

# *Role of heat shock protein 47 in platelet glycoprotein VI dimerisation and signaling*

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## ORIGINAL ARTICLE

# Role of heat shock protein 47 in platelet glycoprotein VI dimerization and signaling

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## Abstract

**Background:** Heat shock protein 47 (HSP47) is an intracellular chaperone protein with an indispensable role in collagen biosynthesis in collagen-secreting cells. This chaperone has also been shown to be released and present on the surface of platelets. The inhibition of HSP47 in human platelets or its ablation in mouse platelets reduces platelet function in response to collagen and the glycoprotein (GP) VI collagen receptor agonist CRP-XL.

**Objectives:** In this study, we sought, through experiments, to explore cellular distribution, trafficking, and influence on GPVI interactions to understand how HSP47 modulates collagen receptor signaling.

**Methods:** HSP47-deficient mouse platelets and SMIH- treated human platelets were used to study the role of HSP47 in collagen mediated responses and signaling.

**Results:** Using subcellular fractionation analysis and immunofluorescence microscopy, HSP47 was found to be localized to the platelet-dense tubular system. Following platelet stimulation, HSP47 mobilization to the cell surface was shown to be dependent on actin polymerization, a feature common to other dense tubular system resident platelet proteins that are released to the cell surface during activation. In this location, HSP47 was found to contribute to platelet adhesion to collagen or CRP-XL but not to GFOGER peptide (an integrin  $\alpha 2\beta 1$ -binding sequence within collagens), indicating selective effects of HSP47 on GPVI function. Dimerization of GPVI on the platelet surface increases its affinity for collagen. GPVI dimerization was reduced following HSP47 inhibition, as was collagen and CRP-XL-mediated signaling.

**Conclusion:** The present study identifies a role for cell surface-localized HSP47 in modulating platelet responses to collagen through dimerization of GPVI, thereby enhancing platelet signaling and activation.

## KEYWORDS

collagen, dimerization, GPVI, HSP47, platelets

## Essentials

- Collagen molecular chaperone heat shock protein 47 (HSP47) contributes to platelet functions.
- HSP47 surface mobilization was increased upon platelet stimulation.
- Platelet adhesion on glycoprotein VI selective ligand, CRP-XL, was impeded by HSP47 inhibition.
- HSP47 supports platelet-collagen interaction and consequent activation via glycoprotein VI dimerization.

## 1 | INTRODUCTION

In the last 20 years, research has unraveled key and vital molecular players that explain how platelets recognize and interact with collagen at sites of vascular damage and consequently trigger hemostasis. Available antithrombotic measures have proven effective in limiting and controlling platelet reactivity; however, the risk of developing bleeding episodes is inevitable. Such side effects have encouraged the continuous search for alternative measures to control platelets, which may include platelet-collagen interactions, noting that none of the currently available measures targets this interaction.

A previous proteomic study led to the discovery of surface protein HSP47, a collagen-specific molecular chaperone, that contributes to the ability of platelets to respond to collagen. The necessity of HSP47 for efficient collagen production is revealed in the embryonic lethality of HSP47 knock-out mice, which display abnormally oriented epithelial tissues and ruptured blood vessels [1]. HSP47 maintains correct folding and configuration of synthesized collagen via its transient interaction with procollagen monomers in the endoplasmic reticulum (ER). The importance of this interaction in preventing aggregation of procollagen likely explains the high correlation of its expression with levels of collagen production in various cells and tissues [2–4]. HSP47 was found to support and stabilize platelet adhesion and subsequent thrombus formation [5,6]. The mechanisms by which HSP47 influences platelet-collagen responses have not been resolved. In the present study, an in-depth examination of HSP47 localization at a subcellular level was revealed for the first time in noncollagen-producing human platelets. The potential role of HSP47 in modulating platelet-collagen responses was explored using a selective HSP47 inhibitor (SMIH). The mechanistic impact of HSP47 on activatory signaling events in response to glycoprotein (GP) VI agonists was demonstrated in HSP47-deficient mouse platelets and SMIH-treated human platelets.

## 2 | RESULTS

### 2.1 | Trafficking of HSP47 towards the plasma membrane upon activation

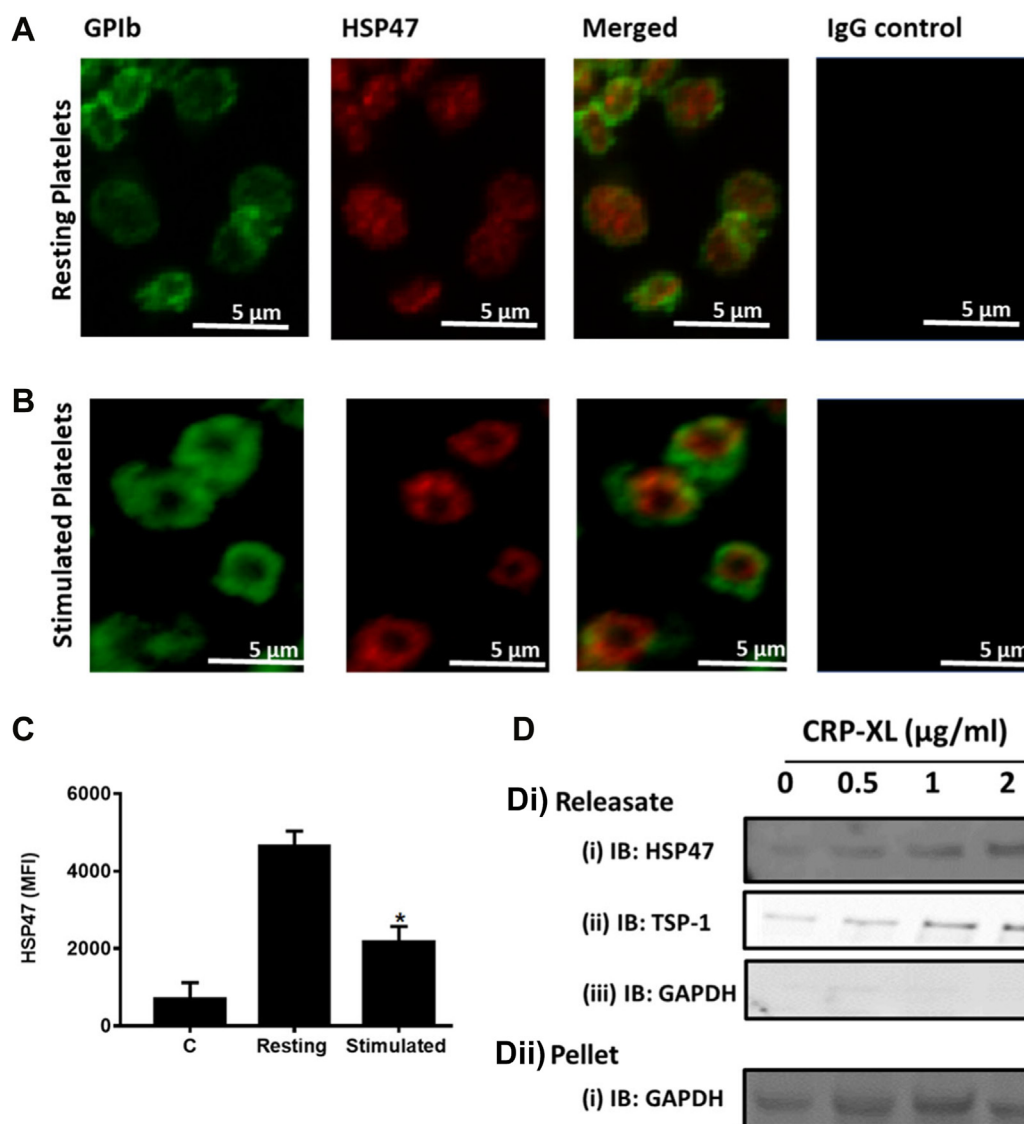
The presence of HSP47 in human and mouse platelets [5,6] is consistent with human and mouse mRNA transcriptome profiling studies that revealed the presence of HSP47 mRNA transcripts in humans and mice [7]. However, the distribution of HSP47 in resting

platelets and whether this distribution is changed upon platelet activation to reflect the location required to influence platelet-collagen responses have not been addressed. Immunofluorescence staining for HSP47 was performed on resting and activated human platelets following permeabilization, and platelets were visualized by confocal microscopy.

