulmonary arterial thrombosis: literature review and clinical significance of a distinct entity. AJR Am J Roentgenol 2023 Jul;221(1):57–68.

[31] Taylor PC, Keystone EC, van der Heijde D, Weinblatt ME, Del Carmen Morales L, Reyes Gonzaga J, et al. Baricitinib versus placebo or adalimumab in rheumatoid arthritis. N Engl J Med 2017;376(7):652–62.
[32] Sandborn WJ, Ghosh S, Panes J, Schreiber S, D'Haens G, Tanida S, et al. Efficacy of upadacitinib in a randomized trial of patients with active ulcerative colitis. Gastroenterology 2020;158(8) 213949.e14.
[33] Blauvelt A, Teixeira HD, Simpson EL, Costanzo A, De Bruin-Weller M, Barbarot S, et al. Efficacy and safety of upadacitinib vs dupilumab in adults with moderate-to-severe atopic dermatitis: a randomized clinical trial. JAMA Dermatol 2021 Sep 1;157(9):1047–55.
[34] White JPE, Coates LC. JAK1 selective inhibitors for the treatment of spondyloarthropathies. Rheumatology (Oxford) 2021;60(suppl 2):ii39–44.
[35] El Jammal T, Gerfaud-Valentin M, Sève P, Jamilloux Y. Inhibition of JAK/STAT signaling in rheumatologic disorders: the expanding spectrum. Joint Bone Spine 2020;87(2):119–29.
[36] Pattison MJ, MacKenzie KF, Arthur JSC. Inhibition of JAKs in macrophages increases lipopolysaccharide-induced cytokine production by blocking IL-10-mediated feedback. J Immunol 2012 Sep 15;189(6):2784–92.
[37] Chen F, Cao A, Yao S, Evans-Marin HL, Liu H, Wu W, et al. mTOR mediates IL-23 induction of neutrophil IL-17 and IL-22 production. J Immunol 2016 May 15;196(10):4390–9.
[38] Giryes S, Wong C, Bridgewood C, Harland M, Altaie A, McDermott N, et al. Paradoxical activation of entheseal myeloid cells by JAK1 and Tyk2 inhibitors via interleukin-10 antagonism. Arthritis Rheumatol 2025. doi: 10.1002/art.43210.
[39] Baker SR, Zabczyk M, Macrae FL, Duval C, Undas A, Ariëns RAS. Recurrent venous thromboembolism patients form clots with lower elastic modulus than those formed by patients with non-recurrent disease. J Thromb Haemost 2019 Apr;17(4):618–26.
[40] Shi Y, Gauer JS, Baker SR, Philippou H, Connell SD, Ariëns RAS. Neutrophils can promote clotting via FXI and impact clot structure via neutrophil extracellular traps in a distinctive manner in vitro. Sci Rep 2021 Jan 18;11(1):1718.
[41] Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 2014;15(12):550.
[42] Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B Methodol 1995 Jan 1;57(1):289–300.
[43] Liberale L, Holy EW, Akhmedov A, Bonetti NR, Nietlispach F, Matter CM, et al. Interleukin-1β mediates arterial thrombus formation via NET-associated tissue factor. J Clin Med 2019 Nov 26;8(12):2072.
[44] Page MJ, Bester J, Pretorius E. Interleukin-12 and its procoagulant effect on erythrocytes, platelets and fibrin(ogen): the lesser known side of inflammation. Br J Haematol 2018 Jan;180(1):110–7.
[45] Kerr R, Stirling D, Ludlam CA. Interleukin 6 and haemostasis. Br J Haematol 2001 Oct;115(1):3–12.
[46] van der Poll T, van Deventer SJ, Pasterkamp G, van Mourik JA, Büller HR, ten Cate JW. Tumor necrosis factor induces von Willebrand factor release in healthy humans. Thromb Haemost 1992 Jun 1;67(6):623–6.
[47] Mackman N. The many faces of tissue factor. J Thromb Haemost 2009 Jul;7(suppl(suppl 1)):136–9.
[48] Grover SP, Mackman N. Tissue factor: an essential mediator of hemostasis and trigger of thrombosis. Arterioscler Thromb Vasc Biol 2018 Apr;38(4):709–25.
[49] Khan MM, Liu Y, Khan ME, Gilman ML, Khan ST, Bromberg M, et al. Upregulation of tissue factor in monocytes by cleaved high molecular weight kininogen is dependent on TNF-alpha and IL-1beta. Am J Physiol Heart Circ Physiol 2010;298(2):H652–8.
[50] Szotowski B, Antoniak S, Poller W, Schultheiss HP, Rauch U. Procoagulant soluble tissue factor is released from endothelial cells in response to inflammatory cytokines. Circ Res 2005 Jun 24;96(12):1233–9.
[51] Chand HS, Foster DC, Kisiel W. Structure, function and biology of tissue factor pathway inhibitor-2. Thromb Haemost 2005 Dec;94(6):1122–30.
[52] Vadivel K, Ponnuraj SM, Kumar Y, Zaiss AK, Bunce MW, Camire RM, et al. Platelets contain tissue factor pathway inhibitor-2 derived from megakaryocytes and inhibits fibrinolysis. J Biol Chem 2014 Nov;289(45):31647–61.
