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ORIGINAL ARTICLE

Constitutive surface expression of the thromboxane A2 receptor is Pim kinase-dependent

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Abstract

Background: The thromboxane A2 receptor (TPαR) plays an important role in the amplification of platelet responses during thrombosis. Receptor activity is regulated by internalization and receptor desensitization. The mechanism by which constitutive surface expression of the TPαR is regulated is unknown. Recently, it has been demonstrated that proviral insertion in murine lymphoma (Pim) kinase inhibitors reduce platelet functional responses in a TPαR-dependent manner.

Objectives: To investigate whether Pim kinases regulate constitutive TPαR surface expression.

Methods: TPαR surface expression was measured in platelets, and human embryonic kidney 293T (HEK293T) cells transfected with tagged TPαRs in the presence and absence of Pim kinase inhibitors using flow cytometry and confocal microscopy. TPαR-dependent calcium flux was assessed using Fluo-4 AM. Site prediction modeling and site-directed mutagenesis were used to identify the TPαR PIM kinase phosphorylation site.

Results: Surface expression of TPαR and calcium responses to U46619 were reduced in platelets and HEK293T cells following Pim kinase inhibition. Overexpression of kinase-dead Pim-1 also reduced TPαR surface expression on HEK293T cells. Reduced surface expression of the TPαR was found to be mediated by increased receptor internalization in a dynamin and β-arrestin-dependent manner. Four putative Pim kinase phosphorylation sites in the TPαR were mutated, and serine 57 in the first intracellular loop of TPαR was identified to be a novel regulatory site important for maintaining TPαR surface expression and thromboxane A2-dependent functional responses.

Conclusion: Pim kinase inhibition may offer a novel therapeutic approach to limit cellular responses to thromboxane A2, independent of cyclooxygenase inhibition and direct antagonism of the receptor.

KEYWORDS
phosphorylation, proto-oncogene proteins pim, receptors, thromboxane A2, thrombosis, blood platelets

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1 | INTRODUCTION

Platelets are key mediators of physiological hemostasis and pathological thrombosis. Platelets rely on G protein–coupled receptors (GPCRs) such as the thromboxane A_2 (TXA_2) receptor (TP), adenosine diphosphate (ADP) receptors (P2Y1 and P2Y12), and thrombin receptors (proteinase-activated receptors [PAR]-1 and PAR4) to mediate platelet activation in response to vessel damage [1,2].

TXA_2 is a member of the prostanoid family, generated by the metabolism of arachidonic acid coordinated by phospholipase A2, cyclooxygenase (COX), and TXA_2 synthase [3]. TXA_2 binds to and activates its cell surface receptor (ie, TP). TXA_2 is an established mediator of cardiovascular (CV) disease, with both biosynthesis of TXA_2 and expression of the TP receptor shown to be elevated in CV and inflammatory diseases [4]. TXA_2 plays a central role in platelet activation and thrombus formation [5] but also regulates vasoconstriction [6] and endothelial and smooth muscle cell migration, proliferation [7], and expression of endothelial adhesion molecules [8,9].

Two isoforms of TP exist. The shorter 343-residue TPαR isoform is the predominant isoform in platelets, while the larger 407 amino acid TPβR isoform is expressed in vascular tissues, including endothelial and smooth muscle cells. Both isoforms are members of the prostanoid subfamily of class A GPCRs [10]. Production and release of TXA_2 amplifies the platelet activatory response via binding to and activation of TPαR. Platelet TPαR couples to both G_q and G_{13} [11,12] to cause phospholipase Cβ-mediated Ca^{2+} release and protein kinase C activation [13], and Rho-mediated platelet activation, respectively [14].

Inhibition of the TPαR signaling pathways represents a key therapeutic target to reduce CV-related thrombotic events and inflammation. The most prescribed antiplatelet drug, aspirin, irreversibly inhibits COX enzymes, reducing platelet TXA_2 generation and, therefore, TXA_2-mediated platelet responses. Despite its widespread use, aspirin is associated with variable patient outcomes and adverse side effects. Some patients demonstrate high on-treatment platelet reactivity, which is associated with an increased risk of acute coronary syndromes [15,16]. Aspirin targets platelet TPαR signaling indirectly by inhibiting COX-1 and synthesis of TXA_2 alongside other prostanoids. However, aspirin has dose-limiting off-target effects on COX-2, an enzyme involved in the synthesis of endogenous inhibitors of platelet function, which can increase the risk of thrombosis when repressed [17,18]. As a result, there is still considerable interest in developing TPαR targeting strategies that work independently of COX activity to preserve the beneficial effects of other prostanoids.

GPCR signaling is regulated by receptor desensitization, internalization, and cycling to and from the cell surface [19]. GPCR cycling is regulated via receptor oligomerization and phosphorylation by GPCR receptor kinases, which facilitate internalization through the recruitment of β-arrestins and clathrins [20–22].

We have previously described the family of proviral insertion in murine lymphoma (Pim) kinases, Pim-1, -2, and -3 [23], as regulators of platelet function via regulation of TPαR signaling that is independent of COX-1 [24]. Pim kinase deletion or pharmacologic inhibition results in reduced thrombus formation and platelet TPαR signaling responses, which appears to be associated with a decrease in surface expression levels of TPαR [24]. The mechanism by which this regulation occurs is, however, unknown. Interestingly, we observe no effect on platelet aggregation following activation by other major platelet GPCR agonists, including ADP, thrombin, and PAR1 peptide, strongly suggesting that Pim kinase inhibitors specifically modulate platelet function by regulating the TPαR through a mechanism that is not shared by all GPCRs. These observations have wider implications for the regulation of TP receptor function in other cell types of the CV system.

In this study, we investigate the mechanism by which Pim kinase regulates TPαR function. Herein, we describe a novel mechanism of TPαR regulation via Pim kinase-mediated phosphorylation of the first TPαR intracellular loop (ICL-1) and identify the ICL-1 S57 residue as necessary to maintain expression of TPαR at the cell surface and facilitate TPαR signaling.

2 | METHODS

2.1 | Materials

AZD1208, SGI-1776, PIM-447, dynasore, barbadin, and U44619 were purchased from Cambridge Bioscience. Pim-1 and Pim-2 antibodies were from Cell Signaling Technology or Santa Cruz Biotechnology. Anti-TPαR antibody and 3,3′,5,5′-tetramethylbenzidine substrate were from Abcam. Biotin beads, anti-FLAG tags, and anti–MYC-tag antibodies were obtained from Cell Signaling Technology. Anti-FLAG HRP, anti-FLAG APC, and anti-FLAG Biotin conjugates were purchased from Miltenyi. Fluorescent secondary antibodies were Alexa Fluor 488, Alexa Fluor 647, and wheat germ agglutinin (WGA) 555 from Invitrogen. Secondary antibodies for Western blot were mouse-HRP conjugate from Santa Cruz Biotechnology, rabbit HRP conjugate from CST, or anti-Rabbit 680 or anti-Mouse 800 from Li-COR. All other reagents were of analytical grade and purchased from Sigma Aldrich.