Platelets displayed diffuse staining of HSP47 throughout the intracellular space (Figure 1A). Upon activation, HSP47 was mobilized towards the plasma membrane, showing concentrated ring-like staining with proportionally lower total levels of immunofluorescence compared to the resting platelet (Figure 1B). This was consistent with HSP47 movement to the cell surface upon platelet activation reported previously [5,6], which likely lies behind its ability to modulate platelet responses to collagens. The fate of the mobilized HSP47 following platelet activation was assessed in resting and activated (1 µg/mL CRP-XL) permeabilized platelets using flow cytometry. Fluorescence intensity in activated platelets was reduced by  $56.62\% \pm 10.69$  when compared with the resting levels (Figure 1C), suggesting a reduction in the level of HSP47 present inside activated platelets, which is consistent with its release. Experiments were therefore performed to determine whether HSP47 could be detected in platelet releasates by immunoblotting. Human-washed platelets were treated with different concentrations of CRP-XL (0–2 µg/mL). The platelet releasate was collected by centrifugation and separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the potential presence of HSP47 was explored by immunoblot analysis. In agreement with flow cytometry studies, immunoblots of platelet releasate showed that stimulated platelets released HSP47 (Figure 1D). This coincided with the secretion of thrombospondin-1 (TSP-1). The absence of GAPDH in the releasate excluded the possibility of HSP47 release due to platelet rupturing during preparation.

### 2.2 | HSP47 is located in the platelet-dense tubular system

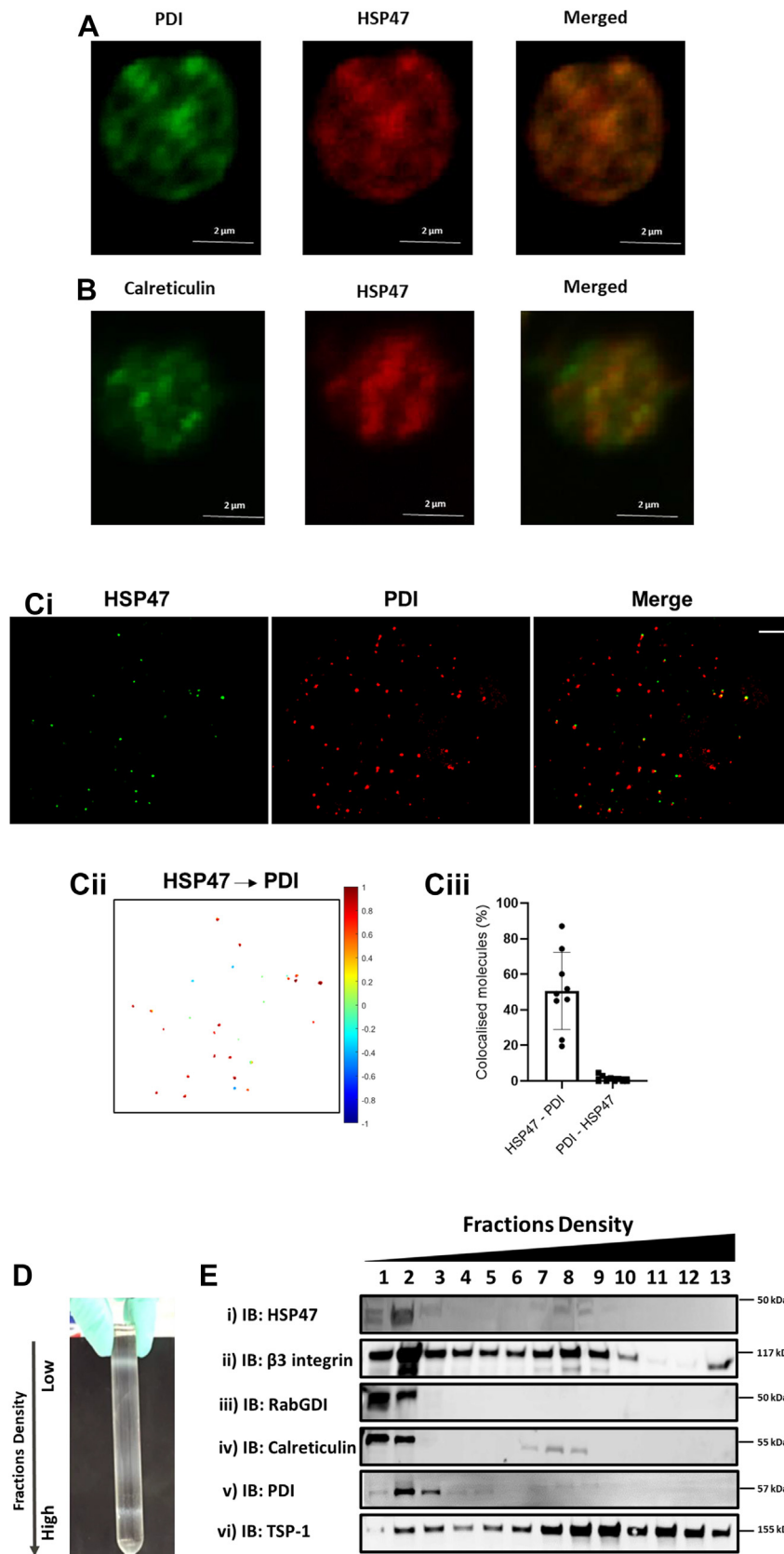
To understand how HSP47 reaches the platelet surface, where it is anticipated to influence platelet collagen adhesion, we explored possible colocalization of HSP47 with different subcellular granules and organelles. In mature platelets, the dense tubular system (DTS) is derived from megakaryocyte endoplasmic reticulum [8], an organelle responsible for hosting HSP47 chaperoning activities in collagen-producing cells [4]. Hence, it was pertinent to ask whether HSP47 is likely to be present in the DTS by examining the distribution of HSP47



**FIGURE 1** HSP47 is translocated and released following stimulation. (A) Resting and (B) stimulated platelets ( $5 \mu\text{M}$  U46619) were fixed with 4% (v/v) formaldehyde and permeabilized using 0.2% Triton X-100. This was followed with multiple washing steps, and washed platelets were subjected to blocking for 1 hour and then incubated with  $1 \mu\text{g/mL}$  anti-HSP47 and anti-GPIb. Binding was detected using Alexa Fluor 488 (glycoprotein [GP] Ib, green) and Alexa Fluor 647 (HSP47, red) conjugated secondary antibodies. The images were obtained using a  $100\times$  oil immersion lens and a Nikon A1-R confocal microscope. Data are representative of  $>3$  separate experiments using platelets from different donors. (C) HSP47 release was investigated following fixation and permeabilization, resting, or ( $1 \mu\text{g/mL}$  CRP-XL) stimulated washed platelets incubated with HSP47 antibodies and detected using Alexa Fluor 647-conjugated antibodies. Appropriate isotype control was included as negative control. Samples were then analyzed by flow cytometry. The HSP47 levels binding in isotype control, resting, and stimulated samples are shown. (D) Washed platelets ( $8 \times 10^8/\text{mL}$ ) were stimulated with the indicated concentrations of CRP-XL under stirring conditions for 3 minutes, and 0.1% sodium azide was added to stop the reaction. (Di) Releasate was collected by centrifugation, separated on sodium dodecyl-sulfate polyacrylamide gel electrophoresis, and immunoblotted to detect (i) HSP47, (ii) thrombospondin-1 (TSP-1), and (iii) GAPDH. Similarly, (Dii) pellet was separated and immunoblotted with (i) GAPDH to exclude platelet rupture during preparation. The presence of TSP-1 confirmed the platelet release, while GAPDH served as a negative control. Immunoblots were scanned using a Typhoon FLA 9500 Fluorescent Imager (GE Healthcare). Data represent mean  $\pm$  SEM ( $n = 3$ ),  $*P \leq .05$  was calculated using 1-way ANOVA. IgG, immunoglobulin G.

along with calreticulin and protein disulfide isomerase (PDI), established residents of this membrane system [9,10]. This was explored using confocal microscopy. HSP47 was distributed in an area that overlapped with PDI (Figure 2A) and calreticulin (Figure 2B) in resting platelets, suggesting possible colocalization with the DTS. Colocalization of HSP47 and PDI was confirmed in platelets spread on

fibrinogen using 2-color stochastic optical reconstruction microscopy and quantified using coordinate-based cluster detection with a degree of colocalization analysis (Figure 2C) [11]. HSP47 molecules are color-coded according to their degree of colocalization with PDI, with a score ranging from -1 to 1; -1 indicating segregation, 0 indicating random distribution, and +1 indicating colocalization (Figure 2Cii).



