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ORIGINAL ARTICLE

Galectin-9 activates platelet ITAM receptors glycoprotein VI and C-type lectin-like receptor-2

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Abstract

Background: Platelets are multifunctional cellular mediators in many physiological and pathophysiological processes such as thrombosis, angiogenesis, and inflammation. Several members of galectins, a family of carbohydrate-binding proteins with a broad range of immunomodulatory actions, have been reported to activate platelets.

Objective: In this study, we investigated the role of galectin-9 (Gal-9) as a novel ligand for platelet glycoprotein VI (GPVI) and C-type lectin-like receptor 2 (CLEC-2).

Methods: Platelet spreading, aggregation, and P-selectin expression in response to Gal-9 were measured in washed platelet suspensions via static adhesion assay, light transmission aggregometry, and flow cytometry, respectively. Solid-phase binding assay and protein phosphorylation studies were utilized to validate the interaction between Gal-9 and GPVI, and immunoprecipitation for detecting CLEC-2 phosphorylation. Wild-type (WT), GPVI-knockout (*Gp6*$^{-/-}$), and GPVI and CLEC-2-double knockout (*Gp6*$^{-/-}$/*Gp1ba-Cre-Clec1b*$^{fl/fl}$) mice were used.

Results: We have shown that recombinant Gal-9 stimulates aggregation in human and mouse washed platelets dose-dependently. Platelets from both species adhere and spread on immobilized Gal-9 and express P-selectin. Gal-9 competitively inhibited the binding of human recombinant D1 and D2 domains of GPVI to collagen. Gal-9 stimulated tyrosine phosphorylation of CLEC-2 and proteins known to lie downstream of GPVI and CLEC-2 including spleen tyrosine kinase and linker of activated T cells in human platelets. GPVI-deficient murine platelets exhibited significantly impaired aggregation in response to Gal-9, which was further abrogated in GPVI and CLEC-2-double-deficient platelets.

Conclusions: We have identified Gal-9 as a novel platelet agonist that induces activation through interaction with GPVI and CLEC-2.

KEYWORDS
C-type lectin-like receptor 2, galectin-9, glycoprotein VI, immunoreceptor tyrosine-based activation motif, platelet

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1 | INTRODUCTION

Platelets, anucleate cytoplasm fragments shed by megakaryocytes, constitute the second most abundant cellular component in human blood following erythrocytes with their classically described role in hemostasis and thrombosis.[1,2] Additionally, the multifunctionality of activated platelets has been proposed to modulate many other physiological processes, for example, wound healing and leukocyte trafficking, and a wide range of pathological diseases such as atherosclerosis, autoimmune diseases, and cancer metastasis.[3,4]

Galectins (Gals) are a family of highly evolutionary-conserved immunoregulatory glycan-binding proteins that have been reported to play a role in autoimmunity, cancer, and numerous cardiovascular diseases.[5–7] To date, 12 members of the galectin family have been identified in humans, most of which are characterized by the capability of binding N-acetyl-lactosamine-based carbohydrate domains of glycans, proteins, and lipids calcium-independently.[8,9]

Over the past decade, several galectins—Gal-1, -3, and -8—have been shown to stimulate various platelet functions *in vitro* such as adhesion, spreading, aggregation, and degranulation, highlighting their potential in platelet biology.[10–12] In this study, we focused on Gal-9, a 34–39 kDa tandem-repeat member composed of a peptide linker with joining N-terminal and C-terminal carbohydrate recognition domains.[13] Since its discovery,[14–17] Gal-9 has been shown to have a positive inflammatory role in certain immune-inflammatory diseases,[13] and to be a therapeutic target in cancer for its role in tumor apoptosis.[7] A recent study reported that the circulating level of Gal-9 in patients with large artery atherosclerotic stroke is significantly higher than in healthy donors.[18] This increase was also observed in patients with stable coronary artery disease; however, lower levels of Gal-9 were detected in the peripheral circulation of patients with non-ST-segment elevation acute coronary syndrome and ST-segment myocardial infarction compared to healthy controls.[19] Collectively these studies highlight an association of Gal-9 with cardiovascular events, in which platelets play a non-negligible role.