2.2 | Platelet preparation

Human whole blood was collected by venepuncture from healthy, aspirin-free, consenting volunteers using procedures approved by the local Ethics Committee into 3.2% sodium citrate and centrifuged at 100 × *g* for 20 minutes at room temperature to isolate platelet-rich plasma. For washed platelets, acid citrate dextrose (29.9 mM trisodium citrate, 113.8 mM glucose, and 2.9 mM citric acid [pH 6.4]) was added to the platelet-rich plasma (1:8 v/v) prior to centrifugation at 350 × *g* for 20 minutes. Washed platelets were prepared by resuspending the platelet pellet in modified Tyrode's-HEPES buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 5 mM glucose, and 1 mM $MgCl_2$, pH 7.3) to 4 × 10^8/mL.

For samples for Western blotting, washed platelets as described above were subsequently pelleted in the presence of acid citrate dextrose (1:8 v/v) and PG12 (125 ng/mL) at 1400 × *g* for 10 minutes before being resuspended to 8×10^8 cells/mL and allowed to rest for 30 minutes.

2.3 | Cell culture, plasmids, and transfections

Human embryonic kidney 293T (HEK293T) cells were cultured in phenol red-free Dulbecco's Modified Eagle's Medium (DMEM) media supplemented with 1% penicillin and streptomycin, L-glutamine, and 10% fetal bovine serum (FBS). The green fluorescent protein (GFP)-tagged TPαR plasmid (NM_001060) and Myc-tagged Pim-1 plasmid (NM_002648) were purchased from Origene, and the FLAG-tagged plasmid was as previously described [25]. Transfections were performed using Mirus Bio TransIT-293 Transfection Reagent (Geneflow) following the manufacturer's instructions.

2.4 | Site-directed mutageneses of TPαR and Pim-1

Predicted sites were identified using GPS 5.0 site prediction software [26]. GFP- and FLAG-tagged TPαR mutant (S→A) plasmids S57A, S61A, S329A, and S331A, and kinase-dead (KD) Myc-tagged Pim-1 (P81→S) [27] plasmids were created using the QuikChange Mutagenesis Kit from Stratagene. Plasmids were expressed in HEK293T cells, and an empty PCDNA3 plasmid was included as a negative control.

2.5 | Flow cytometry

2.5.1 | Platelet TPαR surface expression

Washed platelets were treated with or without Pim kinase inhibitors for 10 minutes before staining with an antibody against the extracellular portion of the TPαR (Abcam) and analyzed by flow cytometry. Data were collected using a Miltenyi MACSQuant Analyzer 16 flow cytometer. Percentage positive events were gated using an immunoglobulin (Ig)G control, and 10 000 events were counted based on platelet forward scatter (FSC) and side scatter (SSC) profiles.

2.5.2 | TPαR surface expression in HEK293T

HEK293T cells transfected with 5 μg extracellular FLAG-tagged TPαR plasmids were treated with or without Pim kinase inhibitors for 60 minutes, and surface expression of the TPαR was determined on live cells, using DAPI as an exclusion marker for dead cells and a fluorescently labeled FLAG-tag antibody at 4 °C in flow cytometry. Data were collected using a Miltenyi MACSQuant Analyzer 16 flow cytometer. The mean fluorescence intensity of transfected cells (% FLAG-positive cells) was calculated by gating transfected cells using an IgG control on empty PCDNA3 plasmid transfected samples; 10 000 events were collected.

2.6 | Fluorescence microscopy

2.6.1 | Platelet TPαR localization

Washed platelets were treated with or without Pim kinase inhibitors for 10 minutes before staining with antibodies for TPαR or the microtubule ring (anti–β-tubulin) as previously described [28]. Samples were analyzed by confocal microscopy using a Leica Stellaris 5 confocal microscope, and colocalization of the TPαR with the microtubule ring (indicating surface expression) was determined.

2.6.2 | TPαR and Pim-1 cellular localization in HEK293T cells

HEK293T cells were plated in 8-well Ibidi dishes and transfected with a combination of either wild-type (WT) or Myc-tagged KD-Pim-1 plasmids or WT or mutant GFP-Tagged TPαR plasmids for 24 hours before treatment with AZD1208 (10 μM) or vehicle (0.1% dimethylsulfoxide [DMSO]) for 60 minutes. Cells were fixed with 4% paraformaldehyde (PFA) for 15 minutes.

For investigation of TPαR localization, the cell membrane was stained with Alexa Fluor 555 WGA (1 μg/mL in serum-free media) for 10 minutes.

For TPαR and Pim-1 colocalization, HEK293T cells were washed and permeabilized with 0.1% Triton X for 5 minutes before being blocked with 5% goat serum and 2% bovine serum albumin (BSA). An anti-Myc primary antibody (1/200 dilution) and anti-rabbit Alexa Fluor 568 secondary antibodies (1/500 dilution) were used to detect Pim-1. Samples were imaged using a 20× or 40× lens on a Leica Stellaris 8 Imaging System. Z stacks were collected with lightening processing, and max intensity projection images were presented. For analysis of TPαR localization, analysis was performed by drawing around the perimeter of the cell on the WGA-555 channel, measuring the plot profile for WGA-555 and GFP (TPαR), and the ratio of GFP to WGA was calculated. The mean fluorescence intensity of the whole image for each channel was measured and assessed for GFP degradation. For colocalization analysis of the TPαR and Pim-1, analysis was performed using the LasX colocalization plug-in to calculate Pearson's coefficient [29]. To ensure a negative control to compare against, the Pim-1 channel was moved 8 μm in both the X and the Y plane, and colocalization analysis was performed again.

2.7 | Cell surface enzyme-linked immunosorbent assay

Cell surface expression of the N-terminal FLAG-tagged TPαR constructs was obtained by following a previously published protocol [30]

with some adaptations. Wells were blocked with 2% BSA for 1 hour at room temperature. Staining was performed using an anti-FLAG HRP tagged antibody (1/1000) in 2% BSA for 1 hour at room temperature, followed by 5 washes in 1x phosphate buffered saline (PBS). Wells were incubated with 3,3′,5,5′-tetramethylbenzidine substrate until a color change was observed, and the reaction was stopped with 1 M HCL. Absorbance was read at 450 nM on a Promega plate reader. Wells were extensively washed before cells were stained with 0.4% Trypan blue for 10 minutes prior to washing 3× with PBS. The last wash was removed before absorbance was read at 600 nm in order to normalize the signal [31].

2.8 | Calcium assays

HEK293T cells were transfected with TPαR constructs, plated in black 96-well plates, and loaded with Fluo-4 AM No Wash Kit (Invitrogen) according to the manufacturer's instructions. Calcium dye-loaded HEK293T were pretreated with AZD1208 (10 μM) or vehicle (0.1% DMSO) for 10 minutes before stimulation with U46619 (1 μM). Calcium mobilization was determined by recording fluorescence measurements with excitation at 475 nm and emission at 510 nm over a period of 2.5 minutes using the GloMax plate reader (Promega).

2.9 | Immunoprecipitation and Western blotting

HEK293T cells were transfected as described above before washing once with cold PBS and lysing with radioimmunoprecipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitors. For immunoprecipitation of TPαR, an aliquot of this lysate was stored as an "input fraction." Samples were incubated rocking overnight with anti-FLAG biotin-tagged antibody at 4 °C. For receptor pulldown, streptavidin beads were incubated with the samples for 2 hours at 4 °C rocking. Beads were washed 5× with RIPA buffer before elution into 3× sample buffer.

Lysates were prepared in Laemmli sample buffer for separation via sodium dodecyl sulfate–polyacrylamide gel electrophoresis and direct analysis by Western blotting, which was performed using standard techniques, as described previously [32]. Proteins were detected using HRP-conjugated secondary antibodies, or IRDye fluorescent secondary antibodies (LI-COR), and visualized using a LI-COR imaging system. Band intensities were quantified, and immunoprecipitated protein was normalized to the levels of the input protein.

