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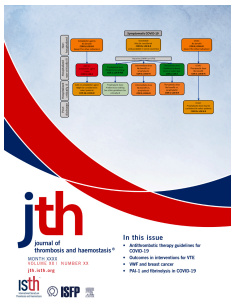

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Title. High FVIII concentrations interfere with GPVI-mediated platelet activation *in vitro*

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Running heads. Binding of FVIII to platelet GPVI

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Abstract

Background. The recruitment of activated FVIII at the surface of activated platelets is a key step towards the burst of thrombin and fibrin generation during thrombus formation at the site of vascular injury. It involves binding to phosphatidyl-serine (PS) and, possibly, to fibrin bound to αIIbβ3. Seminal work had shown the binding of FVIII to resting platelets, yet without a clear understanding of a putative physiological relevance.

Objectives. To characterize the FVIII-platelet interaction and its potential modulation on platelet function.

Methods. FVIII was incubated with washed platelets. The effects on platelet activation (spontaneously or triggered by collagen and thrombin) were studied by flow cytometry and light transmission aggregometry. We explored the involvement of downstream pathways by studying phosphorylation profiles (western blot). The FVIII-GPVI interaction was investigated by ELISA, confocal microscopy and proximity ligation assay.

Results. FVIII bound to the surface of resting and activated platelets in a dose-dependent manner. FVIII at supra-physiological concentrations did not induce platelet activation but rather specifically inhibit collagen-induced platelet aggregation and altered GPVI-dependent phosphorylation. FVIII rid of its chaperon protein, von Willebrand factor (VWF), interacted in close proximity with GPVI at the platelet surface.

Conclusions. We showed that VWF-free FVIII binding to, or close to, GPVI modulates platelet activation *in vitro*. This may represent a yet uncharacterized negative feedback loop to control overt platelet activation. Whether local activated FVIII concentrations achieved during platelet accumulation and thrombus formation at the site vascular injury *in vivo* are compatible with such a function remains to be determined.

Keywords. Factor VIII, platelets, GPVI

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Introduction

The expression of phosphatidylserine (PS) at the surface of activated platelets provides an optimized surface for the assembly of enzymatic complexes of coagulation.[1,2] Thus, thrombin-activated factor VIII (FVIIIa), rid of its B domain and released from its chaperone, von Willebrand factor (VWF), binds to the negatively charged surface-exposed PS via its C1 and C2 domains.[3] PS-bound FVIIIa brings together activated factor IX (FIXa) and factor X (FX) to enhance the generation of activated FX at the platelet surface.[4] This results in a burst of thrombin generation and recruitment of newly activated platelets at the site of injury.[5] Gilbert *et al.* recognized soluble fibrin bound to αIIbβ3 as an additional non-PS FVIII binding site at the surface of activated platelet.[6] Recently, GPVI was identified as a functional platelet receptor for polymerized fibrin, with a potential key role in consolidation of the platelet plug.[7]

Binding of FVIII to resting platelets was documented in the seminal work by Nesheim et al.[8] A key role for the C1 and C2 domains of the FVIII light chain in binding to the surface of non-activated platelets was later reported.[9] Yet, the interaction of FVIII with resting platelets has no known functional repercussion. Here, we describe that FVIII at supra-physiological concentrations binds to resting platelets in close proximity to GPVI and perturbs the GPVI-dependent platelet activation pathway *in vitro*.

Results and Discussion

We first confirmed by flow cytometry that VWF-free FVIII (50 to 200 nM) binds in a dose-dependent manner to washed resting platelets (**Figure 1A**). As expected, the binding of FVIII was further increased at the surface of platelets activated by thrombin (**Figure 1A**). Importantly, none of the concentrations of FVIII did activate platelets, nor did they potentiate thrombin-induced platelet activation, as shown by P-selectin expression studied by flow cytometry (**Figure 1B**). Conversely, FVIII did not inhibit thrombin-induced platelet activation **(Figure**