**FIGURE 2** HSP47 is associated with dense tubular system markers, protein disulfide isomerase (PDI) and calreticulin in platelets. (A and B) Resting platelets were fixed in 4% (v/v) formaldehyde and permeabilized in 0.2% Triton X-100. Samples were then blocked and washed prior to addition of 1  $\mu$ g/mL anti-HSP47 and anti-PDI or anti-calreticulin. Unbound antibodies were washed away, and antibodies bound to platelets were detected using Alexa Fluor 488 (calreticulin or PDI, green) and Alexa Fluor 647 (HSP47, red) conjugated secondary antibodies and images were obtained using a 100  $\times$  oil immersion lens and a Nikon A1-R confocal microscope. Data are representative of 3 separate experiments using platelets from different donors. (C) The colocalization of HSP47 and PDI was also studied using superresolution stochastic optical reconstruction microscopy. (Ci) Direct stochastic optical reconstruction microscopy reconstructions of a platelet spread on 100  $\mu$ g/mL fibrinogen and stained with HSP47 (green) and PDI (red). Scale bar 1  $\mu$ m. (Cii) Clusters were identified using DBSCAN with a radius of 25 nm and a minimum number of points of 3, and a colocalization map was generated using cluster detection with degree of colocalization for HSP47 relative to PDI with -1 indicating segregation, 0 indicating random distribution, and +1 indicating colocalization. (Ciii) The percentage of HSP47 molecules colocalized with PDI and percentage of PDI molecules colocalized with HSP47 per platelet. (D and E) Platelet homogenate was laid on a sucrose density gradient and subjected to ultracentrifugation. (D) Representative picture of the sucrose gradient after centrifugation. Thirteen fractions were then collected from the top of the gradient, with the top 6 fractions representing light-density platelet fractions and the bottom 7 being platelet fractions with heavier density. (E) Fractions were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted for HSP47,  $\beta$ 3 integrin, Rab GDP dissociation inhibitor (RabGDIb), calreticulin, PDI, and thrombospondin-1 (TSP-1). Immunoblots were scanned using a Typhoon FLA 9500 Fluorescent Imager (GE Healthcare). Results are representative of 3 individual experiments.



Supporting the confocal imaging, HSP57 was highly colocalized with PDI, with approximately 50% of the HSP47 molecules colocalized with PDI (Figure 2Ciii). This was further investigated in platelet homogenates fractionated linear sucrose density gradient centrifugation to separate the membrane and cytosolic proteins from the platelet granules and organelles. This approach has proven effective in studying similar colocalization of several proteins in human platelets [10,12]. Washed human platelets were homogenized by nitrogen cavitation using a cell disruption vessel [10,13]. Homogenates were laid over a linear sucrose density gradient and subjected to ultracentrifugation. Fractions collected from the top to the bottom of the gradient, representing the low- to high-density fractions, were separated by SDS-PAGE and western immunoblotted to detect HSP47 and marker proteins that represent distinct subcellular compartments (Figure 2D, E). As previously reported [10,13],  $\beta 3$  integrin represents surface proteins and was present abundantly in the lighter fractions 1 and 2 as well as the heavier fractions 8-10 corresponding to the  $\beta 3$  integrin containing the  $\alpha$ -granules compartment. Calreticulin and PDI are known to be present in the DTS [10] and were concentrated in the light fractions (1, 2, and 3). Similarly, cytosolic marker Rab GDP dissociation inhibitor (RabGDI) was confined to the lighter fractions 1 and 2. Lastly,  $\alpha$ -granule marker TSP-1 was distributed heavily in the higher-density fractions, while its unexpected presence in the lower fractions was due to the possibility of granule rupture during nitrogen cavitation, as previously reported [10] (Figure 2E). In agreement with immunofluorescence studies, HSP47 was highly concentrated in the low-density fractions (1-7) with a distribution pattern similar to that of calreticulin and PDI. Moreover, HSP47 was not detected in the higher-density fractions where platelet granule proteins are expected to reside, suggesting that HSP47 occupies a low-density compartment on the platelet surface or close to a surface such as DTS (Figure 2E).

### 2.3 | Mobilization of HSP47 requires actin polymerization upon platelet activation

A number of studies have reported the importance of actin-dependent cytoskeletal changes in platelet protein mobilization and release [10,14,15]. We, therefore, examined the role of actin polymerization in the mobilization of HSP47 to the platelet surface in the presence or absence of inhibitors of actin polymerization. Latrunculin A was used as an actin polymerization inhibitor at a concentration of 200  $\mu$ M, which was confirmed to be sufficient to reduce  $\alpha$ -granule marker P-selectin exposure on platelet surface [10]. Platelets were stimulated with 5  $\mu$ M thromboxane A2 analog (U46619), which is sufficient to cause platelet secretion without substantial morphologic changes in the presence or absence of latrunculin A and stained for HSP47. Platelets were also costained for P-selectin, whose mobilization and surface exposure are known to be dependent on actin polymerization [10], to confirm the effectiveness of latrunculin A in reducing the release of  $\alpha$ -granules. P-selectin- and HSP47-stained platelets were detected using Alexa Fluor 568 and Alexa Fluor 647-coupled secondary antibodies, respectively, and visualized using confocal microscopy. In the absence of

latrunculin A, P-selectin (Figure 3Aii) and HSP47 (Figure 3Bii) were mobilized to the platelet membrane upon activation. However, latrunculin A reduced the mobilization of both proteins following platelet activation, and platelets displayed the cellular distribution of P-selectin (Figure 3Aiii) and HSP47 (Figure 3Biii), similar to that seen in non-stimulated conditions (Figure 3Ai, Bi). These results indicate that actin polymerization is required for HSP47 mobilization.

### 2.4 | Inhibition of HSP47 attenuates platelet adhesion to collagen and CRP-XL but not GFOGER

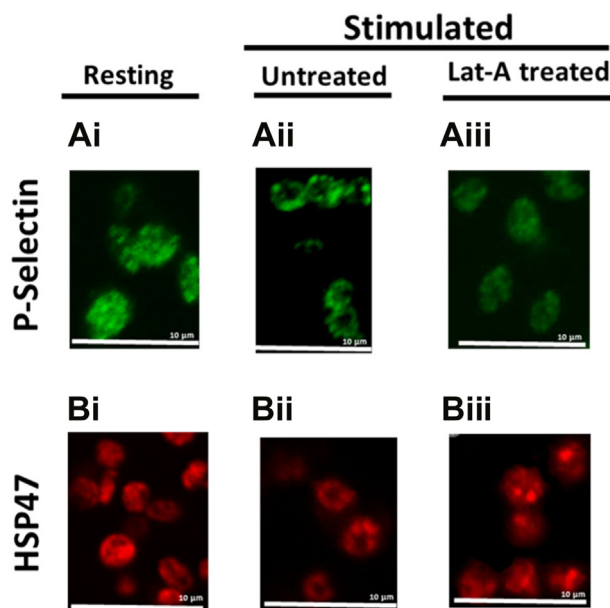
Previous work [6] has reported a reduction in thrombus formation on collagen-coated surfaces under arterial flow conditions in presence of an HSP47 inhibitor, while no effect was observed for thrombi formed on von Willebrand factor-coated surfaces. This indicates that HSP47 supports platelet-collagen adhesion independently of GPIb via other collagen receptors. We therefore explored the effect of HSP47 inhibition on the binding of individual collagen receptors, GPVI and integrin  $\alpha 2\beta 1$ , to their respective immobilized specific ligands CRP-XL and GFOGER peptides, respectively, along with collagen. This was performed under static conditions to allow the evaluation of platelet adhesion on these ligands.