2.10 | Statistical analysis

All experiments were performed to at least $n = 4$. Statistical analyses of the data were carried out using GraphPad Prism software. When comparing 2 sets of data, an unpaired 2-tailed Student's *t*-test (simple) was used. If more than 2 means were present, significance was determined by a 1-way analysis of variance (ANOVA) followed by Dunnett's correction (multiple). When assessing the effect of 2 independent variables on a dependent variable, a 2-way ANOVA was performed, followed by Sidak's multiple comparison test. $P \leq .05$ was considered statistically significant. Unless stated otherwise, values were expressed as mean ± SEM.

3 | RESULTS

3.1 | Pim kinase inhibition reduces TPαR surface expression and signaling

We have previously described reduced platelet functional responses following treatment with Pim kinase inhibitors or genetic deletion of Pim-1, attributed to a reduction in TPαR-dependent signaling responses and direct regulation of TPαR activity [24]. We confirm here that pretreatment of washed platelets with Pim kinase inhibitors leads to a reduction of TP receptor-mediated response, with attenuated platelet aggregation observed in response to U46619 and arachidonic acid following treatment of platelets with AZD1208 (10 μM; Supplementary Figure S1). In other cell types, Pim kinase inhibition has been shown to reduce surface expression of the C-X-C chemokine receptor type 4 (CXCR4) [33,34]. Therefore, to determine whether Pim kinase inhibition alters surface expression levels of the TPαR, the effect of Pim kinase inhibitor treatment on platelet TPαR surface and total expression levels was determined using flow cytometry (Figure 1A) and confocal microscopy (Figure 1B). As shown in Figure 1A, evaluation of surface levels using a TPαR antibody specific for the extracellular portion of the receptor demonstrated that platelets treated with the Pim kinase inhibitors AZD1208 (10 μM), PIM-447 (10 μM), or SGI-1776 (10 μM) show a 20% to 40% reduction in surface expression levels of TPαR compared with vehicle-treated controls in the absence of TPαR agonist. This decrease in surface exposure of TPαR (green) was also confirmed using confocal microscopy (Figure 1B), which revealed a reduction in TPαR colocalization with the microtubule ring (magenta), which sits under the plasma membrane in AZD1208-treated platelets in comparison with vehicle-treated control.

To enable further investigation of the mechanism of TPαR regulation via Pim kinase, cell line studies were performed. HEK293T cells express Pim kinases [35–37] and have previously been used to study TPαR function [25,38,39]. After confirming the expression of both Pim-1 and Pim-2 isoforms by Western blotting (Supplementary Figure S2A, B), HEK293T cells were selected as a cell model to investigate Pim kinase-mediated TPαR regulation.

HEK293T cells were transiently transfected with FLAG-tagged TPαR and GFP-tagged TPαR plasmid constructs. The success of the transfections with the FLAG-tagged TPαR plasmid (65% efficiency) and the GFP-tagged TPαR plasmids (77% efficiency) was confirmed by immunoprecipitation (Supplementary Figure S2C) and microscopy (Supplementary Figure S2D), respectively. Cytotoxicity of AZD1208 at 10 μM was tested by flow cytometry (annexin V binding) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-