1B) or TRAP-induced platelet aggregation, as shown by light transmission aggregometry (100 nM FVIII, **Figure 1C**). In stark contrast, FVIII induced a specific dose-dependent inhibition of collagen-induced platelet aggregation (5 µg/ml collagen, **Figure 1D**) with an estimated IC_{50}=94 nM. There was no inhibition at FVIII concentrations <50 nM and the inhibition reached a plateau for FVIII concentrations ≥150 nM. Accordingly, FVIII at 100 nM, a concentration close to the IC_{50}, reduced by more than 50% the collagen-mediated aggregation of platelets (**Figure 1E**, P<0.0001). Such an effect was not observed in the presence of high concentrations of FIX (used as a control protein involved in the coagulation process at the surface of activated platelets), nor in the presence of the excipient of the therapeutic FVIII used in the latter experiments (i.e., excipient of Novoeight®) (**Figure 1E**). Because PS is an obvious alternative binding site for FVIII and FVIIIa, we investigated whether such an effect of FVIII on platelets is still observed in the presence of other molecules that bind PS. To this end, washed platelets were incubated alone, or with FVIII and/or lactadherin, prior to the addition of collagen. The addition of lactadherin alone had no blocking or enhancing effect on collagen-mediated platelet activation (**Figure 1F**). Lactadherin also failed to modulate the effect of FVIII on collagen-mediated platelet activation.

Our results suggest a specific interactive partnership between FVIII and GPVI. In support of this, there was a positive and statistically significant correlation between the expression of GPVI and binding of FITC-labeled FVIII to washed platelets from 10 healthy individuals (**Figure 1G**, P=0.004). We then studied the GPVI-dependent downstream phosphorylation in the presence of FVIII by western blot. Platelets were stimulated with 2 µg/ml collagen and aggregation was stopped 50 seconds later. Under such conditions, both FVIII (100 nM) and an inhibitory anti-GPVI Fab fragment Fab9012[10] (400 nM) induced a >80% inhibition of platelet aggregation (**Figure 1H**). In support of this finding, FVIII (100 nM) inhibited the tyrosine-phosphorylation mediated by collagen in the absence (52±19%, P=0.018, **Figure 1I**) and in the

presence (72±7%, P=0.023) of eptifibatide (9 µM), a specific antagonist of αIIbβ3 downstream activation.[11] The latter data further suggest a preferential interference of FVIII with the GPVI-dependent activation pathway.

To substantiate an interaction between FVIII and GPVI at the surface of platelets, we compared the distributions of both molecules using labeled specific antibodies and confocal microscopy (**Figure 2**). Washed resting platelets were fixed and incubated with FVIII (400 nM) prior to quantification of FVIII (green) and GPVI (red) colocalization (merge, yellow) (**Figure 2A**). At the surface of resting platelets, 25±8% of FVIII colocalized with GPVI, while 37±5% of GPVI colocalized with FVIII. The close proximity of FVIII and GPVI was further confirmed in a proximity ligation assay that detects molecules separated by less than 40 nm distance (**Figure 2B**).[12] We completed the characterization of the interaction between FVIII and GPVI by ELISA (**Figure 3**) using distinct commercially available FVIII molecules, i.e., a recombinant B domain-deleted FVIII (BDD-FVIII, Novoeight®), a recombinant full-length FVIII (FL-FVIII, Kogenate®) and a plasma-derived FVIII (pd-FVIII, Factane®). Factane® is a B domain-encompassing full-length FVIII which contains substantial amounts of VWF. We also used different types of recombinant GPVI. We thus used a monomeric recombinant GPVI (rGPVI, R&D) that encompasses the Gln21-Lys267 residues and lacks Leu270-Ser339 corresponding to the transmembrane and intracellular portion, as well as a Fc-fused homo-dimeric GPVI covering the GPVI sequence Gln21-L270 (GPVI-Fc).[13] Both molecules lack the transmembrane domain of the protein, thus avoiding any bias consecutive to potential phospholipid contamination during the purification process of GPVI. BDD-FVIII bound identically to both immobilized monomeric rGPVI and dimeric GPVI-Fc (**Figure 3A**). It also bound identically to a recombinant GPVI-Fc that lacks both the transmembrane domain and the highly O-glycosylated stalk region of GPVI (sequence Gln21-Thr203, **not shown**). The data

rule out non-specific binding that would result from the presence of putative phospholipid contaminants.