[53] Miyake R, Yamada Y, Yamanaka S, Kawaguchi R, Ootake N, Myoba S, et al. Tissue factor pathway inhibitor 2 as a serum marker for diagnosing asymptomatic venous thromboembolism in patients with epithelial ovarian cancer and positive Ddimer results. Mol Clin Oncol 2022 Feb;16(2):46.
[54] Yamanaka S, Miyake R, Yamada Y, Kawaguchi R, Ootake N, Myoba S, et al. Tissue factor pathway inhibitor 2: a novel biomarker for predicting asymptomatic venous thromboembolism in patients with epithelial ovarian cancer. Gynecol Obstet Invest 2022;87(2):133–40.
[55] Walker FJ. Protein S and the regulation of activated protein C. Semin Thromb Hemost 1984 Apr;10(2):131–8.
[56] Ndonwi M, Broze G. Protein S enhances the tissue factor pathway inhibitor inhibition of factor Xa but not its inhibition of factor VIIa-tissue factor. J Thromb Haemost 2008 Jun;6(6):1044–6.
[57] Petri A, Sasikumar P, Folgado PB, Jones D, Xu Y, Ahnström J, et al. TFPIα anticoagulant function is highly dependent on protein S in vivo. Sci Adv 2024 Feb 2;10(5):eadk5836.
[58] de Maat S, Maas C. Factor XII: form determines function. J Thromb Haemost 2016 Aug;14(8):1498–506.
[59] Kotyla PJ, Engelmann M, Giemza-Stokłosa J, Wnuk B, Islam MA. Thromboembolic adverse drug reactions in Janus Kinase (JAK) inhibitors: does the inhibitor specificity play a role? Int J Mol Sci 2021 Feb 28;22(5):2449.
[60] Wang H, Brown J, Gao S, Liang S, Jotwani R, Zhou H, et al. The role of JAK-3 in regulating TLR-mediated inflammatory cytokine production in innate immune cells. J Immunol 2013 Aug 1;191(3):1164–74.
[61] Stalder R, Zhang B, Jean Wrobel L, Boehncke WH, Brembilla NC. The Janus kinase inhibitor tofacitinib impacts human dendritic cell differentiation and favours M1 macrophage development. Exp Dermatol 2020 Jan;29(1):71–8.
[62] Noble KE, Harkness D, Yong KL. Interleukin 10 regulates cellular responses in monocyte/endothelial cell co-cultures. Br J Haematol 2000 Mar;108(3):497–504.
[63] Christersson C, Johnell M, Siegbahn A. Tissue factor and IL8 production by P-selectin-dependent platelet-monocyte aggregates in whole blood involves phosphorylation of Lyn and is inhibited by IL10. J Thromb Haemost 2008 Jun;6(6):986–94.
[64] Ernofsson M, Tenno T, Siegbahn A. Inhibition of tissue factor surface expression in human peripheral blood monocytes exposed to cytokines. Br J Haematol 1996 Nov;95(2):249–57.
[65] Lindmark E, Tenno T, Chen J, Siegbahn A. IL-10 inhibits LPS-induced human monocyte tissue factor expression in whole blood. Br J Haematol 1998 Jul;102(2):597–604.
[66] Kamimura M, Viedt C, Dalpke A, Rosenfeld ME, Mackman N, Cohen DM, et al. Interleukin-10 suppresses tissue factor expression in lipopolysaccharide-stimulated macrophages via inhibition of Egr-1 and a serum response element/MEK-ERK1/2 pathway. Circ Res 2005 Aug 19;97(4):305–13.
[67] Marconi VC, Ramanan A V, de Bono S, Kartman CE, Krishnan V, Liao R, et al. Efficacy and safety of baricitinib for the treatment of hospitalised adults with COVID-19 (COV-BARRIER): a randomised, double-blind, parallel-group, placebo-controlled phase 3 trial. Lancet Respir Med 2021;9(12):1407–18.
[68] Majoor CJ, Sneeboer MMS, de Kievit A, Meijers JCM, van der Poll T, Lutter R, et al. The influence of corticosteroids on hemostasis in healthy subjects. J Thromb Haemost 2016 Apr;14(4):716–23.
[69] Orsi FA, Lijfering WM, Geersing GJ, Rosendaal FR, Dekkers OM, le Cessie S, et al. Glucocorticoid use and risk of first and recurrent venous thromboembolism: self-controlled case-series and cohort study. Br J Haematol 2021 Jun;193(6):1194–202.
[70] David P, Hen O, Ben-Shabbat N, Macleod T, Amital H, Watad A, McGonagle DG. Pronounced benefits of JAK inhibition with baricitinib in COVID-19 pneumonia in obese but not lean subjects. RMD Open 2024;10(2):e004045. doi: 10.1136/rmdopen-2023-004045.
[71] Alabbasi S, Tacconelli S, de Vries M, Gunnarsson I, Oke V, Kvarnström M, et al. Janus kinase inhibitors enhance prostanoid biosynthesis in human whole blood in vitro: implications for cardiovascular side effects and prevention strategies. Ann Rheum Dis 2025 May 5;84(9):1549–60.