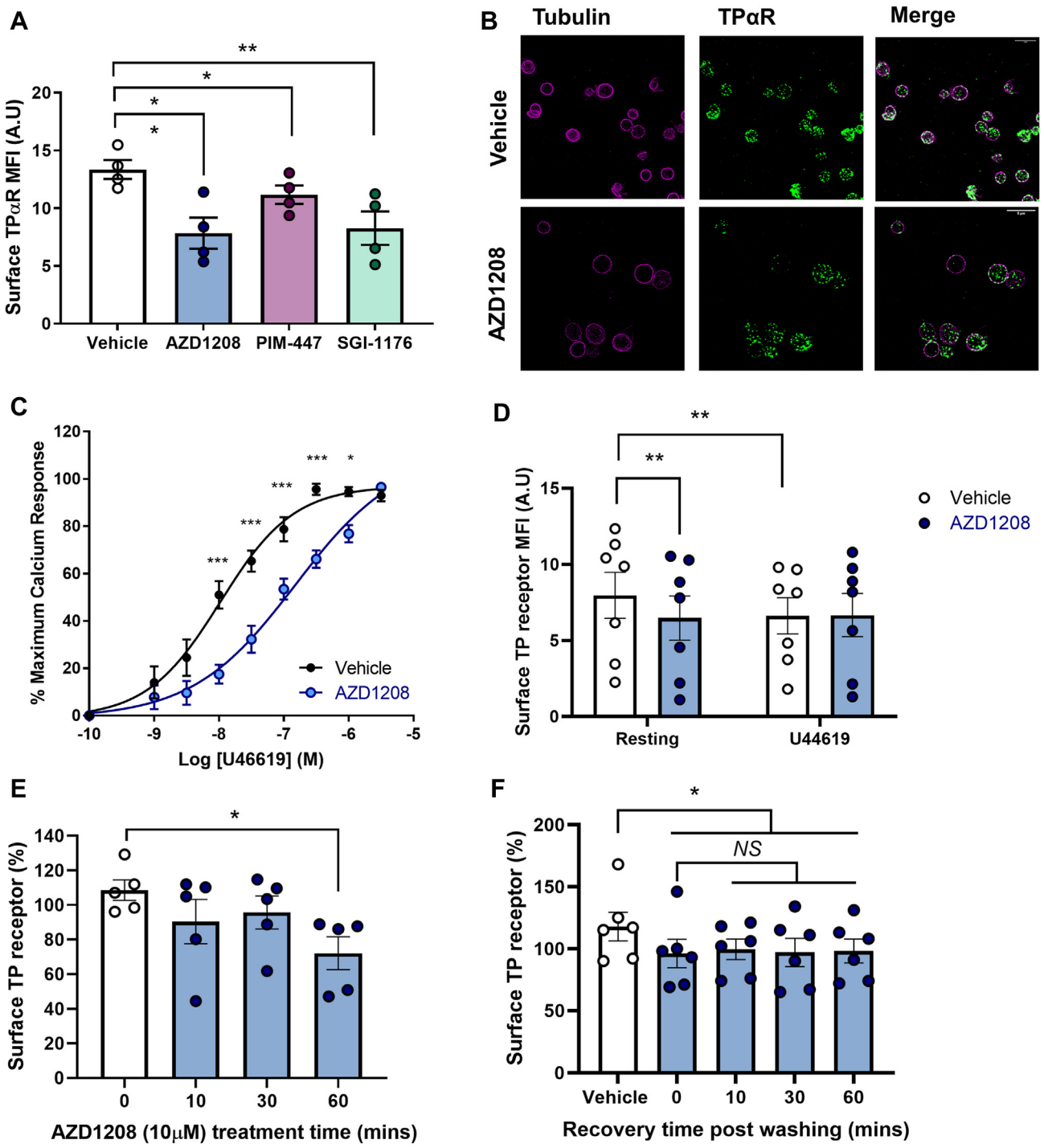

FIGURE 1 Pim kinase inhibition reduces surface expression levels of the thromboxane A_2 receptor (TPαR). (A, B) Washed platelets were incubated with AZD1208, PIM-447, or SGI-1776 (all at 10 μM) or vehicle control (0.1% dimethylsulfoxide [DMSO]) for 10 minutes before analysis of TPαR surface expression by (A) flow cytometry or (B) confocal microscopy. (A) Platelets were stained with an antibody to the extracellular portion of the TPαR for 20 minutes, and samples were analyzed for TPαR surface expression by flow cytometry. (B) Fixed and permeabilized platelets were blocked with bovine serum albumin (BSA) and stained with antibodies to the extracellular portion of the TPαR (green) or tubulin (purple) for confocal microscopy. Images are representative, and the scale bar represents 5 μm. (C–F) FLAG-tagged TPαR-expressing human embryonic kidney 293T (HEK29T) cells were treated with AZD1208 (10 μM) or vehicle control (0.1% DMSO) at 37 °C for (C and D) 1 hour, (E) time points shown, or (F) 1 hour before the cells were washed and allowed to recover. (C) Prior to treatment, cells were loaded with Fluo-4 AM, and calcium mobilization was measured in response to increasing concentrations of U46619. (D) Surface expression of the TPαR was determined using an APC anti–FLAG-tag antibody and flow cytometry. An immunoglobulin G control on empty PCDNA3 plasmid transfected samples was included as a negative control. (E, F) TPαR cell surface expression was measured using a cell surface enzyme-linked immunosorbent assay. AZD1208 values were normalized to vehicle-treated values, and untreated samples were set as 0. Quantified data are shown; $n \geq 5$, all data points are shown; error bars are mean ± SEM. $^{*}P \leq .05$, $^{**}P \leq .01$ $^{***}P \leq .001$. MFI, median fluorescence intensity; A.U, arbitrary units; NS, not significant.

2H-tetrazolium (MTS) assay, confirming that it did not cause cell death or necrosis in HEK293T cells (Supplementary Figure S2A).

We have previously observed reduced signaling effects in platelets downstream of the TPαR in the presence of Pim kinase inhibition [24]. Therefore, the effect of Pim kinase inhibitor treatment on calcium mobilization in TPαR-expressing HEK293T cells was determined following stimulation with the TXA_2 mimetic U46619. Similar to that previously observed in platelets, treatment with AZD1208 caused attenuation of U46619-induced calcium mobilization, increasing the half maximal effective concentration (EC_{50}) of U46619 by ~10-fold (vehicle: 10 ± 1.8 nM vs AZD1208: 151 ± 54 nM), indicating reduced TPαR-mediated signaling (Figure 1C).

To determine whether Pim kinase inhibition results in reduced TPαR surface expression in HEK293T similar to that observed in platelets (Figure 1A, B), extracellular terminal FLAG-tagged TPαR-expressing HEK293T cells were treated with the Pim kinase inhibitor AZD1208 (10 μM). Using an anti-FLAG antibody, AZD1208 treatment was found to cause a 25% reduction in TPαR surface expression (75 ± 5%) in HEK293T cells under basal conditions compared with vehicle-treated (0.1% DMSO) controls (Figure 1D). Interestingly, in the HEK293T cells, AZD1208-induced reductions in TPαR surface expression were not further enhanced following stimulation with U46619, which is known to initiate agonist-induced internalization of the TPαR.

A cell surface enzyme-linked immunosorbent assay (ELISA) protocol [30] was implemented to establish a time course to determine how long Pim kinase inhibitor treatment takes to promote internalization of the TPαR. Transfected cells were plated into 24-well plates and treated with AZD1208 (10 μM) at different time points prior to analysis by ELISA. Internalization of TPαR in HEK293T cells was observed after 60 minutes with AZD1208 (Figure 1E) and sustained for 60 minutes postwashing and inhibitor removal (Figure 1F), indicating that Pim kinase inhibition prevents receptor recovery to the surface for this period.

3.2 | TPαR internalization is mediated by dynamin and β-arrestin

To confirm that the reduction in surface TPαR exposure was due to receptor internalization rather than degradation, microscopy experiments were performed. HEK293T cells expressing GFP-tagged TPαR were incubated with the Pim kinase inhibitor AZD1208 (10 μM) or vehicle control (0.1% DMSO) for 1 hour, stained with Alexa Fluor 555 WGA, a marker of the plasma membrane, and images were taken using confocal microscopy (Figure 2). Treatment of TPαR-expressing HEK293T cells with Pim kinase inhibitors caused a reduction in TPαR surface expression, represented by a reduced colocalization of GFP-TPαR with WGA. No change in the total GFP-WGA ratio was observed, indicating that the decrease in surface GFP-TPαR induced by AZD1208 is not caused by degradation or loss of the receptor (Figure 2Bi) but an increased internalization.

Internalization of GPCRs and receptor endocytosis is regulated by both arrestin-dependent and -independent mechanisms [20]. To evaluate whether Pim kinase activity regulates surface expression levels of the TPαR by altering endocytotic mechanisms, TPαR surface expression in the presence and absence of the Pim kinase inhibitor AZD1208 was determined in TPαR-expressing HEK293T cells following treatment with dynasore (100 μM), a dynamin inhibitor that blocks clathrin-dependent endocytosis, and barbadin (30 μM), an inhibitor of β-arrestin/AP2 [40,41]. Dynasore and barbadin both prevented AZD1208-mediated internalization of TPαR (Figure 2C, D), while TPαR surface levels remained similar to those of vehicle-treated control in the presence of either inhibitor. These findings indicate that Pim kinase maintains TPαR surface expression by blocking both dynamin and β-arrestin-mediated endocytosis.

3.3 | Pim-1 kinase colocalizes with the TPαR and is responsible for the regulation of receptor surface expression

To ensure that our observations using the pharmacologic inhibitor AZD1208 were specifically mediated by Pim kinase, WT and KD-Pim-1 (P81→S) [27] were overexpressed in HEK293T cells prior to transient expression of FLAG-tagged TPαR. Mutation of the kinase active site did not disrupt cell viability (Supplementary Figure S2B) or total protein expression, as no significant difference in total Pim-1 protein expression levels of WT vs KD was observed (Figure 3A). Moreover, overexpression of either form of Pim-1 did not alter the total amount of TPαR expressed per cell (Figure 3B). In support of our observations made following treatment with Pim kinase inhibitor, surface expression levels of the TPαR were found to be reduced in HEK293T cells expressing KD-Pim-1 compared with cells overexpressing WT (active) Pim-1 kinase (Figure 3C). Furthermore, AZD1208 treatment of cells overexpressing WT-Pim-1 reduced TPαR surface expression but did not alter TPαR surface levels in cells expressing the KD-Pim-1 mutant (Figure 3D). Together, these data indicate that Pim kinase activity maintains cell surface expression of the TPαR.

Further evidence that Pim-1 plays a role in the regulation of the TPαR is its interaction with the receptor. Pim-1 colocalization with the TPαR was verified in HEK293T cells transfected with both Pim-1 (Myc-tagged) and GFP-TPαR plasmids using an anti-Myc antibody and GFP fluorescence. Analysis of Pearson's value was performed, and both WT and KD-Pim-1 were observed to significantly colocalize with the TPαR in HEK293T cells with a positive correlation (Pearson's correlation coefficient ≥ .5) of the 2 proteins observed (WT: 0.51 ± 0.04, KD: 0.55 ± 0.07; Figure 3E).