Glycoprotein VI (GPVI) is a critical receptor of platelet signalling in hemostasis expressed exclusively in the megakaryocyte lineage,[4,20] while C-type lectin-like receptor 2 (CLEC-2) has been demonstrated to be crucial for the development of cerebrovascular and lymphatic systems as well as the maintenance of post-development vascular integrity, with a comparatively minor contribution to hemostasis.[21,22] Nonetheless, both GPVI and CLEC-2 have been proposed to participate in thrombo-inflammation.[4] Signal transductions of GPVI and CLEC-2 both rely on the presence of the immunoreceptor tyrosine-based activation motif (ITAM).[23] Upon binding to collagen, GPVI clusters[24,25] and transduces the signal via its association with dimeric Fc receptor γ-chain causing a signalling cascade which involves phosphorylation of the Src family kinases (SFKs) and spleen tyrosine kinase (Syk), assembly of a downstream signalosome comprising linker of activated T cells (LAT) and diverse effectors including phospholipase Cγ2, and results in platelet degranulation, inside-out activation of integrins.[4,23] CLEC-2 signals through a similar axis to GPVI, with Syk crosslinking two receptors through a phosphorylated hemITAM sequence in the cytosolic tail.[22] To date, several other ligands for GPVI have been reported, for example, laminin,[26] adiponectin,[27] fibronectin,[28] vitronectin,[29] basigin,[30] fibrin,[31–33] and fibrinogen,[34] whereas only two endogenous ligands, podoplanin[35] and heme/hemin,[36] have been proposed to interact with CLEC-2.

More recently, Gal-3 was demonstrated to be a novel binding partner of GPVI inducing platelet adhesion and ATP release.[12] Interestingly, the N-terminal carbohydrate recognition domain of Gal-9 shares considerable similarities with the structure of Gal-3 carbohydrate recognition domain.[37] Furthermore, endothelial cells have been proposed to be a source of Gal-9,[38,39] with externalization of Gal-9 observed *in vitro* upon endothelial cell activation.[38] Endothelial cells from inflamed tissue and after interferon-γ treatment *in vitro* overexpress Gal-9.[40–42] However, to the best of our knowledge, whether Gal-9 contributes platelet activation remains unclear.

Here, we have expanded on the current knowledge of Gal-9 biology by identifying it as a novel endogenous ligand in the vasculature system, interacting with GPVI and CLEC-2 and inducing activation of human and murine platelets.

Essentials

- Galectin-9 (Gal-9) is a glycan binding protein with a range of immunomodulatory functions.
- Responses of human, wild-type, and genetically modified murine platelets to Gal-9 was assessed.
- Gal-9 was identified as a novel platelet agonist mediating multiple platelet functions.
- Gal-9 activates platelets through interaction with glycoprotein VI and C-type lectin-like receptor 2.

2 | MATERIALS AND METHODS

2.1 | Reagents

Human recombinant galectin-9 (GA-2045) was purchased from R&D Systems, bio-techne, UK. Recombinant human dimeric GPVI-Fc construct (GPVI residues 1-183), its variant GPVI (NQ)-Fc fusion, and the C-terminal human IgG1 Fc segment were generated by the Sigplg^{+} mammalian expression vector (R&D Systems) as previously described.[33,43] Nanobody 2 (Nb2), raised against GPVI through VIB Nanobody core (VIB Nanobody Service Facility), was expressed in pMECS plasmid and purified as formerly reported.[43] Fab fragments of monoclonal anti-GPVI antibody JAQ1 were kindly provided by Prof. Bernhard Nieswandt. Rhodocytin was kindly provided by Dr.

Johannes A. Elbe. Further detailed information on other materials used in the present study can be found in Table S1 in supporting information.

2.2 | Generation of AYP1

The monoclonal anti-human CLEC-2 antibody AYP1 IgG1 was produced from mouse hybridoma cells. The cell culture supernatant was harvested, centrifuged, and filtered to remove cell debris and went through protein G affinity chromatography. After IgG binding, AYP1 was eluted by Tris-glycine buffer and then dialyzed into phosphate buffered saline (PBS). $F(ab)_2$ fragments of AYP1 were made with Pierce Fab preparation kit (Thermo Fisher Scientific). The whole IgG and fragments were then characterized by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) to verify molecular weight and purity. Biological activities of AYP1 whole IgG and $F(ab)_2$ fragments were also verified by CLEC-2 binding and rhodocytin blockade on human platelet aggregation.