FL-FVIII demonstrated an up to 10-fold stronger binding to GPVI than BDD-FVIII, while pd-FVIII showed only marginal binding (**Figure 3B**). Digestion of FVIII by thrombin, that removes the B domain and frees FVIII from VWF, restored the binding of pdFVIII to GPVI to levels reached by BDD-FVIII (**Figure 3B**). Focusing on the specific effect of VWF, we confirmed that VWF induced a dose-dependent inhibition of FVIII binding to GPVI, which was not observed when a mutated FVIII^{Y1680C} that lacks VWF-binding capacity was used (**Figure 3C**).[14,15] These results indicate that VWF prevents the FVIII-GPVI interaction, potentially by shielding key residues in the C1 and C2 domains that were shown be important for binding to resting platelets.[9]

The digestion of FVIII by thrombin also removes the B domain of FVIII, which has no known function for FVIII pro-coagulant activity. The B domain however facilitates the intracellular trafficking and secretion of FVIII and improves its thermostability.[16–18] Conversely, binding of asialylated oligosaccharide structures on the FVIII B domain to the asialoglycoprotein receptor potentiates FVIII catabolism.[19] More particular to platelets, the B domain of FVIII was shown to decrease the binding of FVIII to activated platelets.[20] In our experiments, the up to 10-fold stronger binding of FL-FVIII to GPVI returns to levels similar to that observed with BDD-FVIII after digestion by thrombin (**Figure 3B**). Digestion by thrombin did however not alter the binding of BDD-FVIII to GPVI. These results suggest that the B domain of FVIII potentiates with other FVIII moieties for binding to GPVI, at least *in vitro*. Importantly, the removal of N-linked glycans oligosaccharides from FL-FVIII using PNGase F did not reduce the binding of FVIII to immobilized GPVI (data not shown), suggesting that N-linked glycans clustered within the B domain of FVIII are not responsible for the increased interaction with GPVI. In order to get an insight into the apparent equilibrium binding affinity of FVIII for

GPVI and confirm the specificity of the interaction, we performed competitive ELISA wherein Fc-fused FVIII (rFVIIIFc, 20 nM) was co-incubated with increasing concentrations of soluble BDD-FVIII, FIX or human serum albumin (HSA). Soluble FVIII inhibited the binding of Fc-fused FVIII to GPVI in a dose-dependent manner with an apparent K_D of ~141 nM (**Figure 3D**). The value is in line with the IC_{50} determined in platelet aggregation experiments (94 nM, **Figure 1D**). In contrast, neither FIX nor HSA competed with FVIII for binding to GPVI.

Taken together, our results indicate that FVIII at high concentrations specifically interferes with GPVI-dependent platelet activation and downstream phosphorylation. The results also suggest that GPVI is a potential new platelet-binding site for activated FVIIIa, albeit in the absence of VWF. This observation potentially enriches the list of FVIII-binding catabolic and non-catabolic receptors (LRP, ASGPR, CLEC4M, STAB2, SIGLEC-5, CD206) and platelet ligands (PS and αIIbβ3-bound fibrin).[21] The fact that FVIII interaction with GPVI is blocked upon engagement with VWF and that the apparent affinity that characterizes the interaction is low (about 100-fold lower than the circulating FVIII concentration) raises the question of the physiological relevance of the observed phenomenon. FVIII circulates in blood at an approximate concentration of 0.5 nM.[22] The local concentration of FVIII at the site of bleeding has, to our knowledge, never been determined. One may hypothesize that, at the site of bleeding and following activation by thrombin and dissociation from VWF, FVIII shifts from a volume of blood characterized by a capillary diameter of about 3 µm and concentrates at the vicinity of the activated cell surface in a layer of ~80 Å.[23] In such a situation, FVIII would be present at 0.5 x 3 / 0.008 = 188 nM. While this model is obviously oversimplified since it assumes that 100% of the FVIII in the 3D space is activated locally, dissociates from VWF, and concentrates at the surface of the activated cells, it suggests a local FVIII concentration compatible with that showing reduction of platelet activation in our assays.

The platelet plug includes super-activated platelets (e.g., platelets obtained *in vitro* following stimulation with thrombin plus collagen), that are located at sites of collagen exposure and along the clot surface, and demonstrate enhanced PS exposure, high levels of surface-bound FV, FVIII, FIX and FX, and enhanced procoagulant activity.[24] Super-activated platelets are surrounded by layers of activated platelets characterized by lower PS exposure (e.g., thrombin-activated platelets).[24] The apparent low affinity of FVIII for GPVI may favor interaction of FVIIIa with GPVI at the surface of the latter platelet population, rather than at the surface of super-activated platelets. In such a scenario, GPVI may work in concert with fibrin-bound-αIIbβ3 at the surface of thrombin-activated platelets to sequester FVIIIa, thus stabilizing the tenase complex and fostering FXa and thrombin generation. This association may be further stabilized by GPVI-bound polymerized fibrin.[7] Conversely, the interference of FVIII/FVIIIa with GPVI-mediated platelet activation may synergize with the regulatory effect of super-activated platelets on thrombus growth.[25] Platelet GPVI has also been shown to contribute to the maintenance of vascular homeostasis as well as vascular integrity at the site of inflammation.[26] Whether local concentrations of FVIII/FVIIIa at sites of vascular injury or in specific vascular beds are compatible with physiological roles for FVIII in these different contexts remains however to be experimentally determined.

