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Abstract:
Platelet factor 4 (PF4) is an abundant chemokine that is released from platelet α-granules upon activation. PF4 is central to the pathophysiology of vaccine-induced immune thrombocytopenia and thrombosis (VITT) in which antibodies to PF4 form immune complexes with PF4, which activate platelets and neutrophils through Fc receptors. In this study, we show that PF4 binds and activates the thrombopoietin (TPO) receptor, c-Mpl on platelets. This leads to the activation of Janus kinase 2 (JAK2) and phosphorylation of signal transducer and activator of transcription (STAT) 3 and STAT5, leading to platelet aggregation. Inhibition of the c-Mpl-JAK2 pathway inhibits platelet aggregation to PF4, VITT serum, and to the combination of PF4 and IgG isolated from VITT patient plasma. The results support a model in which PF4-based immune complexes activate platelets through binding of the Fc domain to FcγRIIA and PF4 to c-Mpl.

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Key points

- PF4 activates the thrombopoietin receptor c-Mpl in platelets resulting in JAK2-STAT3/5 signaling.
- Inhibition of kinase activity of JAK2 abrogates platelet aggregation by PF4.

Abstract

Platelet factor 4 (PF4) is an abundant chemokine that is released from platelet α-granules upon activation. PF4 is central to the pathophysiology of vaccine-induced immune thrombocytopenia and thrombosis (VITT) in which antibodies to PF4 form immune complexes with PF4, which activate platelets and neutrophils through Fc receptors. In this study, we show that PF4 binds and activates the thrombopoietin (TPO) receptor, c-Mpl, on platelets. This leads to the activation of Janus kinase 2 (JAK2) and phosphorylation of signal transducer and activator of transcription (STAT) 3 and STAT5, leading to platelet aggregation. Inhibition of the c-Mpl-JAK2 pathway inhibits platelet aggregation to PF4, VITT serum, and to the combination of PF4 and IgG isolated from VITT patient plasma. The results support a model in which PF4-based immune complexes activate platelets through binding of the Fc domain to FcγRIIA and PF4 to c-Mpl.

Introduction

Platelet factor 4 (PF4), also known as C-X-C motif ligand 4 (CXCL4), is a 7.8 kDa chemokine that is released from platelet α-granules.[1] PF4 forms tetramers with a compact globular structure and a strong equatorial positive charge,[2,3] and binds strongly to negatively charged molecules including endothelial proteoglycans and infectious agents.[4,5] Antibodies to PF4 underlie the pathogenesis of heparin-induced thrombocytopenia (HIT)[6,7] and vaccine-induced immune thrombocytopenia and thrombosis (VITT)[8,9] by forming immune complexes with PF4.

The direct effect of PF4 on platelets has not been extensively studied. PF4 potentiates activation of platelets to threshold doses of thrombin[10] and has recently been shown to induce platelet aggregation[11] but the underlying mechanism has not been investigated. In this study, we show that PF4 binds the thrombopoietin (TPO) receptor, c-Mpl, inducing activation of Janus kinase 2 (JAK2). The JAK2 inhibitor, ruxolitinib, blocks platelet aggregation to PF4 and to VITT antibodies.

Methods

For a full description of all reagents and methods, see supplemental Methods.

Ethical approval

Approval for collecting blood from healthy volunteers was granted by the University of Birmingham's Internal Ethical Review Committee (ERN_11-0175APP22). Collection of serum and plasma from patients with VITT at University Hospitals Birmingham NHS Foundation Trust was authorized by the University of Birmingham Human Bioresource Centre (approval 15/NW/0079). Samples were obtained in accordance with the Declaration of Helsinki.

Key reagents

PF4 was from ChromaTec GmbH (Greifswald, Germany), collagen-related peptide (CRP) from CambCol Ltd (Ely, UK), recombinant human thrombopoietin (TPO) from Proteintech (Illinois, USA), ruxolitinib from Stratech Scientific (Ely, UK), recombinant c-Mpl (TpoR) (4444-TR) from Biotechne (R&D Systems, Minneapolis, USA), and c-Mpl polyclonal blocking antibody PA5-47042) from Invitrogen (ThermoFisher Scientific, Waltham, USA).

Light transmission aggregometry (LTA)

Washed platelets ($2x10^8$/mL) were prepared as described.[12] Platelets were pre-warmed to 37°C and pre-incubated with PF4 and/or inhibitors for 5 min. Agonists were added under stirring conditions (1200 rpm) and aggregation measured for 7-30 min.