3.4 | Four putative Pim kinase phosphorylation sites identified in TPαR ICL-1 and C-terminal tail

We next sought to identify which sites within the TPαR may be phosphorylated and targeted by Pim kinase. Phospho-kinase site prediction

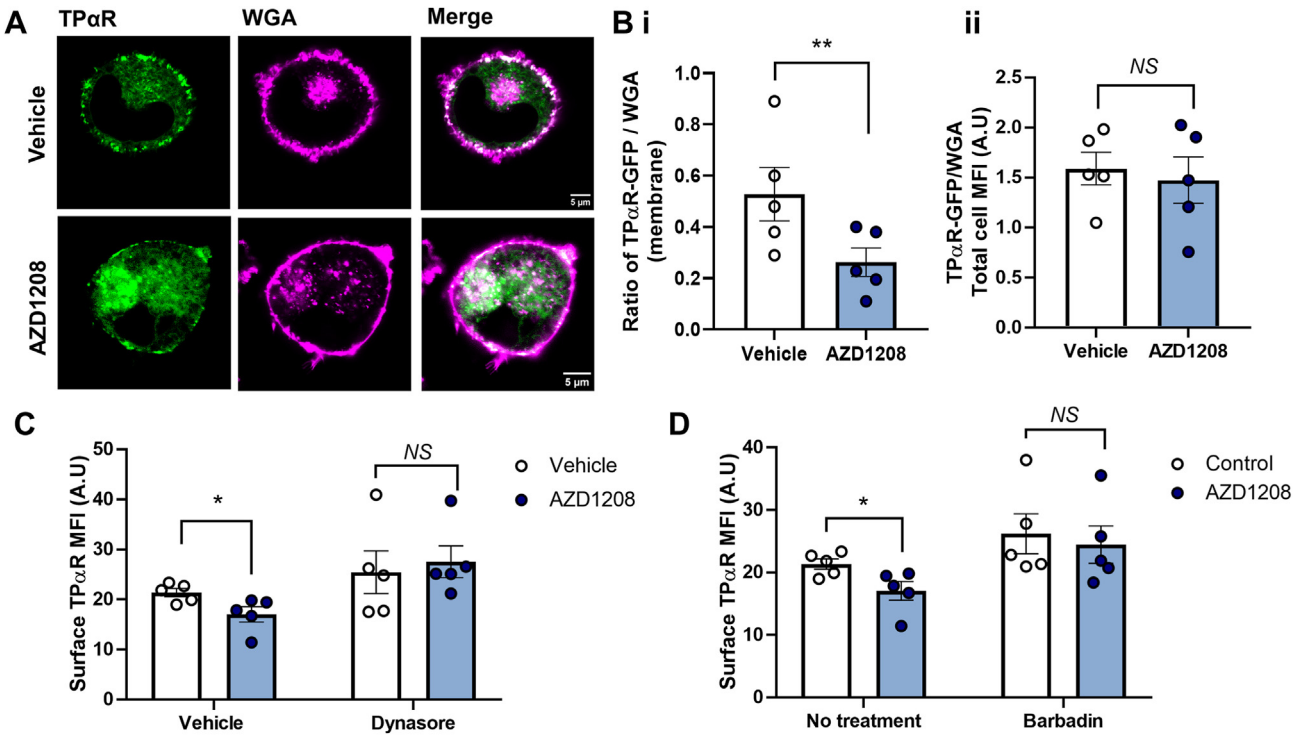

FIGURE 2 Pim kinase inhibition reduces surface expression levels of the thromboxane A_2 receptor (TPαR) in human embryonic kidney 293T (HEK293T) cells via receptor internalization, which is dynamin and β-arrestin-dependent. (A, B) green fluorescent protein (GFP)-tagged or (C, D) FLAG-tagged TPαR-expressing HEK293T cells were treated with AZD1208 (10 μM) or vehicle control (0.1% dimethylsulfoxide, DMSO) for 60 minutes. Cells were fixed and stained with Alexa Fluor 555 wheat germ agglutinin (WGA) for 10 minutes to stain the cell membrane and imaged using confocal microscopy. (A) Representative images are shown. (B) Analysis was performed by drawing around the perimeter of the cell on the WGA-555 channel, measuring the plot profile, and then measuring the GFP plot profile. (Bi) The ratio of GFP to WGA was analyzed, and (Bii) the mean fluorescence intensity of the whole cell region of interest (ROI) for each channel was measured and assessed for GFP degradation. (C, D) Prior to treatment with AZD1208, cells were treated with or without (C) dynamin inhibitor dynasore (100 μM) or (D) β-arrestin inhibitor barbadin (30 μM) for 30 minutes at 37 °C before fixation with 4% paraformaldehyde (PFA) and subjected to cell-based enzyme-linked immunosorbent assay. $n = 5$, error bars are SEM. $^{*}P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$. MFI, median fluorescence intensity, A.U, arbitrary units; NS, not significant.

software GPS [26,42] identified 4 possible phosphorylation sites on serine residues within the TPαR sequence: 2 in ICL-1 (S57 and S61) and 2 in the intracellular C-terminal tail (S329 and S331). To investigate the potential role of each of the phosphorylation sites in TPαR surface expression and Pim kinase regulation, a QuikChange site-directed mutagenesis kit was used to mutate each serine residue of interest to alanine (S57A, S61A, S329A, and S331A) to mimic the dephosphorylated state. Mutation of each residue individually was found to not alter total receptor expression or transfection efficiency into HEK293T cells (Figure 4) or the cell viability (Supplementary Figure S2C) compared with WT control. Flow cytometry was subsequently used to investigate the total and surface TPαR expression of the mutants. The analysis demonstrated reduced surface receptor expression levels in the S57A mutant (Figure 4), while the surface expression of all other mutants, S61A, S329A, and S331A, were similar to WT TPαR, indicating that the ICL-1 serine (S57) residue is required for surface expression of the receptor (Figure 4B, C).

3.5 | Pim kinase regulates TPαR surface expression and signaling via phosphorylation of S57 of the receptor

Having identified the S57 residue in the ICL-1 of TPαR as a key regulator of TPαR surface expression levels and signaling, we next investigated whether this regulation is mediated via Pim kinase. TPαR mutant-expressing HEK293T cells were therefore treated with and without Pim kinase inhibitor AZD1208 (10 μM), and surface expression of the S57A mutant TPαR was determined and compared with WT and other TPαR serine mutants (Figure 5A). WT, S61A, S329A, and S331A mutants all showed reduced surface expression levels of TPαR following treatment with AZD1208, indicating that, while they may be Pim kinase phosphorylation sites, they are not involved in Pim kinase-mediated regulation of TPαR surface expression. In contrast, while reduced TPαR surface expression levels were observed in S57A-expressing cells compared with WT