Treatment with the selective HSP47 inhibitor SMIH resulted in a significant reduction of platelet adhesion to collagen-coated coverslips (Figure 4A). In comparison with vehicle-treated control, platelet adhesion was inhibited by 77% and 90% following treatment with 5 and 10  $\mu$ M SMIH, respectively (Figure 4B).

Incubation with SMIH (5 and 10  $\mu$ M) inhibited platelet adhesion to CRP-XL-coated coverslips by approximately 40% and 51%, respectively, when compared with adhesion levels in vehicle-treated samples (Figure 4C, D). Inhibition of platelet binding to immobilized CRP-XL indicates that HSP47 supports platelet binding via GPVI. Moreover, pretreatment of platelets with SMIH (5 and 10  $\mu$ M) did not alter platelet adhesion to GFOGER, the integrin  $\alpha 2\beta 1$  binding sequence present in collagens, when compared with vehicle-treated samples (Supplementary Figure S1).

### 2.5 | HSP47 supports glycoprotein VI dimerization

Several lines of evidence suggest that GPVI functions as a dimer, and such configuration elevates receptor affinity for collagen [16-22]. Using noninhibitory, recombinant dimer-specific 204-11 Fab, GPVI dimer formation was assessed in resting and stimulated platelets following treatment with SMIH. Treatment of resting platelets with SMIH (5 and 10  $\mu$ M) significantly reduced the basal level of dimerization by 50% and 74%, respectively (Figure 5A). Likewise, activated platelets pretreated with SMIH (5  $\mu$ M) showed a 34% decrease in dimerization compared with vehicle-treated platelets and treatment with a higher concentration of SMIH (10  $\mu$ M) further inhibited the level of dimerization by 66% (Figure 5B). This result points to an important role of HSP47 on GPVI-collagen affinity and explains



**FIGURE 3** HSP47 mobilization upon platelet activation requires actin polymerization. Platelets were stimulated with modified Tyrode's N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Ai and Bi) or 5  $\mu$ M U46619 in the absence (Aii and Bii) or presence (Aiii and Biii) of 200  $\mu$ M latrunculin A (incubated with platelets for 10 minutes) were fixed in 4% (v/v) formaldehyde and permeabilized using 0.2% Triton X-100. Samples were then blocked and incubated with 1  $\mu$ g/mL (A) anti-P-selectin or (B) anti-HSP47. Multiple washing cycles were performed on the samples to ensure the removal of unbound antibodies. Alexa Fluor 568 (P-selectin, green) and Alexa Fluor 647 (HSP47, red) were then added to detect antibody-bound platelets. The images were obtained using a 100  $\times$  oil immersion lens and a Nikon A1-R confocal microscope. Data are representative of 3 separate experiments using platelets from different donors.

attenuated collagen and CRP-XL adhesion events downstream of dimeric GPVI in HSP47-inhibited platelets (Figure 5).

## 2.6 | HSP47 treatment of collagen does not modulate platelet adhesion to collagen

Our data suggest that HSP47 may support GPVI dimerization and hence contribute to firmer and stabilized platelet collagen binding. Given the chaperone function of HSP47 in collagen-producing cells [23], we examined whether HSP47 would modulate collagen and, hence, influence its binding by platelet receptors. For this, potential enhancement of platelet adhesion under static conditions on collagen pretreated with HSP47 recombinant protein was evaluated. The presence of HSP47 staining in unwashed control confirmed its binding and hence proposed collagen treatment (Figure 6A). Removal of the protein below the detection level was achieved in a washed coverslip, as indicated by the lack of staining for HSP47 (Figure 6B). Exposure of precoated collagen to 2 concentrations of HSP47 recombinant proteins (2.5 and 5  $\mu$ g/mL) did not enhance platelet binding to collagen

when compared to the levels of platelet adhesion to untreated collagen (Figure 6C–F). Together, we conclude that HSP47 does not contribute to collagen-platelet interaction via modulating collagen structure in this experimental system.

## 2.7 | SMIH down-regulates platelet signaling mediated by collagen

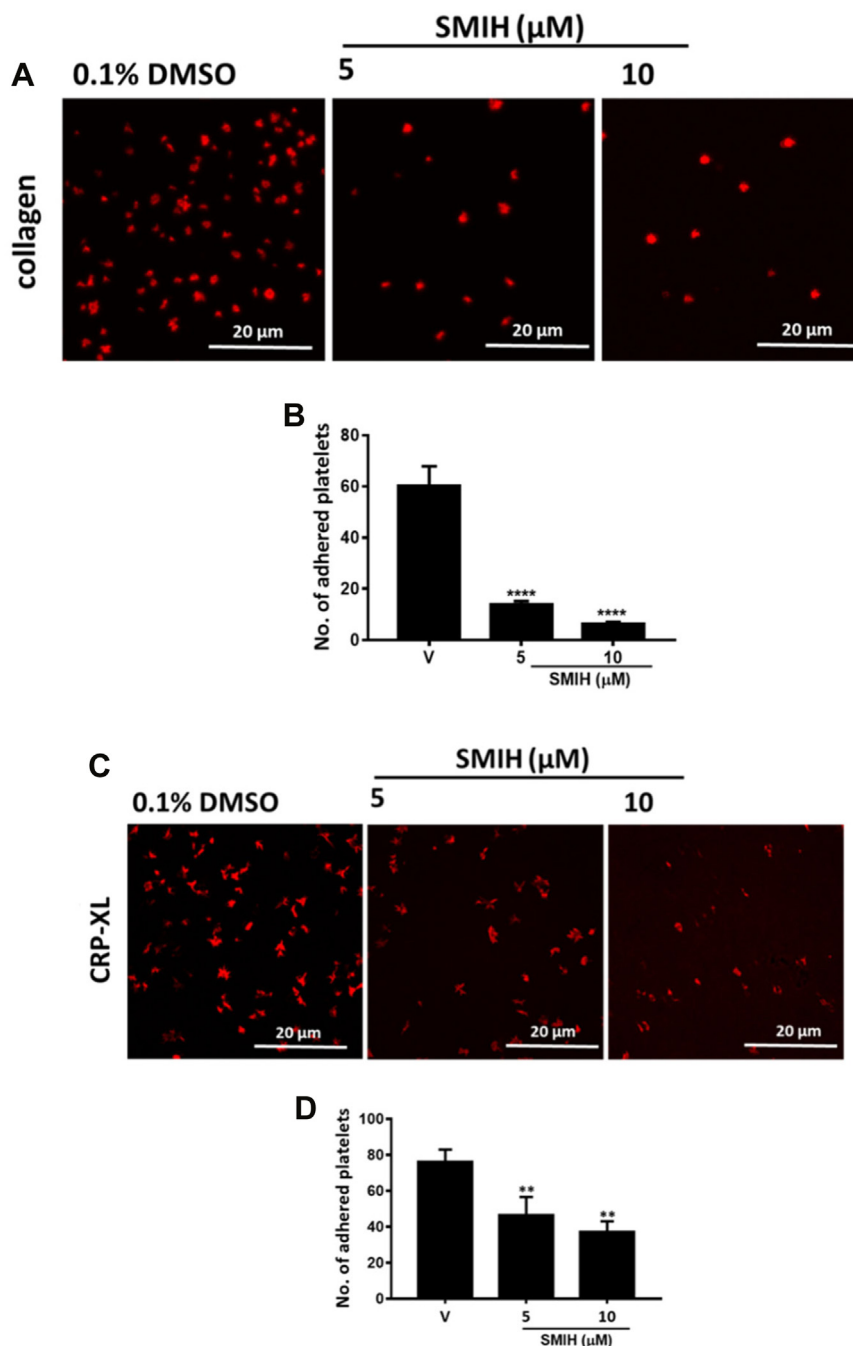
Having demonstrated the role of HSP47 in supporting platelet-collagen interaction, we examined the impact of SMIH on the modulation of signaling events downstream of collagen receptors. Human-washed platelets ( $4 \times 10^8$  cell/mL) were incubated with vehicle (containing 0.12% dimethylsulfoxide) or SMIH (5 or 10  $\mu$ M) for 10 minutes before activation with collagen (25  $\mu$ g/mL) for 90 seconds in nonaggregating conditions. Finally, the total tyrosine phosphorylation of proteins stimulated by activation of GPVI was observed by immunoblotting (Figure 7Ai). Approximately 61% and 84% lower phosphorylation levels were observed after treatment with 5 and 10  $\mu$ M SMIH, respectively (Figure 7Aii).