Protein phosphorylation

Platelets ($4x10^8$/mL) pre-warmed to 37°C were pre-treated with eptifibatide (9 μM). Reactions were terminated after 10 min, samples separated by sodium docecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), electro-transferred, and western blotted. Mass spectrometry was performed by excision of material at ~95 kDa on SDS-PAGE gel. Full details are included in supplemental Methods.

Data presentation

Results were expressed as mean ± standard deviation (SD) and were considered significant at $p<0.05$. Details on statistical packages can be found in supplemental Methods.

Data sharing statement

Original data are available on reasonable request to the corresponding authors. Mass spectrometry data are deposited to the PRoteomics IDEntifications Database (PRIDE) and are available via www.ProteomeXchange.org (identifier PXD043558).

Results and Discussion

PF4 stimulated biphasic platelet aggregation over 3-100 μg/mL (0.4-12.8 μM) with a gradual initial phase followed by a rapid second phase (Figure 1A). In some donors, the dose response curve was bell-shaped with the second phase often absent at higher concentrations of PF4 (Figure 1A). Aggregation was abrogated in the presence of eptifibatide demonstrating that it was mediated through activation of integrin GPIIb/IIIa (supplemental Figure 1).

These concentrations are higher than the plasma concentration of PF4 which is in the region of 0.8 – 50 ng/mL.[13] However, the vast majority of PF4 is contained in platelet α-granules. Based on data reporting 500,000 copies of PF4 per platelet,[14,15] the whole blood concentration is ~1.5 μg/mL, with higher local concentrations formed on α-granule release and being concentrated at surfaces through glycosaminoglycan binding. In addition, these are typical concentrations that are used in assays that are widely used in studies of VITT and HIT, as well as in the diagnosis of HIT.[16,17]

To investigate the mechanism of activation, we measured tyrosine phosphorylation of whole cell lysates by western blotting using the anti-phosphotyrosine monoclonal antibody, 4G10. PF4 stimulated a concentration dependent increase in tyrosine phosphorylation of a prominent doublet at 95 kDa (Figure 1Bi, arrows). Analysis by mass spectrometry revealed the presence of a STAT5a/b peptide phosphorylated at Tyr 694/699 (supplemental data file). These findings were confirmed by western blot using a phosphospecific antibody to STAT5a/b (Figure 1Bi). STAT3 was also identified by mass spectrometry and shown to be phosphorylated on Tyr704 using a phosphospecific antibody (Figure 1Bi).

The STAT family of transcription factors are phosphorylated in platelets downstream of the c-Mpl-JAK2 pathway.[18] In line with this, PF4 stimulated dose-dependent phosphorylation of JAK2 (Figure 1Bii) and c-Mpl (Tyr626) (Figure 1Biii). The dose response relationships for phosphorylation of all four proteins were similar to that for aggregation (Figure 1Biv).

Direct binding of PF4 to c-Mpl was shown by surface plasmon resonance with an estimated affinity constant (K_D) of PF4 flowed over recombinant c-Mpl of 744±19 nM (corresponding to 5.8 μg/mL) (Figure 1C). The affinity constant represents a net sum of affinity and avidity due to the tetrameric nature of PF4. Modelling of the binding of PF4 to c-Mpl by AlphaFold[19,20] (mean pLDTT 69 for both PF4 and TPO models, indicating a reasonable measure of confidence for the models) suggests that the interaction occurs at a site adjacent to that of TPO (supplemental Figure 2A). Consistent with this, we observed reduced but not complete blockade of binding of TPO to-c-Mpl in the presence of PF4 by enzyme linked immunosorbent assay (ELISA) (supplemental Figure 2B).

The JAK2 inhibitor ruxolitinib inhibits platelet activation by PF4

The effect of the JAK2 inhibitor, ruxolitinib,[21] on platelet activation by PF4 was investigated. For these studies, we used a concentration of ruxolitinib (100 nM) that had no effect on platelet activation by CRP or thrombin to minimize off-target effects (supplemental Figure 3). In high responding donor platelets, ruxolitinib inhibited aggregation by PF4 50 μg/mL (Figure 2A) and TPO (supplemental Figure 4A), and blocked potentiation of aggregation to a sub-threshold concentration of CRP by PF4 (10 μg/mL) (Figure 2B) and TPO (10 ng/mL) (supplemental Figure 4B). The same concentration of ruxolitinib also inhibited phosphorylation of STAT3 and STAT5a/b by PF4 and TPO (Figure 2C). In contrast, aggregation induced by PF4 was not altered in the presence of the Src kinase inhibitor

dasatinib (1 μM) or the Syk inhibitor PRT-060318 (1 μM), whereas both blocked aggregation to CRP (supplemental Figure 5).