2.3 | Animals

All animal experiments were designed and performed in accordance with Home Office regulations under the licence PP9677279. *Gp6*$^{-/-}$ mice on Bl6J background were bred as homozygotes with purchased wild-type (WT) mice from Charles River on the same background as controls. *Gp6*$^{-/-}$/*Gp1ba-Cre-Clec1b*$^{fl/fl}$ (further referred to as *Gp6*$^{-/-}$/*Clec-2*$^{-/-}$) mice were bred as previously described.[21] In line with the ARRIVE guidelines and the principles of 3Rs on the use of minimal number of animals required for experimentation we performed a power calculation prior to our experiments. We used previous data generated from our lab to determine our desired effect size and based on the power calculation a sample size of three to four mice per group was required to achieve a statistical significance of 5%.

2.4 | Preparation of human washed platelet suspension

The present study was approved by the local research ethics committee (QMERC2014/61). Healthy donors who had not taken any anti-platelet medication 10 days prior to donation were included and provided informed written consent in accordance with the Declaration of Helsinki. Human and mouse washed platelets were retrieved from platelet-rich plasma by centrifugation with the presence of prostacyclin (Caymen Chemicals) and resuspended with modified Tyrode's buffer (134 mM NaCl, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 12 mM $NaHCO_3$, 20 mM HEPES, 1 mM $MgCl_2$, 5 mM glucose; pH 7.3) as previously described.[31,44] Before being tested, freshly washed platelets were left for 30 min.

2.5 | Light transmission aggregometry

Aggregation was assessed in human and murine washed platelet suspensions (2×10^8/ml) via a Born Aggregometer (Model 700, CHRONO-LOG) under the stirring condition of 1200 rpm at 37°C. Human washed platelets were prewarmed for 5 min (2 min for mice) at 37°C before measurement and left being stirred for 1 min on the aggregometer before agonist stimulation. In some conditions, pretreatment with PP2 (Selleck Chemical), PRT (SYNkinase), Nb2, JAQ1 Fab, and AYP1 $F(ab)_2$ were carried out for 10 min, while apyrase (Sigma) and indomethacin (Sigma) were added 5 min before agonist addition. Light transmission of platelets was monitored for 6 min after addition of agonists. Aggregation traces together with their maximal amplitude (Amax) and area under the curve (AUC) were recorded and calculated on Aggrolink 8 (CHRONO-LOG).

2.6 | Flow cytometry

Washed platelets (2×10^7/ml) were stimulated with vehicle, 300 nM Gal-9 (R&D Systems, Bio-techne) or 10 μg/ml collagen-related peptide (CRP; CambCol Laboratories) and incubated with fluorescein isothiocyanate (FITC)-conjugated α-human CD41/CD61 antibody (clone: PAC-1; BioLegend) and phycoerythrin (PE)-conjugated α-human CD62P antibody (BioLegend), or with PE-conjugated α-mouse CD41/CD61 antibody (clone: JON/A; Emfret Analytics) and FITC-conjugated α-mouse CD62P antibody (Emfret Analytics) for 30 min at 37°C. The platelets were then fixed with 10% neutral buffered formalin (CellPath Ltd.) for 20 min at room temperature (RT) after incubating with the antibodies. Samples were assessed on BD Accuri C6 Plus flow cytometer (BD Biosciences). Histograms were plotted on FlowJo version 10.0.7.

2.7 | Platelet spreading assay

Following incubation with 600 nM human recombinant Gal-9 or 10 μg/ml Horm collagen (Takeda Austria) at 4°C overnight, coverslips (VWR) were blocked in 5 mg/ml fatty acid–free and heat-denatured bovine serum albumin (BSA) in PBS. Pre-treatments with PP2 (Selleck Chemicals) and PRT (SYNkinase) in washed platelet (2×10^7/ml) in some conditions were carried out for 10 min at 37°C. After washing, the coverslips were incubated with washed platelets for 30 min (human specimens) or 45 min (mouse specimens) at 37°C and then washed again with PBS. Adherent platelets on the coverslips were fixed with 10% neutral formalin (CellPath) for 10 min, incubated for 10 min in 50 mM ammonium chloride, permeabilized for 5 min using 0.1% Triton X-100, and stained with Alexa Fluro® 488-Phalloidin (Cell Signaling) for 45 min at RT. Each of the former four processes was followed by three wash steps with PBS. Coverslips were mounted on slides (VWR) with Hydromount (National Diagnostics). Digital images of platelet spreading were taken under Axio Observer 7 inverted

epifluorescence microscope (×630; Carl Zeiss Microscopy) and Zen 2.3 Pro software. Analysis of spreading parameters was carried out with the open-source platforms KNIME[45] version 4.0.1 and ilastik[46] version 1.3.2 as described by Pike et al.[47]