The secondary mediators, inositol-1,4,5-trisphosphate and 1,2-diacylglycerol, synthesized from the cleavage of phosphatidylinositol-4,5-bisphosphate in the  $G_{\alpha q}$  induced activation pathway, in turn, mediate mobilization of calcium and an increase in protein kinase C (PKC) activity, respectively [24–26]. Given the inhibition of CRP-XL-mediated  $Ca^{2+}$  mobilization observed upon HSP47 inhibition [6], the phosphorylation of PKC substrates was also evaluated in presence of SMIH. An antibody raised against phosphorylated PKC substrate recognition sequence was used to assess the effect of SMIH on PKC activity. PKC substrate phosphorylation was reduced by approximately 59% and 85% in the presence of 5 and 10  $\mu$ M SMIH, respectively (Figure 7B).

Experiments were expanded to determine the impact of SMIH treatment on signaling upstream of PKC by evaluating the phosphorylation of PLC $\gamma$ 2. A similar reduction was also observed in the phosphorylation of PLC $\gamma$ 2 at Y1217, a site for phosphorylation by Bruton's tyrosine kinase [27], with 60% and 67% inhibition in 5 and 10  $\mu$ M SMIH-treated samples, respectively (Figure 7C). PLC $\gamma$ 2 is brought to the vicinity of its substrate at the plasma membrane via interaction with tyrosine-phosphorylated LAT [28]. Pretreatment with 5 and 10  $\mu$ M SMIH also inhibited LAT phosphorylation at site Y200 by 52% and 74%, respectively (Figure 7D). LAT phosphorylation is mediated by the action of Syk [29]. Likewise, the autophosphorylation sites on Syk at the Y525/526 site [30] were impacted by SMIH treatment. When compared to vehicle control treated samples, pretreatment with 5 and 10  $\mu$ M SMIH for 10 minutes inhibited collagen-stimulated tyrosine phosphorylation of Syk by 50% and 62%, respectively (Figure 7E).

The Src-family tyrosine kinases (SFKs)-mediated Fc $\gamma$ R phosphorylation provides a docking site for Syk, which binds specifically via tandem Src-homology 2 domains, triggering Syk phosphorylation and activation [31,32]. Given the inhibition observed in the phosphorylation of Syk and downstream events, the impact of SMIH on collagen-

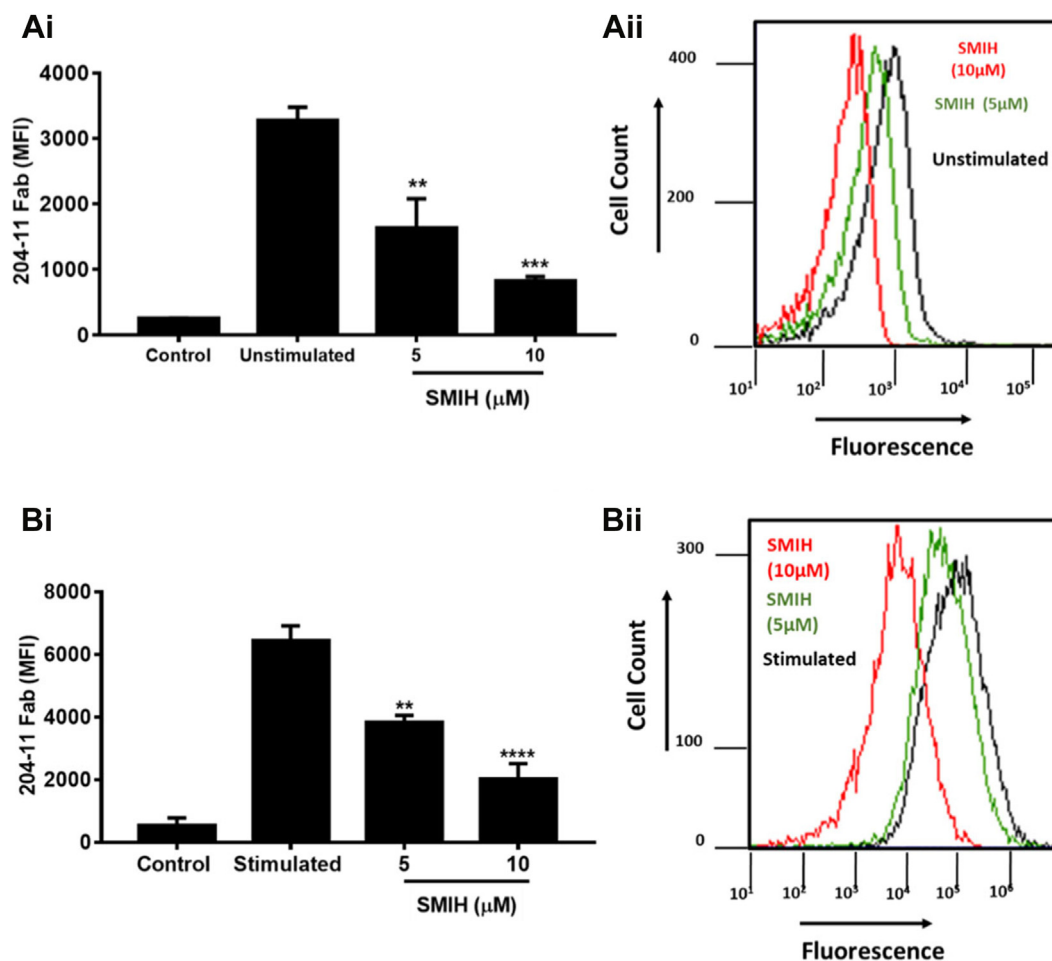




**FIGURE 4** HSP47 inhibition reduces platelet adhesion on collagen and CRP-XL. SMIH (5 and 10  $\mu$ M) or vehicle-control (containing dimethylsulfoxide 0.12% [v/v]) was incubated with human-washed platelets ( $2 \times 10^7$  cells/mL) for 10 minutes at 37 °C. Glass coverslips were precoated with (A) collagen (100  $\mu$ g/mL) or (C) CRP-XL (10  $\mu$ g/mL) and platelets treated with SMIH and vehicle control were added onto coated coverslips for 45 minutes at 37 °C. Unbound platelets were removed, and adhered platelets were fixed with 0.2% (w/v) paraformaldehyde, followed by a permeabilization step with 0.2% (v/v) Triton X-100 and staining for 1 hour with Alexa Fluor conjugated 647 phalloidin. Coverslips were mounted on glass slides using Prolong Gold Antifade mounting media. (A and C) The visualization of adhered platelets was performed using a Nikon A1-R confocal microscope with a 100  $\times$  oil immersion lens, and 5 random images were captured of each sample. (B and D) An average number of adhered platelets in-vehicle control and SMIH-treated samples are shown. Data represent mean  $\pm$  SEM ( $n = 3$ ), \*\* $P \leq .01$  and \*\*\*\* $P \leq .0001$  were calculated by 1-way ANOVA.

mediated regulation of SFKs was explored. Tyrosine phosphorylation of Src at pY418, its autophosphorylation site [33], was reduced by 62% and 75% in 5 and 10  $\mu$ M SMIH pretreated platelets, respectively (Figure 7F). Pretreatment with 5 and 10  $\mu$ M SMIH inhibited

phosphorylation levels of Lyn (Y396) by 56% and 90%, respectively (Figure 7G). These data are consistent with reduced activation of GPVI and, therefore, all signaling events expected downstream of this receptor.