Studies were designed to investigate whether PF4's interaction with c-Mpl contributes to platelet activation in VITT. In this disorder, immune complexes consisting of PF4 and anti-PF4 IgG activate platelets through FcγRIIA.[8,22] In most, but not all cases, strong platelet activation by VITT serum requires the addition of PF4 (10 μg/mL).[8,22] This is illustrated using an IgG fraction from the plasma of a patient with VITT, with activation observed only in the presence of PF4 (Figure 2D) and blocked by Syk, Src, and Bruton tyrosine kinase (BTK) inhibitors, and the FcγRIIA blocking F(ab), IV.3, confirming a critical role for the low affinity immune receptor in mediating activation (supplemental Figure 6A). In contrast, and as expected, IV.3 blockade had no effect on PF4-mediated platelet aggregation or its ability to potentiate the response to a threshold dose of CRP (supplemental Figure 6B).

Strikingly however, aggregation to VITT IgG and PF4 was also reduced by ruxolitinib (100 nM) suggesting that activation is mediated through synergy between FcγRIIA and c-Mpl (Figure 2D). Moreover, ruxolitinib blocked PF4-enhancement of aggregation to VITT serum samples from two other patients (Figures 2E and 2F), and reduced aggregation to a VITT serum sample that occurred without exogenous PF4 (Figure 2E). Confirmation that activation of JAK2 is downstream of c-Mpl in this model was shown using a polyclonal c-Mpl blocking antibody. In high responding donor platelets that aggregated to 10 μg/mL PF4, the c-Mpl antibody completely blocked aggregation (Figure 2Gi-ii). The c-Mpl antibody also reduced platelet aggregation to VITT IgG and PF4 (Figure 2Giii-iv). These results provide evidence that activation of the c-Mpl-JAK2 pathway can contribute to platelet aggregation by anti-PF4 immune complexes.

Conclusions

These results demonstrate that high concentrations of PF4 (> 10 μg/mL) can induce robust platelet aggregation through the TPO receptor, c-Mpl, and that lower concentrations can potentiate platelet activation through the same pathway. In platelets, c-Mpl signals through the tyrosine kinase JAK2 which lies upstream of STAT3 and STAT5a/b, and several other effectors, namely p38-MAPK,[23] ERK-cPLA2,[24] and PI3K.[25] The relative contribution of each of these to platelet activation by PF4 is not known.

The findings have significance for the mechanism of platelet activation by VITT and HIT sera, with the binding of PF4 to c-Mpl supporting activation. In the context of VITT, binding of PF4 to c-Mpl might increase the functional affinity of immune complexes for platelet binding and increase the avidity of the antibody-FcγRIIA interaction. These findings may have a similar relevance for HIT, where in addition there may be synergy with the activation of PEAR1 by heparin.[26] The interactions of these pathways are shown schematically in supplemental Figure 7 and may contribute to the variation in sensitivity of donor platelets to VITT and HIT serum. JAK2 inhibitors such as ruxolitinib however are not recommended in the treatment of VITT or HIT due to the associated thrombocytopenia and their partial action relative to the full blockade induced agents that target FcγRIIA such as the monoclonal antibody, IV.3. The present findings may have significance for the ability of PF4 to inhibit megakaryopoiesis,[27] which has been attributed to an interaction with low-density lipoprotein receptor-related protein 1 (LRP1) but with the underlying mechanism unknown.[28] Further studies are required to determine the interplay of PF4 with TPO, c-Mpl, and LRP1.

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Authorship Contributions

R.J.B. and S.J.M. performed experiments, analyzed and interpreted data, and wrote the manuscript. L.A.M., and E.M.M. performed experiments, analyzed and interpreted data, and revised the manuscript. A.S. interpreted data and revised the manuscript. S.P.W designed the study, interpreted data, and wrote the manuscript. P.L.R.N designed the study, recruited patients, analyzed and interpreted data, and wrote the manuscript.

Disclosure of Conflicts of Interest

R.J.B. and P.L.R.N are named investigators on an unrelated research grant from AstraZeneca. The other authors report no relevant conflicts of interest.