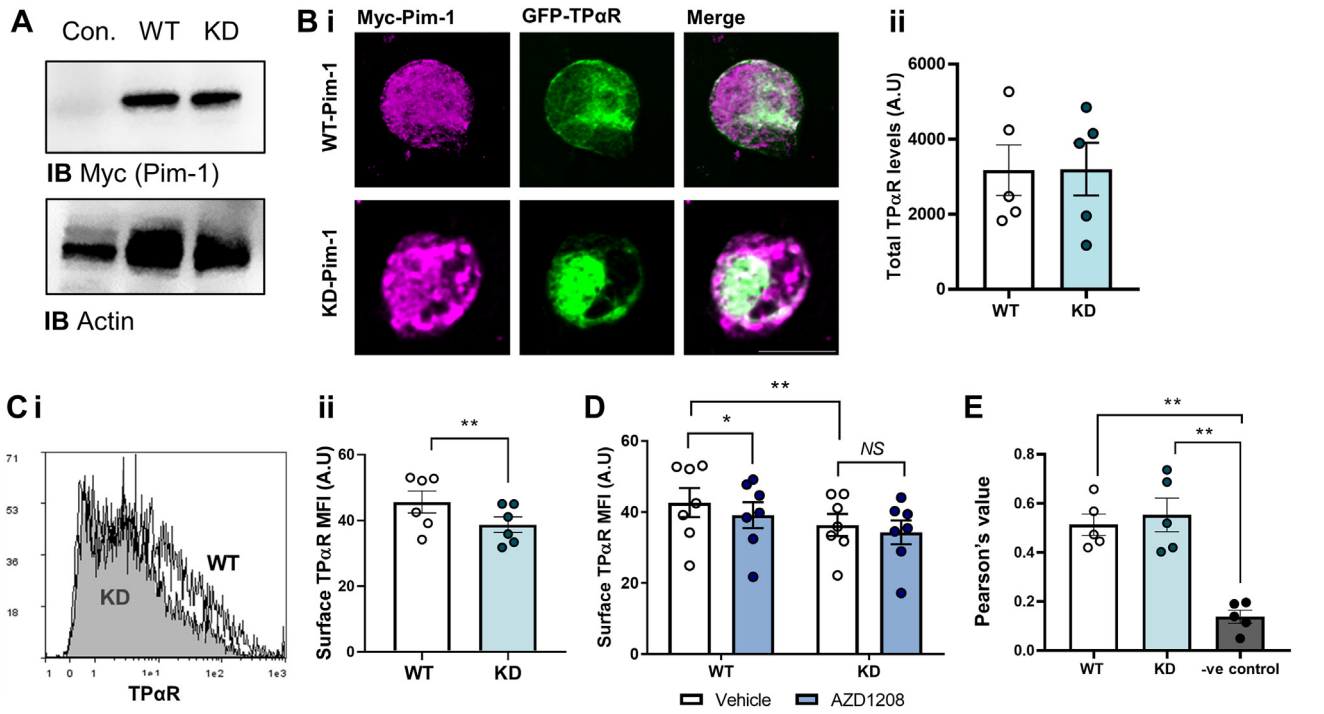

FIGURE 3 Pim-1 kinase colocalizes with the thromboxane A_2 receptor (TPαR) and regulates receptor surface expression. Human embryonic kidney 239T (HEK293T) cells were transiently transfected with wild-type (WT) or kinase-dead (KD; P81→S) Pim-1 plasmids (MYC-tagged) along with either a green fluorescent protein (GFP)-tagged or FLAG-tagged TPαR or empty PCDNA3 plasmid for 48 hours. (A) Transfected cells were lysed, and Western blots were performed to confirm equal expression of WT and KD-Pim-1 within the cells. Control (Con.) = empty PCDNA3 (no_myc tag). (B) Total expression of GFP-tagged TPαR determined using confocal microscopy and median fluorescence intensity (MFI), (Bi) shows representative images for Pim-1-anti-Myc (purple) and TPαR- GFP (green), and (Bii) quantified MFI data are shown. (C, D) Surface expression of the TPαR determined on live cells (DAPI used as an exclusion marker for dead cells) using a FLAG-tagged antibody with staining at 4 °C in flow cytometry. (Ci) A representative histogram is shown. (Cii, D) The mean fluorescence intensity of transfected cells was calculated by gating transfected cells using an immunoglobulin G Con. on empty PCDNA3 plasmid transfected samples; quantified data are shown. (D) Prior to staining, cells were treated with vehicle or AZD1208 (10 μM for 1 hour). (E) HEK293T cells expressing WT or KD-Pim-1 plasmids and GFP-TPαR were analyzed by confocal microscopy for colocalization of Pim-1 and TPαR using Pearson's correlation. Data are presented as Pearson's colocalization values. To act as a negative Con., images within the Pim-1 channel were moved by 8 μm in the X and Y planes, and colocalization with TPαR determined (Con.). n = 5, error bars are mean ± SEM, $^{*}P \leq .05$, $^{**}P \leq .01$. A.U, arbitrary units; IB, immunoblot; NS, not significant.

controls, no further reduction in TPαR surface expression was observed following treatment with AZD1208 in S57A TPαR-expressing cells, suggesting S57 is the site of Pim kinase-dependent regulation (Figure 5A).

To further support these observations, site-directed mutagenesis was used to mutate the TPαR ICL-1 serine 57 to glutamate (S57E) to mimic a constitutively phosphorylated state, which should be resistant to Pim kinase inhibition. The S57E TPαR mutant-expressing HEK293T cells were found to have a normal surface expression of TPαR compared with WT controls; however, they were resistant to AZD1208 (10 μM)-induced internalization observed with WT TPαRs (Figure 5B), confirming that S57 in ICL-1 regulates TPαR surface expression levels in a Pim kinase-dependent manner.

4 | DISCUSSION

Our findings demonstrate that Pim kinase activity is required for constitutive surface expression of the TPαR. We show that Pim kinase activity regulates the TPαR via phosphorylation of the ICL-1 at S57. This is a novel mechanism that has implications for our current understanding of GPCR biology, the regulation of TPαR in multiple cell types, and creates a path for novel therapeutic approaches based on the regulation of TPαR signaling by altering the internalization of the receptor.

The TPαR, which is the predominant variant expressed in platelets, has a shorter C-terminal intracellular tail compared with the TPβR and is a member of the prostanoid GPCR family [5,43]. TPαR activity is regulated by coupling to G proteins following ligand binding, which is responsible for the transmission of downstream signaling, and finally, receptor desensitization to terminate signaling responses [3,43]. Desensitization of the TPαR can typically be divided into 2 stages. Stage 1 involves the uncoupling of receptors from G proteins, and stage 2 involves a loss of receptor sites from the plasma membrane [3,44]. Phosphorylation of the TPαR by protein kinases [45] regulates its desensitization, specifically within the C-terminal tail of the receptor [46,47]. Key serine residues in the C-terminal tail, shared by both TPαR and TPβR isoforms, sites S324, S329, and S331, have all been identified