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Authors Contributions
Designed research: JR, YB, MJP, VP, SLD
Performed research: RS, AM, MBJ, SD, SL, VD, PB, AB, VP
Contributed essential material: YB, MJP
Analyzed data: RS, SL, KV, YB, VP, SLD
Wrote the paper: RS, VP, SLD

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Conflict of Interest. Authors declare no competing financial interests.

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Figure legends

Figure 1. FVIII inhibits collagen-mediated platelet aggregation. Panels A and B. FITC-coupled FL-FVIII (Kogenate®) was incubated with washed platelets ($3x10^7$/ml) alone or with bovine thrombin (1 IU/ml; FVIII 200 nM). The binding of FITC-coupled FL-FVIII was studied by flow cytometry (Panel A, black dotted curve: resting platelets incubated alone; blue curve: 50 nM FVIII; orange: 100 nM; green: 200 nM; red: 200 nM+FIIa). P-selectin expression was investigated as a read-out for platelet activation by flow cytometry, and is depicted as mean fluorescent intensity (Panel B: MFI, mean±SD, duplicates). **Panel C.** Washed resting platelets ($3x10^8$/ml) from 4 healthy donors were pre-incubated alone or in the presence of BDD-FVIII (Novoeight®, 100 nM) for 5 min at room temperature prior to the addition of 25 µM TRAP. Platelet aggregation was monitored by light transmission aggregometry (mean±SD). **Panels D and E.** Washed resting platelets were pre-incubated alone or in the presence of BDD-FVIII (Novoeight®) for 5 min prior to the addition of 5 µg/ml collagen. **Panel D**. Dose-dependent inhibition of platelet aggregation (expressed as %) as a function of FVIII concentration in 4 to 11 healthy donors (mean±SEM). Inset: data from one representative donor. **Panel E.** Maximal collagen-induced platelet aggregation in the absence or presence of 100 nM FVIII, 100 nM FIX or excipient of Novoeight®. Individuals symbols depict individual platelet donors and lines represent mean±SD. **Panel F.** Washed platelets from 4 healthy donors (mean±SEM) were incubated alone or with FVIII (100 nM, Novoeight®) and/or lactadherin (100 nM) for 5 min prior to the addition of collagen (5 µg/ml). Maximal collagen-induced platelet aggregation was then measured. **Panel G.** Washed platelets from 10 healthy individuals were incubated with 200 nM FVIII-FITC and a PE-labeled anti-human GPVI antibody (clone 1G5). MFI were correlated using the Spearman rank correlation test (Rs: Spearman correlation coefficient). **Panel H.** Washed platelets ($5x10^8$/ml) were incubated alone, with FVIII (Novoeight®, 100 nM) or with Fab9012 (400 nM) for 5 min prior to addition of 2 µg/ml collagen, after preincubation

alone or with 9 µM eptifibatide. The graph depicts the % of platelet aggregation monitored during 50 s in one of 3 representative healthy donors. **Panel I.** Proteins in platelet lysates (20 µg per lane) were separated in a reducing 10% SDS-PAGE and blotted. Total phosphorylation was detected using the anti-phosphotyrosine antibody 4G10. Total density profiles were recorded for each lane. Total areas under the curves were computed and normalized against ß-actin (mean±SD, n=3 donors) are shown on top. Differences were systematically assessed by a two-sided non-parametric Mann-Whitney test or a one-way ANOVA for panel G (ns: not significant).