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Figure legends

Figure 1. PF4 induces aggregation through binding to c-Mpl and activation of the JAK2-STAT3/5 pathway. (A) PF4 dose response assessed by light transmission aggregometry. Pre-warmed platelets ($2x10^8$/mL) at 37°C were stirred at 1200 rpm for 1 min before addition of PF4. (Ai) Representative platelet aggregation traces; (Aii) Quantification of aggregation (AUC; area under the curve per minute) for 30 min (n=4-13). (B) Phosphorylation of STAT3, STAT5, JAK2, and c-Mpl in PF4-stimulated platelets. Washed platelets ($4x10^8$/mL) were pre-incubated with eptifibatide (9 μM) for 10 min before PF4 addition. After 10 min, platelets were lysed. Protein was separated by SDS gel electrophoresis and analyzed for pan-phosphotyrosine (4G10), phospho-STAT3 (pSTAT3; Tyr705), phospho-STAT5a/b (pSTAT5a/b; Tyr694/699), phospho-c-Mpl (pc-Mpl; Tyr626), and Syk. For JAK2, samples were immunoprecipitated (IP) prior to western blotting for pan-phosphotyrosine with 4G10 (pJAK2). (Bi) Representative western blots using monoclonal antibodies to phosphotyrosine (4G10) and to pSTAT3 and pSTAT5a/b; (Bii) Representative western blot using 4G10 after JAK2 IP; (Biii) Representative western blot using an antibody to pc-Mpl; (Biv) Quantification of pixel lane intensities for phosphorylation of STAT3 (blue triangles), STAT5 (red circles), c-Mpl (black diamonds), and JAK2 (green squares) measured as fold change relative to resting (n=3). Values are normalized for loading controls. (C) Surface plasmon resonance (SPR) showing binding of PF4 to c-Mpl. c-Mpl was conjugated directly onto the chip and PF4 flowed over. All sensograms shown are double reference subtracted and at least two replicates were injected per cycle with experimental replicates of n=3. Statistical analyses by one-way ANOVA; *P<0.05, ***P<0.001.

Figure 2. JAK2 and c-Mpl blockade inhibit platelet responses to PF4. (A) Inhibition of PF4-induced platelet aggregation with ruxolitinib (Rux). Pre-warmed platelets ($2x10^8$/mL) at 37°C were incubated with Rux (100 nM) or vehicle (DMSO 0.01%) for 5 min and stirred at 1200 rpm before addition of PF4 (50 μg/mL). (Ai) Representative aggregation traces to PF4 (50 μg/mL) and Rux (100 nM); (Aii) Quantification of aggregation (AUC per minute) for 30 min (n=8) and analyzed by paired T-test. (B) Enhancement of platelet aggregation to CRP by PF4 and inhibition by Rux. Pre-warmed platelets were incubated with Rux (100 nM) for 5 min followed by PF4 (10 μg/mL) for 5 min before CRP (0.03 μg/mL) addition. (Bi) Representative aggregation traces; (Bii) Quantification of aggregation (AUC per min) for 15 min and analyzed by one-way ANOVA. (C) Rux inhibits STAT3 and STAT5a/b phosphorylation induced by PF4 and TPO. Pre-warmed platelets ($4x10^9$/mL) were pre-incubated with eptifibatide (9 μM) for 10 min, then Rux (10-100 nM) for 5 min before stirring for an additional minute and PF4 50 μg/mL or TPO 100 ng/mL addition. After 10 min, platelets were lysed. Protein was separated by SDS gel electrophoresis and analyzed for pan-phosphotyrosine (4G10), phospho-STAT3 (pSTAT3; Tyr705), phospho-STAT5a/b (pSTAT5a/b; Tyr694/699), phospho-c-Mpl (pc-Mpl; Tyr626), and Syk. (Ci) Representative western blots. (Cii-iii) Quantification of pixel lane intensities for phosphorylation of (Cii) STAT3 and (Ciii) STAT5a/b measured as % change relative to resting (n=3). Values are normalized for loading controls. (D) PF4 enhancement of platelet aggregation to IgG isolated from a patient with VITT (IgG) and inhibition by Rux. Pre-warmed platelets ($2x10^8$/mL) at 37°C were incubated with Rux (100 nM) or vehicle (DMSO 0.01%) for 5 min, PF4 (10 μg/mL) or vehicle (phosphate buffered saline [PBS]) for 5 min, then stirred at 1200 rpm before IgG (100 μg/mL) addition. (Di) Representative platelet aggregation traces; (Dii) Quantification of aggregation (AUC per minute) for 30 min (n=7) and analyzed by one-way ANOVA. (E) PF4 enhancement of platelet aggregation to VITT serum (Serum) and inhibition by Rux. Platelets were prepared as for (D) with the addition of serum instead of IgG. (Ei) Representative platelet aggregation traces; (Eii) Quantification of aggregation (AUC per minute) for 30 min

(n=7) and analyzed by one-way ANOVA. (F) As for (E) but with serum from a different patient. (G) Effect of polyclonal anti-c-Mpl blocking antibody on platelet aggregation to PF4 and PF4 + VITT IgG. (Gi-ii) Washed platelets were incubated with anti-c-Mpl antibody (10 µg/mL) for 5 min and stirred for an additional minute before PF4 10 µg/mL addition. (Gi) Representative platelet aggregation traces; (Gii) Quantification of aggregation (AUC per minute) for 30 min (n=6) and analyzed by paired T-test. (Giii-iv) As for Gi-Gii but pre-incubated with PF4 10 µg/mL for 5 min after incubation with c-Mpl antibody prior to stirring and IgG addition. *P<0.05, **P<0.01, ***P<0.001.