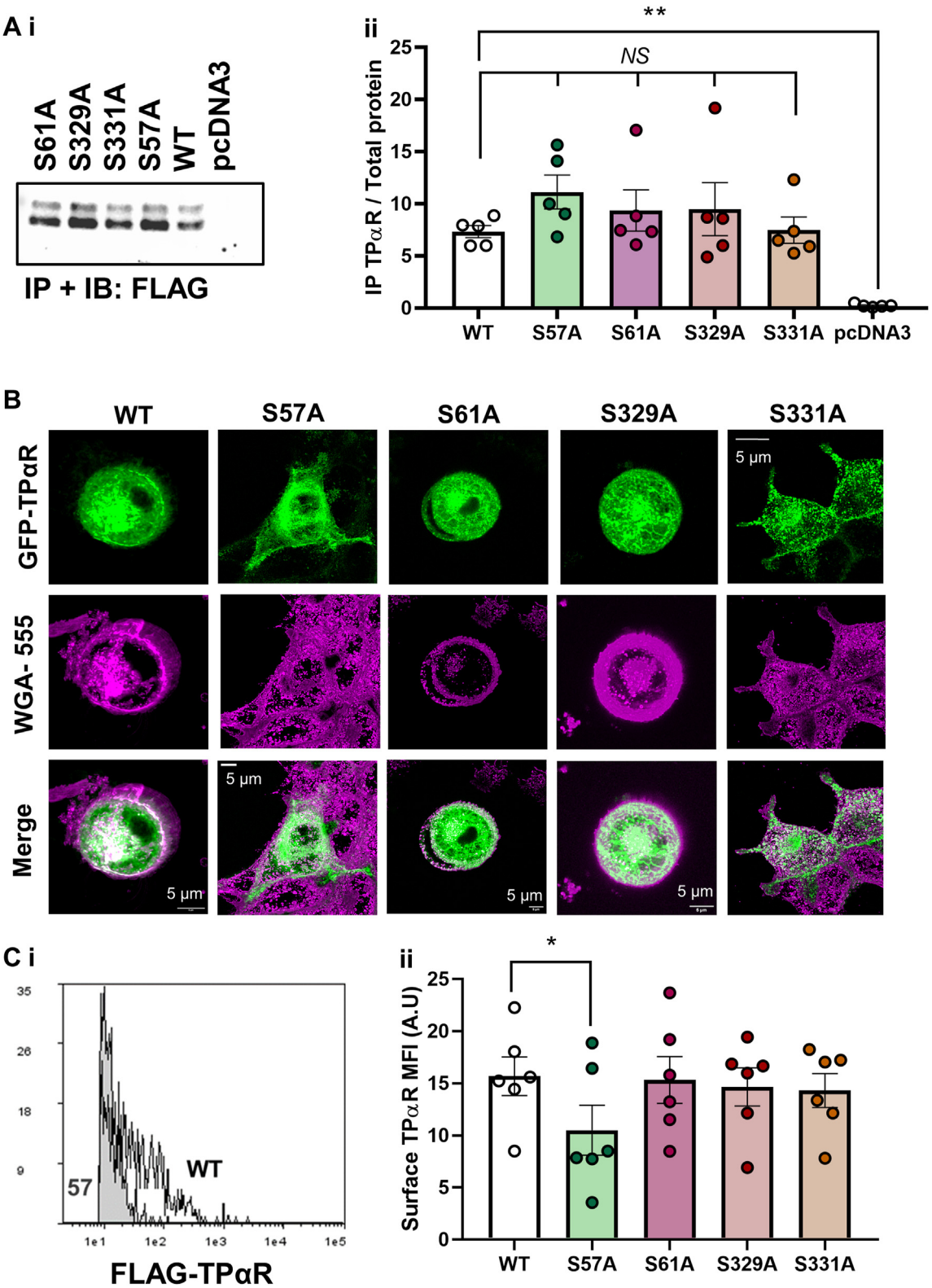

FIGURE 4 S57A thromboxane A_2 receptor (TPαR) mutant shows reduced surface expression levels. Human embryonic kidney 293T (HEK293T) cells were transfected with FLAG-tagged TPαR mutant (S→A) plasmids (S57A, S61A, S329A, and S331A) to mimic dephosphorylation of the serine residue, or an empty PCDNA3 plasmid for 24 hours and (A) levels of protein expression were determined and compared via immunoprecipitation using a biotin anti–FLAG-tagged antibody and streptavidin beads. A total protein (input) control confirmed equal loading. (Ai) Representative blots and (Aii) quantified data are shown. (B) HEK293T cells were transfected with green fluorescent protein (GFP)-TPa2R mutants before staining with Alexa Flour 555 wheat germ agglutinin (WGA; 1 μg/mL), and Z stack images were performed using confocal microscopy. Max intensity projection images are presented with green, representing TPαR, and magenta, representing WGA

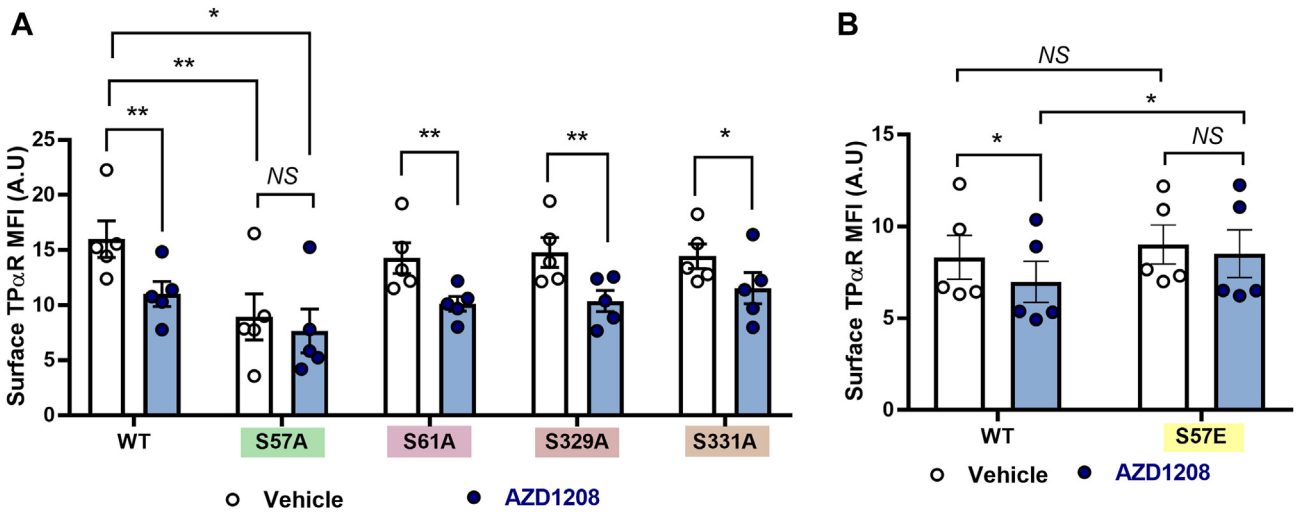

FIGURE 5 S57 thromboxane A_2 receptor (TPαR) mutants are resistant to Pim kinase inhibition-mediated internalization. Human embryonic kidney 293T (HEK293T) cells were transfected with FLAG-tagged TPαR mutant S57 (A; S→A) to mimic the dephosphorylated state or (B; S→E) to mimic the phosphorylated state plasmids or an empty PCDNA3 plasmid for 24 hours and treated with vehicle (0.1% dimethylsulfoxide [DMSO]) or AZD120 (10 μM) for 60 minutes at 37 °C before TPαR surface expression was determined by flow cytometry and mean fluorescence intensity (MFI) calculated. Quantified data are shown. $n \geq 5$. $^{*}P \leq .05$, $^{**}P \leq .01$. A.U, arbitrary units; NS, not significant.

to be phosphorylated in TPαR in resting platelets [48]. All are phosphorylation sites for protein kinase A (PKA), protein kinase G (PKG), and protein kinase C (PKC), with phosphorylation associated with receptor desensitization [49–51]. In addition, S145 phosphorylation by PKC has also been demonstrated to lead to partial TP desensitization [46].