Figure 2. Colocalization of FVIII with GPVI. Fixed washed resting platelets ($2x10^7$/ml) were spinned-down on 0.01% poly-L-lysine-coated dishes and incubated with FL-FVIII (Kogenate®, 400 nM). **Panel A**: GPVI was recognized with the mouse monoclonal anti-GPVI IgG 9012 (green). FVIII was recognized with the human monoclonal anti-FVIII IgG BOIIB2 (red). Images were acquired using Zeiss LSM confocal microscopy with 63x objective. Co-labeling is shown as merge (yellow) and platelet morphology in a transmitted light image. The data are representative of two independent experiments performed using platelets from two different donors with the analysis of 7-10 fields per experiment. **Panel B**. Washed resting platelets were incubated alone (left) or with FVIII (right), stained with the mouse anti-GPVI IgG 9012 and human anti-FVIII IgG BOIIB2, and incubated with the Duolink Plus anti-mouse and Minus anti-human probes and substrate. Each red dot depicts a positive signal in the proximity ligation assay. Data are representative of 3 experiments performed using platelets from 2 different donors.

Figure 3. Binding of FVIII to immobilized GPVI. Panel A. ELISA plates were coated with 13.3 nM GPVIFc or 9 nM recombinant GPVI (R&D systems) and blocked with PBS-3% bovine

serum albumin (BSA). BDD-FVIII (Novoeight®) diluted in blocking buffer was incubated. Bound FVIII was revealed using a biotinylated polyclonal anti-FVIII antibody (SAF8C, 1 µg/ml) and streptavidin-HRP. The binding of FVIII to GPVI is depicted as optical density (OD measured at 492 nm). **Panel B.** BDD-FVIII, FL-FVIII (Kogenate®) and plasma-derived (pdFVIII, Factane®) were pre-incubated alone or with bovine thrombin (1 IU/ml, FIIa) prior to transfer to GPVIFc-coated plates. **Panel C.** Home-made $FVIII^{HSQ}$ and $FVIII^{Y1680C}$ (5 nM) were pre-incubated with varying concentrations of VWF (Wilfactin®, decomplemented at 56°C for 30 min) and transferred to GPVIFc-coated plates. The binding of FVIII to GPVI is depicted as the % of maximum binding in the absence of competitor. All data are representative of 2 to 3 independent experiments (mean±SD of triplicates). **Panel D.** ELISA plates were coated with rGPVI (13.3 nM) in PBS and blocked with PBS-BSA 3%. BDD-FVIII, factor IX (FIX) or human serum albumin (HSA) were co-incubated at different concentrations with rFVIIIFc (20 nM, Eloctate®) in blocking buffer on ELISA plates coated with recombinant GPVI (R&D systems). The residual bound rFVIIIFc was recognized with a goat anti-human Ig Fc conjugated to HRP and OPD substrate. The binding of rFVIIIFc to GPVI is expressed as the percentage of binding as compared to rFVIIIFc incubated alone.