Figure 1

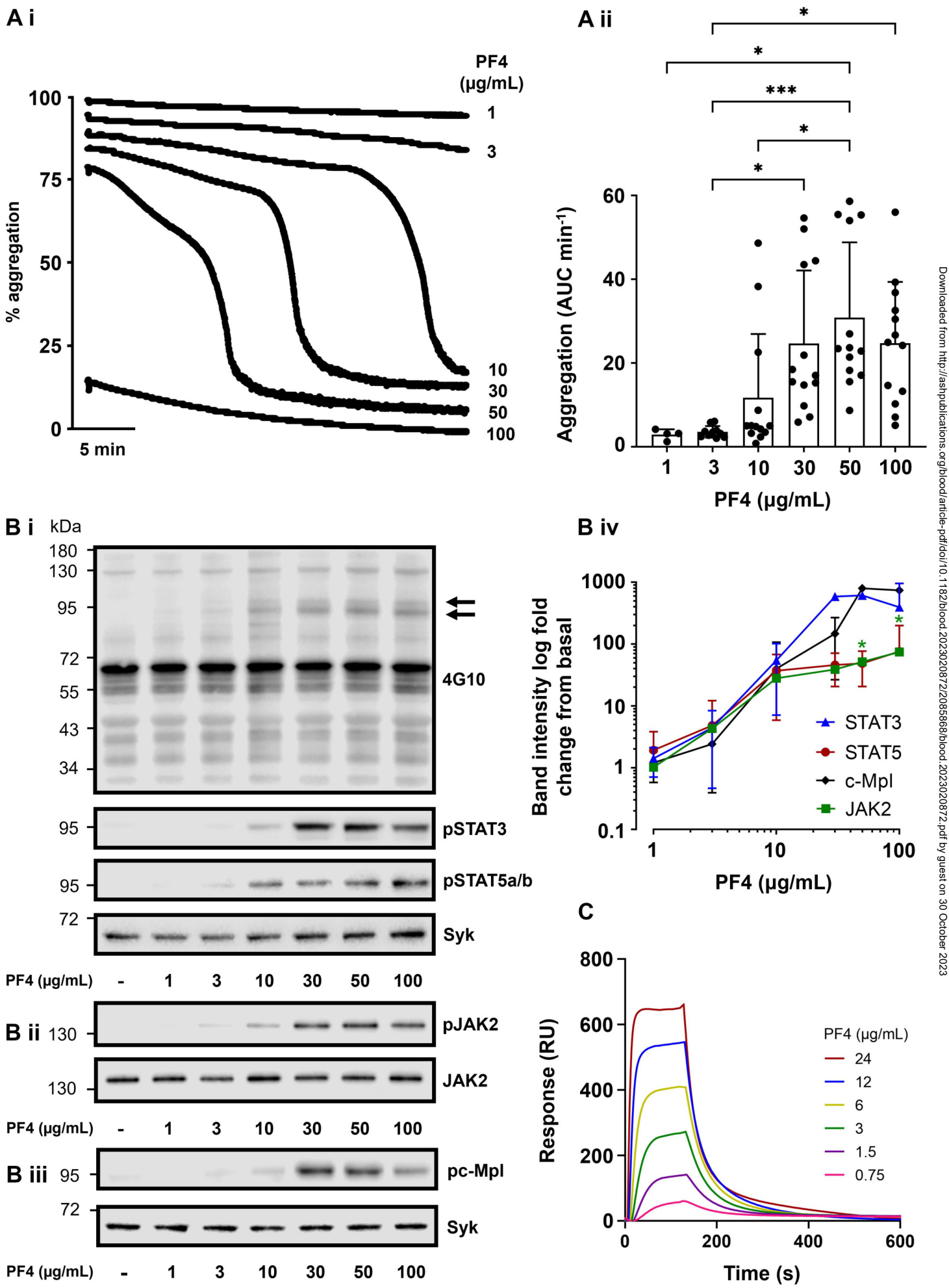

Figure 2