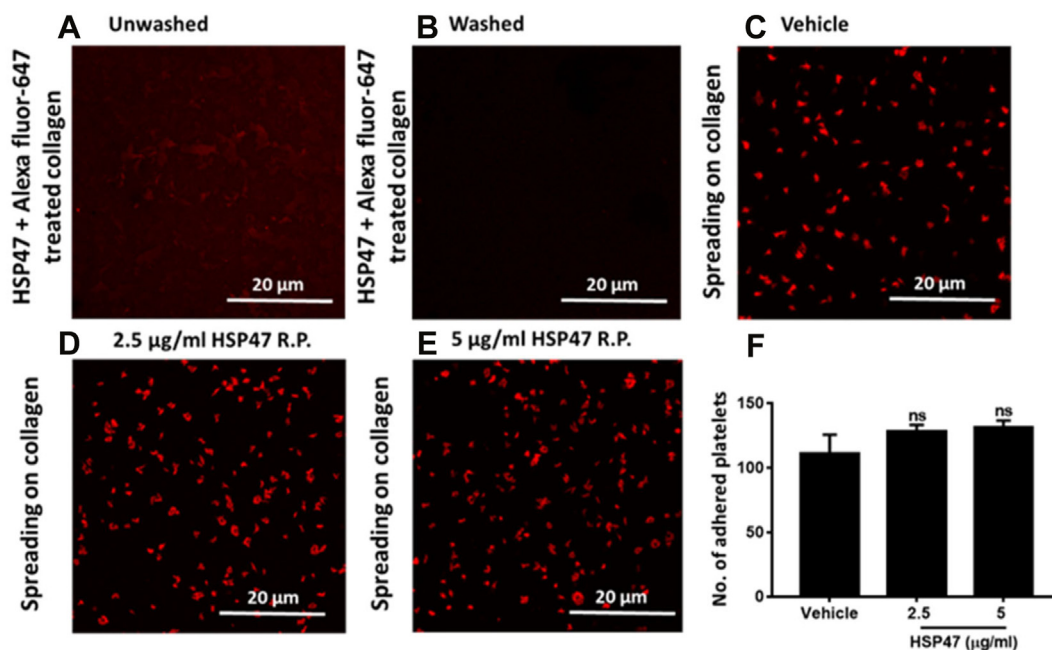
**FIGURE 5** HSP47 supports GPVI dimerization in resting and stimulated platelets. Whole blood was treated with SMIH (5 and 10 μM) or vehicle for 10 minutes and treated with Tyrode's N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer unstimulated or stimulated with CRP-XL in the presence of EDTA (2 mM). Dimer-specific m Fab 204-11 or control human Fab was added, and fluorescein isothiocyanate-labelled anti-m Fab was added to detect antibody binding using flow cytometry. Binding of FITC-labeled 204-11 Fab to the vehicle and SMIH (5 and 10 μM) pretreated (Ai) unstimulating and (Bi) stimulated platelets are shown. FITC-labeled control mouse Fab was used as a control. Representative histograms of dimerization in (Aii) unstimulating and (Bii) stimulated platelets in the presence of vehicle or SMIH (5 and 10 μM). Data represent mean ± SEM ( $n = 3$ ), \*\* $P \leq .01$ , \*\*\* $P \leq .001$ , and \*\*\*\* $P \leq .0001$  were calculated by 1-way ANOVA. MFI, mean fluorescent intensity.

## 2.8 | HSP47 supports glycoprotein VI-mediated signaling

To confirm that the actions of HSP47 are mediated through modulation of GPVI activation and signaling, the effects of HSP47 deficiency or inhibition on CRP-XL-mediated signaling were explored. HSP47-deficient mouse platelets displayed a reduction in the tyrosine phosphorylation levels of several signaling proteins in response to CRP-XL stimulation (Figure 8A). In agreement with the reduction targeting an early signaling phase observed in SMIH-treated platelets upon collagen stimulation, phosphorylation levels of Src (Y418) and Lyn (Y396) were reduced by 74% and 55%, respectively, in comparison with wild-type platelets (Figure 8B, C). Accordingly, other signaling proteins downstream of Src family kinase (Lyn) were also studied. Phosphorylation levels of LAT and PLCγ were decreased significantly

in CRP-stimulated platelets by 50% and 54%, respectively, when compared with wild-type platelets (Figure 8D, E).

Consistent with this, pretreatment of human platelets with SMIH (5 and 10 μM) inhibited an early event downstream of CRP-XL-mediated signaling with reduced tyrosine phosphorylation of key proteins by 32% and 42%, respectively, compared with vehicle (Supplementary Figure 2A). Consistent with CRP-XL-mediated signaling findings in mice, HSP47 inhibition negatively affected an upstream key regulator in human platelets, as shown in the reduced phosphorylation levels of Src (Y418), Lyn (Y396), and FcR γ-chain (Supplementary Figure S1B–D). Subsequently, activation of Syk (Y525/526), LAT (Y200), and PLCγ (Y1217) was similarly reduced in SMIH-treated platelets (Supplementary Figure S1E–G). Lastly, PKC activity was also inhibited following CRP-XL stimulation in SMIH-treated platelets (Supplementary Figure S2H). In addition,



**FIGURE 6** Platelet adhesion is not enhanced by the pretreatment of collagen with HSP47. Precoated collagen (100 µg/mL) coverslips were treated with BSA or HSP47 recombinant protein (2.5 and 5 µg/mL), followed by multiple washing steps. Human-washed platelets ( $2 \times 10^7$  cells/mL) were then added onto collagen-coated coverslips at 37 °C and after 45 minutes, the supernatant was removed, and adhered platelets were fixed and permeabilized with 0.2% (w/v) paraformaldehyde and 0.2% (v/v) Triton X-100, respectively. Alexa Fluor 647 phalloidin was used to stain platelets' actin cytoskeleton for 1 hour, and coverslips were mounted onto glass slides using Prolong Gold Antifade mounting media. As controls for HSP47 treatment and washing procedure, (A) unwashed and (B) washed treated collagen was stained for HSP47 and detected using Alexa Fluor 647. A Nikon A1-R confocal microscope with a 100 × oil immersion lens was used to aid in the visualization of samples, and 5 randomly selected images were captured of each sample. Representative image of platelet adhesion on (C) untreated vehicle and (D and E) treated collagen. (F) An average number of platelets adhered in each sample is shown. Images of (A) unwashed and (B) washed HSP47-treated collagen-coated coverslip controls. Data represent mean ± SEM ( $n = 3$ ); 1-way ANOVA was used for statistical analysis. ns, not significant; R.P., recombinant protein.