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Figure 1

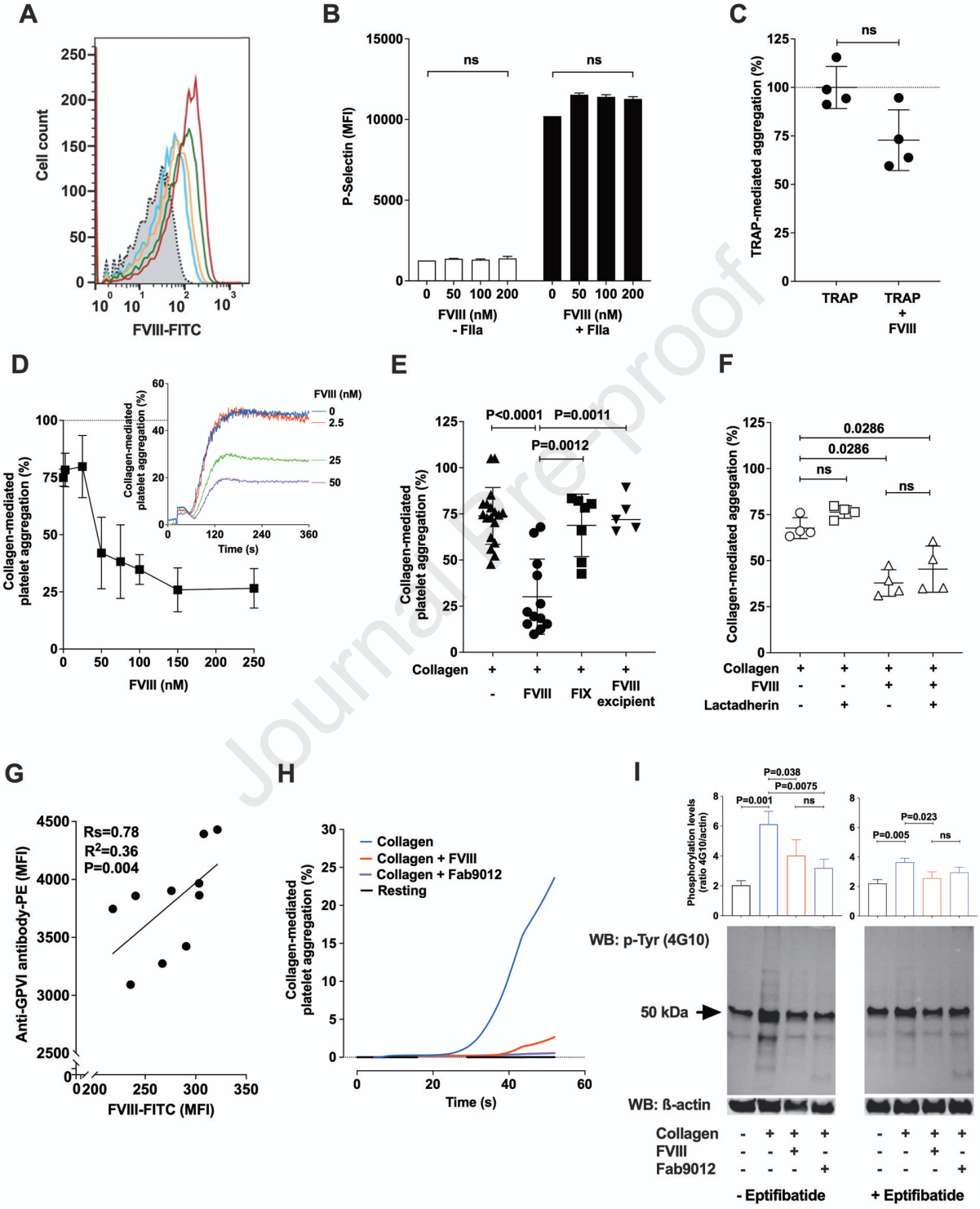

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Figure 2