2.8 | Avidity-based extracellular interaction screening assay

GPVI and other bait proteins were incubated in streptavidin-coated 96-well microtiter plates (Nunc) for 1 h at 22°C. The bait protein-coated plate was incubated with recombinant human Gal-9 for 1 h at 22°C. The bound Gal-9 was detected with goat anti-human Gal-9 antibody (R&D Systems) followed by horseradish peroxidase (HRP)-conjugated anti-goat IgG (Invitrogen). Three wash steps were performed between each incubation using 0.1% (v/v) PBS-Tween 20. After addition of 1-Step™ Slow TMB substrate (Thermo Fisher Scientific) and incubated for 1 h at 22°C, the reaction was stopped by addition of a sulfuric or phosphoric acid. Absorbance was measured at 485 nm on a VersaMax microplate reader (Molecular Devices).

2.9 | Solid-phase binding assay

Both direct and competitive methods were carried out in the present study. During the competitive binding assay, 96-well microplates were coated in the presence of 4 μg/ml Horm collagen overnight at 4°C. After blocking the collagen-coated wells with fatty acid–free and heat-denatured 3% (w/v) BSA for 1 h, plates were washed and human recombinant Gal-9 at different concentrations (10–3000 nM), together with 25 nM recombinant human dimeric GPVI-Fc fusion were added and incubated at RT. In the direct binding assay, the plates were coated in the presence of 10 nM Gal-9, blocked, and incubated with recombinant GPVI-Fc, GPVI (NQ)-Fc, and Fc segment at the same concentrations from 10 to 3000 nM, respectively. Wells were then labelled with HRP-conjugated anti-human IgG antibody (Invitrogen) for 1 hour at RT. After washing, 3, 3′, 5, 5′-tetramethylbenzidine liquid substrate (Sigma-Aldrich) was added for 30 min at RT before the addition of the stop solution (1M H_2SO_4). Absorbance at 450 nm was measured with a VersaMax microplate reader (Molecular Devices).

2.10 | Protein phosphorylation

Human washed platelet suspension (4×10^8/ml) pre-incubated with 9 μM eptifibatide was stimulated with 300 nM Gal-9 or 10 μg/ml CRP for 5 min under 1200 rpm stirring condition at 37°C, in some conditions pre-treatment with 20 μM PP2 or 10 μM PRT were carried out. Protein lysates were subsequently obtained by the addition of SDS sample buffer being further stirred for 30 s and then boiled for 5 min. Before being transferred onto polyvinylidene fluoride membranes (Bio-Rad), proteins in the lysates were separated via SDS-PAGE on 4%–12% (w/v) pre-cast Bis-Tris gels (NuPAGE, Invitrogen) under reducing conditions. Following transfer, the membranes were blocked by 4% BSA for 1 h and incubated with anti-phosphotyrosine (clone 4G10; Millipore), phospho-Syk (Tyr 525/526; Cell Signaling), and phospho-LAT (Tyr 200; Abcam) antibodies at 4°C overnight, respectively. Species-relevant HRP-conjugated secondary antibodies were added to membranes for 1 h at RT. All incubations were carried out on a shaker and with three washing intervals between each incubation. Membranes were developed by an enhanced chemiluminescence detection system (Thermo Scientific), and re-probed using pan-anti-Syk antibody (Santa Cruz) and pan-anti-LAT antibody (Millipore). Western blots were all imaged with autoradiographic film.

2.11 | Immunoprecipitation

Human washed platelets (4×10^8/ml) in the presence of 9 μM eptifibatide were stimulated by 300 nM Gal-9 for 5 min 1200 rpm stirring condition at 37°C and subsequently lysed with equal volume of 2× Nonidet P-40 Extraction buffer (20 mM Tris-HCl, 300 mM NaCl, 2 mM EGTA, 2 mM EDTA, 2% Nonidet P-40, 2 mM AEBSF, 5 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin; pH 7.5). The whole lysates were precleaned with GammaBind™ Plus Sepharose™ (GE Healthcare). Immunoprecipitation was carried out by incubating the cleaned lysates with 2 μg/ml of AYP1 whole IgG for 30 min and GammaBind Plus Sepharose for 1 h at 4°C, and cooling centrifugation at 16 000 g for 15 min. Precipitated proteins were resuspended with SDS sample buffer, separated by SDS-PAGE, western blotted against anti-phosphotyrosine antibody (clone 4G10; Millipore), and re-probed with pan-anti-CLEC-2 antibody (R&D Systems) as described above.