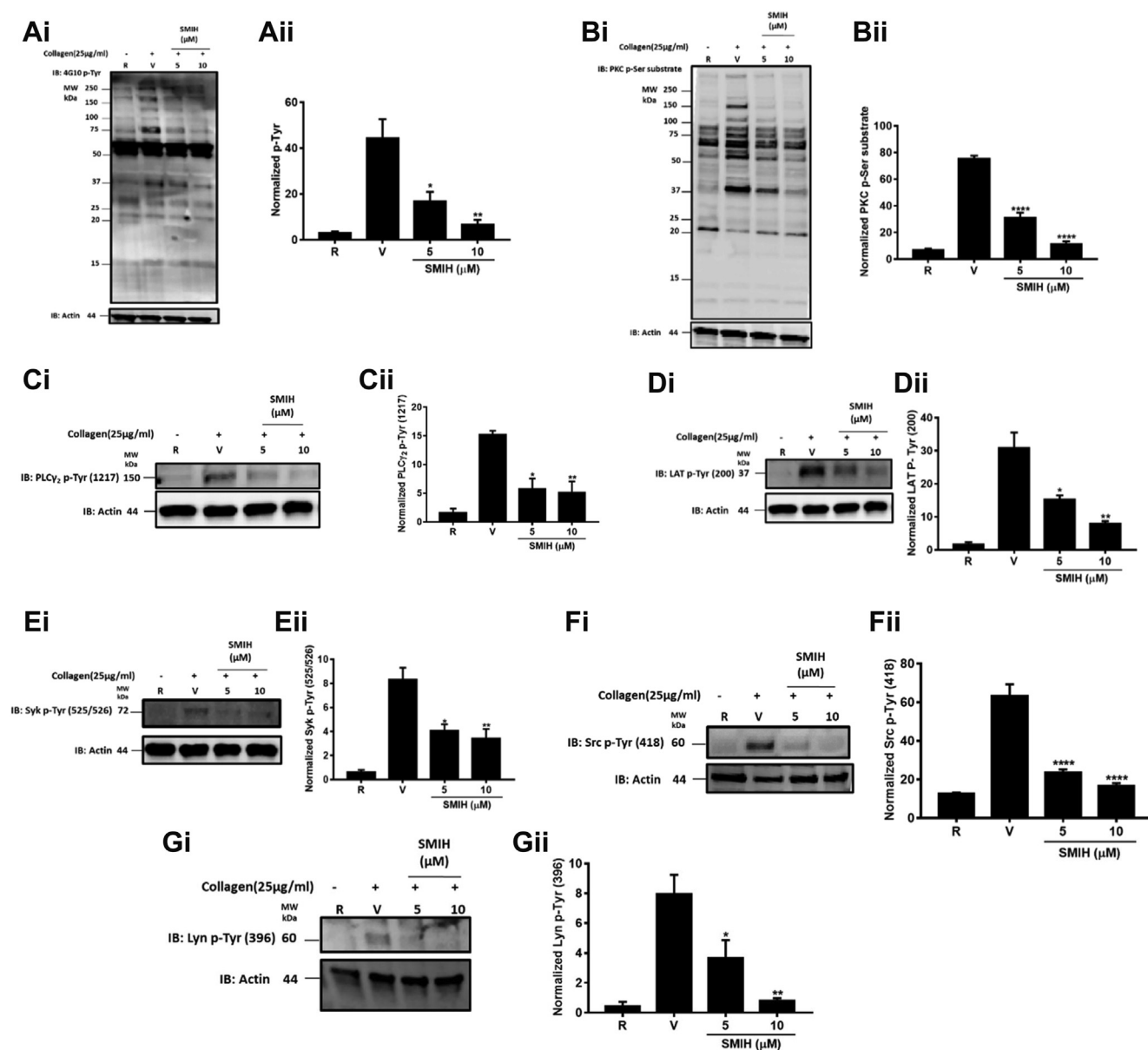
pretreatment of platelets with SMIH did not affect total tyrosine phosphorylation levels following exposure to GFOGER, an integrin  $\alpha 2\beta 1$  selective binding sequence (Supplementary Figure S3). Taken together, these findings implicate HSP47 in modulating GPVI dimerization and, therefore, initiation of GPVI signaling.

### 3 | DISCUSSION

The discovery of HSP47 expression in platelets and its role in supporting collagen-mediated platelet functions warrant investigation of this protein as a potential novel antithrombotic target. HSP47 was first discovered in a platelet proteomic study that aimed to identify platelet proteins recruited to the membrane following GPVI stimulation [5]. It was then shown that targeting HSP47 with an inhibitor resulted in attenuated platelet aggregation in response to collagen [5,6]. A recent study has extended the role of platelet HSP47 to include a contribution to neutrophil-mediated venous thrombosis through activation of TLR2. Notably, lowering platelet HSP47 expression levels following extended duration immobility, for example, due to spinal cord injury or hibernation of brown bears, was found to explain an immobility-associated thromboprotection mechanism [34].

In the present study, the hypothesis that HSP47 contributes to platelet-collagen interaction and activation and, hence, regulation of arterial thrombosis and hemostasis was tested. We first examined the subcellular localization of HSP47 in platelets. The cellular events responsible for HSP47 movement to the platelet surface on activation, where it is anticipated to participate in a platelet-collagen encounter, were investigated. HSP47 was noted to be localized to the cytoplasm of resting platelets. To further explore such localization, the colocalization of HSP47 with distinct structures inside platelets was investigated using immunofluorescence imaging studies. HSP47 exhibited similar colocalization with DTS markers, and spread platelets were confirmed to colocalize with PDI using stochastic optical reconstruction microscopy superresolution microscopy. These findings were further confirmed through subcellular fractionation analysis of platelet lysates. HSP47 is known to reside in the ER of nucleated cells.

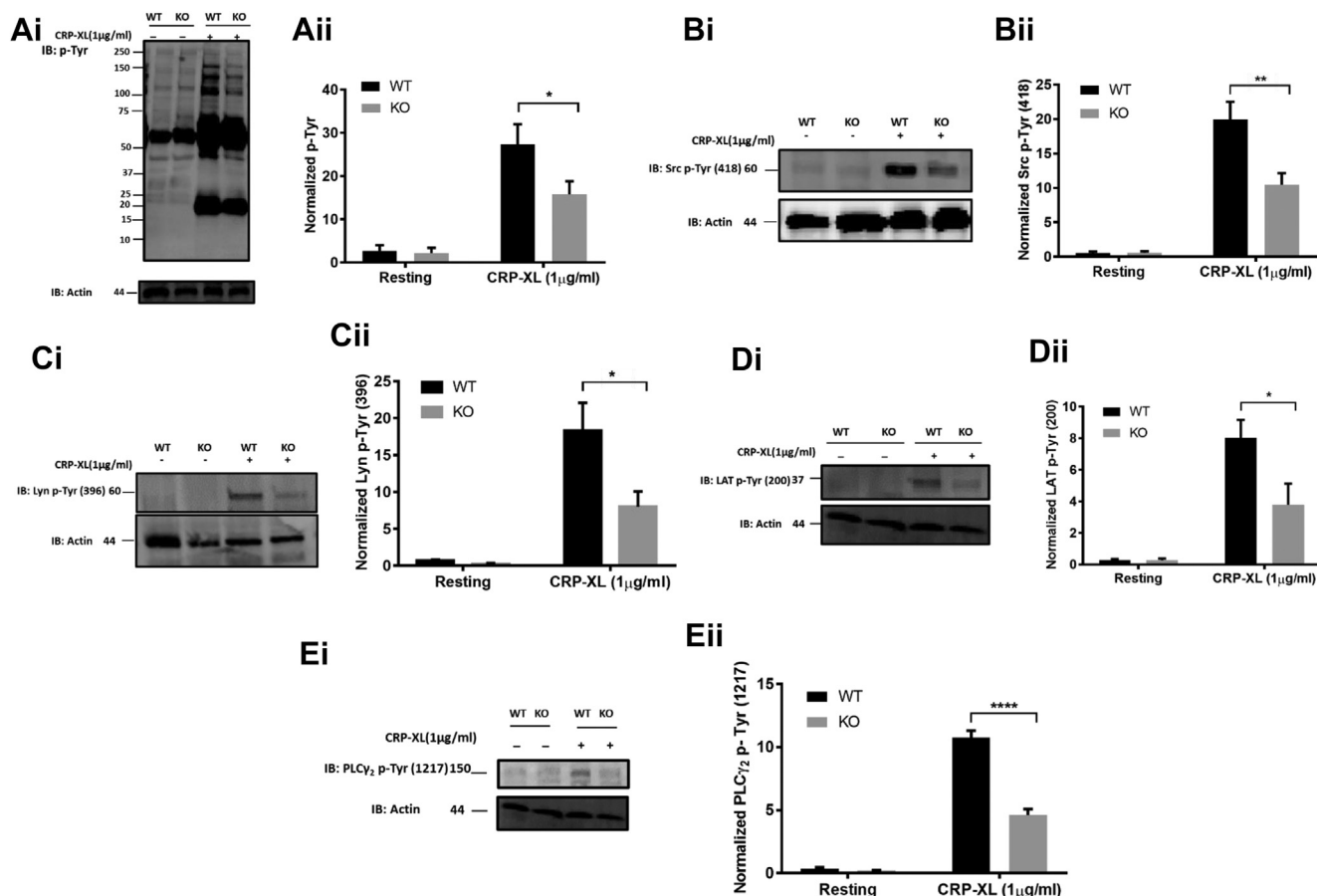
Our immunofluorescence studies demonstrated increased surface mobilization of HSP47 upon activation, consistent with previous studies [5,6]. Given the DTS localization of this chaperone, HSP47 may utilize a mobilization route toward the surface other than the classical route exhibited by  $\alpha$ -granule cargo, as has been shown for other KDEL ER-retention sequence-bearing proteins, such as PDI, ERp57, and ERp5. Actin polymerization was required for HSP47 to be mobilized to