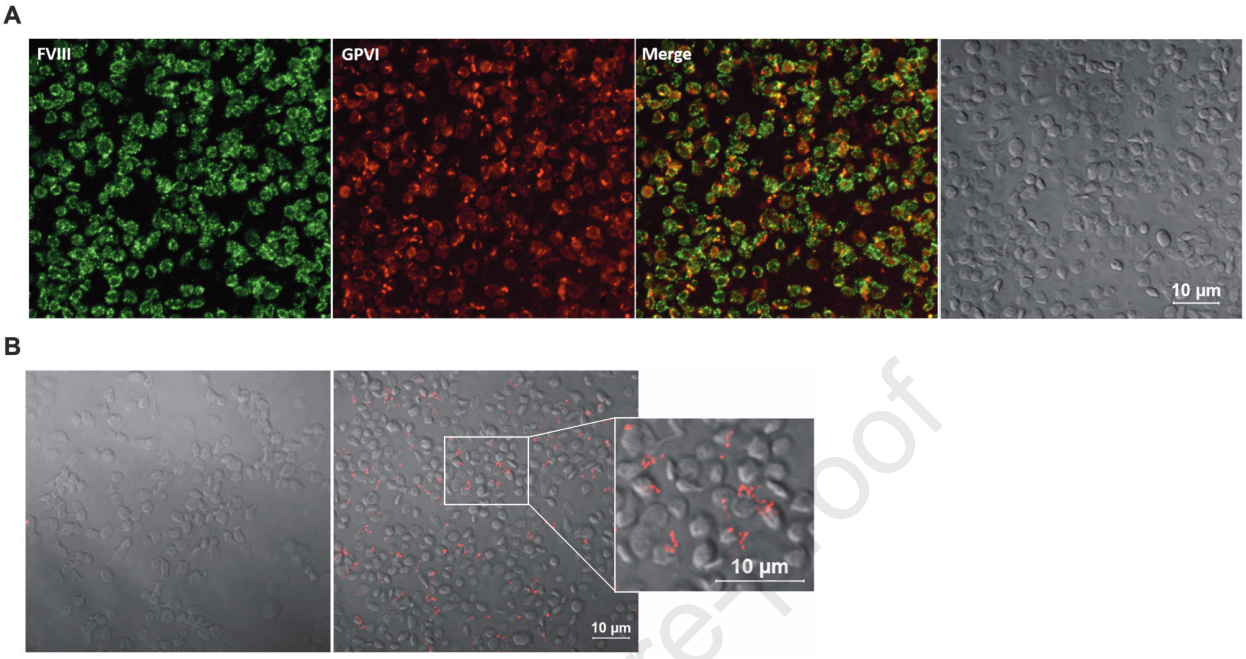

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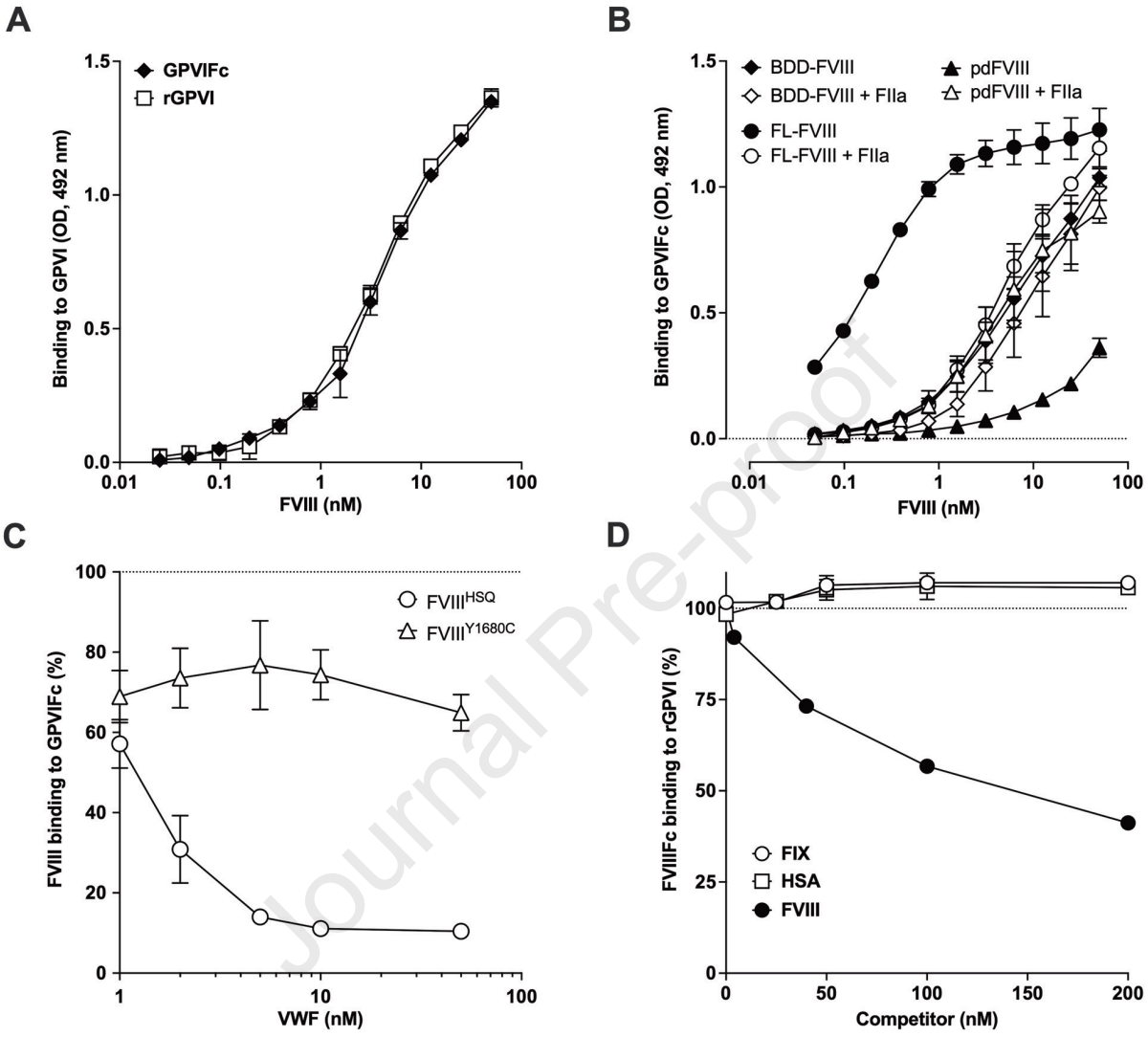
Figure 3



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