2.12 | Statistical analysis

The data are presented as mean ± standard deviation, unless otherwise stated. Student's *t*-test was used for the comparison between two groups, while multiple comparisons were conducted via one-way analysis of variance with Tukey's or Dunnett's post hoc test. *P*-values <.05 were considered significant ($^*P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$). Logarithmic dose-response/inhibition curves were generated through four-parameter nonlinear regression analysis with variable slopes. All statistics were analyzed on Prism version 8.0.2 (GraphPad Software).

3 | RESULTS

3.1 | Galectin-9 induces aggregation and secretion in human washed platelets

To validate whether Gal-9 has a role in platelet activation, we first investigated its effects on platelet aggregation. Recombinant human Gal-9 induced aggregation of human washed platelets in a

dose-dependent manner (Figure 1Ai). Gal-9 had an EC_{50} of ~124 nM and stimulated maximal platelet aggregation at 300 nM (Figure 1Aii). From 100 to 300 nM, Gal-9 mediated prominent biphasic aggregation in human washed platelets, suggesting participation of feedback agonists (Figure 1Ai), which is further corroborated by the substantial reduction in Gal-9-induced aggregation following administration of ADP scavenger enzyme apyrase and cyclo-oxygenase inhibitor indomethacin (Figure S1 in supporting information). The GPIIb/IIIa antagonist eptifibatide blocked platelet aggregation to Gal-9 at 300 nM (Figure 1B), indicating active aggregation rather than platelet agglutination. A similar blockade pattern was also observed following CRP-induced platelet aggregation (Figure S2 in supporting information). Platelets stimulated by Gal-9 (300 nM) showed a significant increase in the percentage of GPIIb/IIIa activation assessed using CD41/CD61 antibody (PAC-1; Figure 1Cii), and an increasing trend in medium fluorescent intensity (Figure 1Ciii; $P = .06$). Gal-9 also induced P-selectin expression on the surface of human platelets assessed by flow cytometry (Figure 1D), indicating release of α-granule contents. Platelets stimulated with CRP were used as positive control (Figure 1C-D).

3.2 | Gal-9 mediates human platelet aggregation and spreading via an ITAM-based signalling pathway

Collectively, the data presented here indicate that Gal-9 can activate human platelets. We subsequently sought to identify signalling pathways involved in this interaction. The effect of Gal-9 on human platelet aggregation was blocked by 25 mM lactose but not by sucrose at the same concentration (Figure 2A), consistent with lactose binding to Gal-9.[13] As a control, lactose (25 mM) had no effect on aggregation to a collagen mimetic, CRP (Figure S3 in supporting information). This result demonstrates that β-galactoside binding is essential for Gal-9–induced platelet aggregation. Administration of SFK inhibitor (PP2) and Syk inhibitor (PRT) substantially blocked platelet aggregation in response to Gal-9 (Figure 2B). We next measured tyrosine phosphorylation of Syk and LAT, which are downstream of the ITAM-based signalling pathway, in human platelet lysates following stimulation with Gal-9 (Figure 2C). Gal-9 induced a similar pattern of tyrosine phosphorylation to CRP, with an increase in both phospho-Syk and phospho-LAT. PP2 and PRT significantly reduced the signals of phospho-Syk and phospho-LAT triggered by Gal-9 (Figure 2C). Moreover, spreading of human platelets on immobilized Gal-9 (Figure S4 in supporting information) was substantially inhibited by pre-treatment with PP2 and PRT compared to the vehicle (Figure 3B). These results illustrate that Gal-9 activates human platelets through an ITAM receptor–based pathway.