Pim kinase, like PKA, PKG, and PKC, is a serine-threonine kinase. However, unlike the other kinases that require activation by upstream effectors, the Pim kinase isoforms are constitutively active and controlled by their expression and degradation [52–55]. We have previously reported and shown here that treatment of platelets with Pim kinase inhibitors or genetic deletion of Pim-1 kinase leads to reduced TPαR-mediated platelet aggregation and secretion responses to arachidonic acid and U46619 (Supplementary Figure S1) [24]. In contrast, platelet functional responses were normal to other platelet GPCR agonists [24]. In this study, we demonstrate that this inhibition of platelet TPαR function and signaling is related to changes in receptor surface expression.

It has been previously shown in nucleated cells that the long-form 44 kDa variant of Pim-1 kinase, Pim-1L, is localized to the plasma membrane and associated with the phosphorylation and regulation of several membrane proteins [33,56–60]. In support of Pim-1 kinase-mediated positive regulation of the TPαR, we observed reduced surface expression of TPαR and reduced downstream signaling events in platelets [24]. Further mechanistic investigations in platelets were hindered by a lack of suitable reagents following the discontinuation of the N-terminus TPαR antibody. To overcome this, we used HEK293T cells transiently expressing GFP or FLAG-tagged TPαR. Similar to the observations made in platelets, we demonstrated reduced TPαR surface expression and downstream signaling in the presence of Pim kinase inhibitors and the following expression of a KD-Pim-1 in the HEK293T cells. Furthermore, in our cell-based studies, immunofluorescence microscopy (GFP-TPαR) and cell surface ELISA (FLAG-TPαR) identified intracellular internalization and retention of the TPαR following Pim kinase inhibition, with the receptor internalization found to be dynamin and β-arrestin-dependent.

Our investigations are not the first description of Pim kinase-dependent regulation of GPCR signaling and receptor function. Studies investigating the role of Pim kinase in the pathogenesis of chronic lymphocytic leukemia have identified that Pim kinase phosphorylates the CXCR4 receptor at S339 [33,34], and we have shown this is also true for megakaryocytes and platelets [61]. This residue is within the intracellular cytoplasmic tail and regulates CXCR4 surface expression via the prevention of receptor recycling, subsequently reducing signaling responses to CXC motif chemokine ligand 12 (CXCL12)/stromal derived factor 1α (SDF1α), similar to the observations made here with TPαR (Figure 1). Using the Pim kinase substrate recognition sequence (RXRHXS) and phosphorylation site prediction software, we identified S329 and S331 in the intracellular tail, known sites of PKA and PKG phosphorylation, respectively [46,50,51], and 2 other putative Pim kinase phosphorylation site serine residues S57

(membrane). The scale bar represents 5 μm, and representative images are shown. (C) Surface expression of the TPαR was determined using flow cytometry for each mutant using a FLAG-tagged antibody to recognize the extracellular portion of the TPαR. Data are expressed as median fluorescence intensity (MFI) of TPαR positive cells. (Ci) Representative histogram of wild-type (WT) TPαR (white) and S57A mutant (gray), and (Cii) quantified data are shown. $n \geq 5$, error bars are SEM. $^{*}P \leq .05$, $^{**}P \leq .01$. A.U, arbitrary units; IB, immunoblot; IP, immunoprecipitation; NS, not significant.

and S61 in the ICL-1. The involvement of S57 and S61 amino acids in TPαR regulation and function has not been previously described. Interestingly, mutation of neighboring R60, also located in ICL-1 of TPαR, has also been shown to reduce signaling responses to TPαR agonists [62,63].

Due to the lack of phospho-specific antibodies that recognize the Pim kinase substrate recognition sequence or site-specific serine residues of the TPαR, we expressed TPαR phosphorylation site mutants (S57A, S61A, S329A, and S331A) in HEK293T cells. We identified site S57 located within the TPαR ICL-1, and not the intracellular cytoplasmic tail residues, as a key residue required for the maintenance of TPαR surface expression and the site of Pim kinase-mediated regulation. We show reduced TPαR surface expression levels in the S57A mutant TPαR, which cannot be phosphorylated, compared with WT controls that are resistant to Pim kinase inhibition and regulation. In further support of the phosphorylation of the S57 residue being a key regulator of TPαR surface expression, mutation of the S57 to glutamate (S57E), mimicking phosphorylation, renders the receptor resistance to Pim kinase inhibition and receptor internalization. Development of site-specific phospho-antibodies targeting the TPαR is needed to facilitate further investigation of this mechanism of TPαR regulation.

S57 residue lies within the ICL-1 of the TPαR between transmembrane domains 1 and 2. The ICL-1 of TPαR comprises 14 residues (residues 56-70) [64]. Nuclear magnetic resonance (NMR) and structural studies of the TPαR ICL-1 indicate a possible β-turn from residues 56 to 59 and a short, stable, helical structure in the C-terminal segment of the loop from residues 63 to 66 [64]. The remaining 4 C-terminal residues (67-70) are hydrophobic in nature. Recombinant and structural protein studies have identified ICL-1 as important for G protein coupling and signal transduction, and topological analysis of group A GPCRS demonstrates that ICL-1 is likely positioned near the C-terminal tail [65]. Molecular modeling indicates that Arg60 within ICL-1 interacts via hydrogen bonds with Met126 and Arg130 in the third transmembrane domain 3 [66]. Arg130 is part of the highly conserved D/ERY motif, which has been identified to be critical for TPαR activation and G protein coupling [67]. It is possible that constitutive phosphorylation of S57 facilitates these interactions, and loss of phosphorylation disrupts the structure of ICL-1, including the β-turn and receptor topology, altering the ability of the TPαR to interact with its G proteins, which triggers receptor desensitization via receptor internalization.

Of note are observations that the receptor for activated protein kinase-1 (RACK1) has been shown to bind to ICL-1 of the TPβR in an agonist-dependent manner and regulate receptor function and surface expression [68]. Similar observations have also been made for other GPCRs, including CXCR4, where reduced RACK1 expression leads to reduced GPCR trafficking and reduced surface expression [68]. Whether or not Pim kinase regulates TPαR surface expression and function via recruitment of RACK1 remains to be elucidated, although evidence using a nonspecific PKC inhibitor GF109203X [68], which can also inhibit Pim-1 kinase, suggests this is a possible mechanism of action that could be investigated further.

Our findings demonstrate the importance of S57 in the ICL-1 of TPαR and its phosphorylation by Pim-1 kinase for the regulation of constitutive TPαR surface expression and receptor function.

Altered thromboxane receptor signaling is associated with several different pathophysiologies, particularly CV disease. Current therapeutic strategies target this pathway either via inhibition of COX-1 or direct receptor antagonism, both of which have the potential to, or do, increase bleeding risk in patients. Increased expression of Pim-1 has been identified in patients with pulmonary hypertension, diabetes, and venous thrombosis, but its deletion is not associated with altered hemostasis in mouse models [23,69–71]. Pim kinase inhibitor-mediated reduction of TPαR levels and signaling could, therefore, offer a novel TPαR targeting strategy that is not associated with bleeding.

AUTHOR CONTRIBUTIONS

Conceptualization: S.H.N., J.L.H., K.N., S.J., S.J.M., and A.J.U.; Resources: A.J.U., Writing – Original Draft Preparation: S.H.N., M.B.L., and A.J.U.; Writing – Review & Editing: S.H.N., J.L.H., M.B.L., K.N., S.J., S.J.M., and A.J.U.; Visualization: S.H.N. and A.J.U.; Supervision: S.J.M. and A.J.U.; Project Administration: A.J.U.; Funding Acquisition: A.J.U., K.N., and S.J.M. All authors have read and agreed to this version of the manuscript.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

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