**FIGURE 7** HSP47 supports collagen-stimulated signaling. In the presence of indomethacin (20  $\mu$ M), ethyleneglycol-*bis*( $\beta$ -aminoethyl)-N,N,N',N'-tetraacetic acid (1 mM), cangrelor (1  $\mu$ M), and MRS2179 (100  $\mu$ M), washed platelets ( $4 \times 10^8$  cells/mL) pretreated with vehicle (containing 0.12% dimethyl sulfoxide) or SMIH (5 and 10  $\mu$ M) were activated with collagen (25  $\mu$ g/mL) and after 90 seconds, Laemmli sample buffer was added to lyse the samples. Whole-cell lysates were then resolved by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. Membranes were probed with antiphosphotyrosine antibody (4G10), an antibody directed against phosphorylated protein kinase C (PKC) substrate recognition sequence, site-specific phospho-antibodies for PLC $\gamma$ 2 (Y1217), LAT (Y200), Syk (Y525/526), Src (Y418), and Lyn (Y396) to determine phosphorylation levels. Representative immunoblots for the phosphorylation levels of (Ai) 4G10, (Bi) PKC substrates, (Ci) PLC $\gamma$ 2, (Di) LAT, (Ei) Syk, (Fi) Src, and (Gi) Lyn are shown following treatment with vehicle or SMIH. As a loading control, membranes were reprobed with antibodies against actin. Bar charts are mean phosphorylation values of (Aii) 4G10, (Bii) PKC substrates, (Cii) PLC $\gamma$ 2, (Dii) LAT, (Eii) Syk, (Fii) Src, and (Gii) Lyn in samples treated with SMIH relative to actin. Results represent the mean  $\pm$  SEM ( $n = 3$ ), \* $P \leq .05$ , \*\* $P \leq .01$ , and \*\*\*\* $P \leq .0001$  were calculated by 1-way ANOVA. IB, immunoblot; R, resting; V, vehicle.

the platelet periphery, as shown in the reduction of HSP47 mobilization in presence of latrunculin A (actin polymerization blocker [10,35]). Notably, the reliance on actin dynamics for release was also reported to be required for the thiol isomerase (PDI) [10], suggesting a common mechanism for release of DTS-resident proteins from platelets.

Previous studies established a potential role for HSP47 in collagen-induced platelet activation, but further elucidation of the mechanisms by which such chaperone modulates platelet functions required clarification [5,6]. In this study, this was approached using different forms of collagen and receptor-specific synthetic collagen peptides such as GFOGER and CRP-XL. Adhesion to CRP-XL- and



**FIGURE 8** Ablation of HSP47 attenuates glycoprotein VI signaling. Platelets ( $2 \times 10^8$  cells/mL) from HSP47<sup>+/+</sup> and HSP47<sup>-/-</sup> mouse were activated with CRP-XL (1 µg/mL). Stimulation was carried out for 90 seconds in the presence of indomethacin (20 µM), cangrelor (1 µM), MRS2179 (100 µM), and ethyleneglycol-bis(β-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA acid, 1 mM) before the addition of Laemmli sample buffer. Whole-cell lysates were then separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. Total tyrosine phosphorylation, tyrosine phosphorylated Src, Lyn, LAT, and PLCγ<sub>2</sub> were detected by antiphosphotyrosine antibody (4G10), a site-specific phospho-antibodies for phosphorylated Src (Y418), Lyn (Y396), LAT (Y200), and PLCγ<sub>2</sub> (Y1217), respectively. Representative immunoblots are shown for the phosphorylation levels of (Ai) 4G10, (Bi) Src, (Ci) Lyn, (Di) LAT, and (Ei) PLCγ<sub>2</sub> in mouse platelets. Phosphorylation levels of (Aii) 4G10, (Bii) Src, (Cii) Lyn, (Dii) LAT, and (Eii) PLCγ<sub>2</sub> are presented as the mean relative to actin that was also used as a control for protein loading. Results are mean  $\pm$  SEM ( $n = 3$ ), \* $P \leq .05$ , \*\* $P \leq .01$ , and \*\*\*\* $P \leq .0001$  were calculated by 1-way ANOVA. IB, immunoblot; KO, knock-out; R, resting; WT, wild-type.

GFOGER-coated surfaces (GPVI- and integrin  $\alpha 2\beta 1$ -selective ligands, respectively) was assessed with and without HSP47 inhibition using SMIH. Pretreatment with SMIH reduced platelet adhesion to CRP-XL but not GFOGER. Such findings suggest a direct or indirect effect of HSP47 on GPVI function.

To explore the mechanisms underlying attenuated platelet adhesion to and activation by CRP-XL, we explored the potential for HSP47 to modulate GPVI dimerization. GPVI dimerization has been shown to increase the collagen binding competency and affinity for collagen [20]. Subsequently, collagen binding provokes GPVI dimerization or clustering [22,36]. Levels of dimeric GPVI on the surface of resting and stimulated platelets were reduced upon HSP47 inhibition. Since this dimeric form of GPVI is required for both platelet adhesion to collagen and subsequent activation [19,20], the present study suggests that the previously established role of HSP47 in collagen-mediated functions and GPVI binding to collagen may be due to its

capability to support GPVI dimerization. Notably, there was no difference in HSP47 surface translocation in presence of SMIH (data not shown), which indicates that the observed reduced collagen response is more likely caused by inhibited function of HSP47 rather than reduced availability on the surface. Moreover, platelet adhesion to fibrin is not supported by HSP47 [6], while a recent study has found that fibrin does not bind dimeric GPVI [37]. Based on the present findings, we propose a role for HSP47 in supporting platelet collagen responses via GPVI dimerization.

We also considered the chaperoning nature of HSP47 toward collagen [38,39] and the possibility that this may modulate the competency of collagen to support interactions with platelets. While we cannot rule out a role for HSP47 in modulating collagen structure in a manner that impacts platelet interactions, for example, following burns or trauma, we found no evidence to support this in our studies. These observations, along with our report that HSP47 does not regulate von Willebrand



factor-GPIb interactions [6], indicate that this initial entrapment and activation of platelets at sites of injury is GPVI-dependent. Following entrapment, released HSP47 may modulate responses of platelets to other agonists, such as thrombin, and enhance venous thrombosis through actions like stimulation of neutrophils [34].

Clustering GPVI receptors via collagen or CRP-XL induces trans/autophosphorylation of SFKs such as Lyn and Fyn [40]. This is followed by SFK-dependent phosphorylation of FcR $\gamma$ -chain-associated immunoreceptor tyrosine-based activation motif. Consequently, the tyrosine kinase Syk is recruited and autophosphorylated [41,42]. Inhibition of HSP47 attenuated platelet phosphorylation levels by hindering these early phases in collagen-mediated signaling, as observed in the reduced phosphorylation of SFK Lyn. Notably, a similar effect on GPVI-mediated signaling was observed in HSP47-deficient mouse platelets. Since HSP47 deficient mice have been characterized to possess normal platelet count and normal levels of platelet receptors, including collagen receptors [6], this further suggests that HSP47 functions in supporting GPVI-mediated function. As mentioned, HSP47 inhibition did not affect platelet adhesion on the integrin  $\alpha 2\beta 1$  selective ligand peptide GFOGER. Hence, we then sought to explore the impact of SMIH on the generation of integrin  $\alpha 2\beta 1$ -mediated phosphorylation events in platelets [43]. No difference was recorded in outside-in signaling events generated upon the ligation of integrin  $\alpha 2\beta 1$  with its selective agonist peptide, GFOGER.

Collectively, these findings are consistent with a role for HSP47 in the modulation of platelet function through GPVI. Evidence provided in the present study identifies the collagen molecular chaperone HSP47 as an important player in platelet-collagen interaction and provides insight into the underlying mechanisms through regulation of GPVI dimerization.

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## AUTHOR CONTRIBUTIONS

S.A. led the study, performed experiments, analyzed data, and prepared the manuscript. P.S., A.P., T.A., F.A., S.K., K.S., and M.A. performed experiments and data analysis. S.J. designed the study. J.M.G. designed the study and prepared the manuscript.

## RELATIONSHIP DISCLOSURE

There are no competing interests to disclose.

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## SUPPLEMENTARY MATERIAL

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