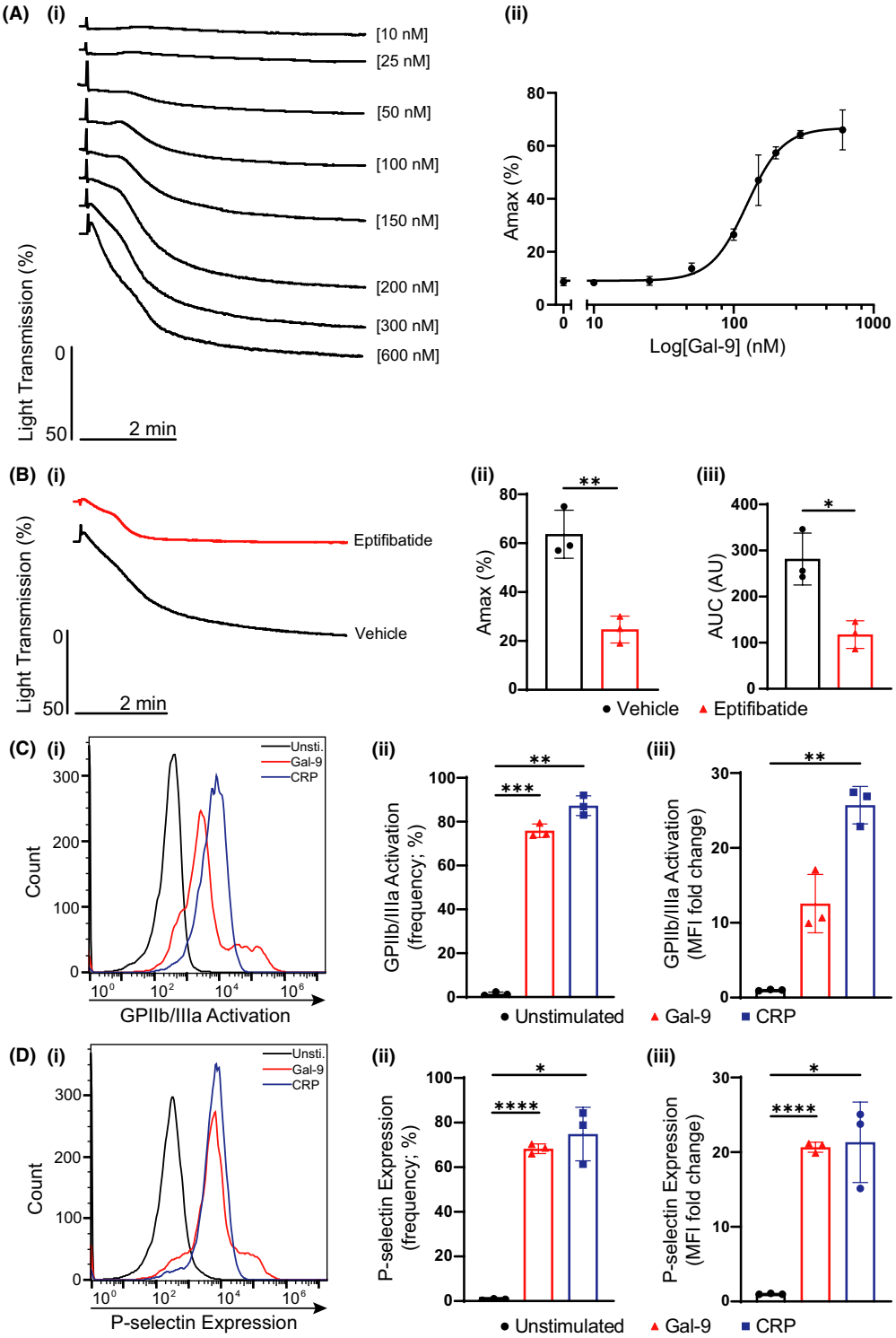
3.3 | Activation of human platelets by Gal-9 is partially blocked by antibodies to GPVI

To identify potential counter-receptors of Gal-9 on human platelets, we used the Avidity-based Extracellular Interaction Screening Assay,[48] and found the platelet collagen receptor GPVI as a significant binding partner of Gal-9 *in vitro* (Figure S5 in supporting information). Gal-9 competitively inhibited the binding of recombinant dimeric GPVI-Fc (25 nM) to a Horm collagen (10 nM)–coated surface in the solid-phase binding assay, with an approximate IC_{50} of 220 nM (Figure 4Ai), suggesting that the Gal-9 binding site may overlap with the collagen binding site found within D1. No prominent difference in the binding of Gal-9 (10 nM) to native GPVI-Fc ($EC_{50} \approx 153$ nM) and its un-glycosylated variant GPVI (NQ)-Fc ($EC_{50} \approx 257$ nM) was observed in the direct binding assay (Figure 4Aii), which indicates a protein–protein interaction between Gal-9 and GPVI. The Fc segment of GPVI constructs was also independently expressed and tested in the binding assay as a control; it did not exhibit binding affinity for Gal-9 (Figure 4Aii).

To further study the interaction between Gal-9 and GPVI, we tested whether the aggregation response caused by recombinant human Gal-9 was interfered with by the anti-GPVI blocking biologics, Nb2[43] and Fab fragments of the monoclonal antibody JAQ1, both of which completely blocked CRP-mediated platelet aggregation (Figure S6A,B in supporting information). JAQ1 Fab but not Nb2 partially attenuated the aggregation triggered by Gal-9 (Figure 4B,C and Figure S7), implying that platelet activation by Gal-9 is partially mediated by GPVI but at a site that is distinct from the binding site of Nb2. The partial inhibition suggests that a secondary receptor that also signals though Src and Syk tyrosine kinases may also contribute to activation. Human platelets express two other ITAM receptors, FcγRIIA and CLEC-2.[23] Hence, the washed platelets were also pre-treated with AYP1 $F(ab)_2$, dimeric Fab fragments of the monoclonal antibody AYP1 against CLEC-2, which completely abolishes the effect of rhodocytin on platelet aggregation (Figure S8 in supporting information). Administration of AYP1 $F(ab)_2$ did not induce a significant reduction in aggregation triggered by Gal-9 compared to vehicle (Figure 4Cii, $P = .92$; Figure 4Ciii, $P = .92$). Combined treatment with JAQ1 Fab and AYP1 $F(ab)_2$ also showed no significant difference from JAQ1 Fab alone (Figure 4Cii, $P = .63$; Figure 4Ciii, $P = .80$). However, phosphorylation of CLEC-2 induced by Gal-9 was detected in platelet lysates (Figure 4D). Hence, the lack of effect of AYP1 could be due to Gal-9 binding a distinct site from which it interacts with rhodocytin and podoplanin.

3.4 | Galectin-9 induces mouse platelet activation through GPVI and CLEC-2

To further address the interaction between Gal-9 and platelet ITAM receptors GPVI and CLEC-2, we employed genetically deficient mouse strains including *Gp6*$^{-/-}$ and *Gp6*$^{-/-}$/ *Clec-2*$^{-/-}$. Similar to human platelets, Gal-9 stimulated WT platelet aggregation (Figure 5A), the conformational change in GPIIb/IIIa to the activated form of the integrin (Figure 5B) and expression of P-selectin (Figure 5C). WT platelets also adhered and spread on recombinant Gal-9–coated surfaces under static conditions compared to BSA (Figure S9 in supporting information). Pre-treatment of WT



platelets with SFK (PP2) and Syk (PRT) inhibitors completely inhibited Gal-9–induced aggregation (Figure 6A) and spreading (Figure 6B). Most WT platelets fully spread on immobilized Gal-9 being characterized by formation of lamellipodial sheets and actin stress fibers (Figure 6Bi–ii). PRT significantly reduced the percentage of fully spread platelets (Figure 6Bii) and mean coverage area of WT platelets (Figure 6Biii) on immobilized Gal-9. PP2-treated platelets also showed a decreasing trend in the proportion of fully spread platelets (Figure 6Bii; $P = .18$) and mean platelet area (Figure 6Biii; $P = .14$). A much higher proportion of platelets underwent partial spreading and formed filopodia and actin nodes on Gal-9 in both inhibitor-treated groups (Figure 6Bi–ii). The results demonstrate that WT platelet spreading, but not adhesion (Figure 6Biv), on immobilized Gal-9 is dependent upon ITAM receptor signalling.

Gp6$^{-/-}$ platelets showed significantly impaired aggregation in response to Gal-9 compared to WT controls (Figure 7A). Consistent

FIGURE 1 Galectin-9 (Gal-9) stimulates platelet aggregation and secretion. A, The dose-dependent effect of Gal-9 on human platelet aggregation. Ai, Representative aggregation traces. Aii, The concentration-response curve of Gal-9 in platelet aggregation ($EC_{50} \approx 124$ nM; $n = 3$). Data are presented as mean ± standard deviation (SD). B, The effect of eptifibatide (9 μM) on human platelet activation triggered by Gal-9 (300 nM). Bi, Representative aggregation traces. Bii, Maximal amplitude (Amax) and (Bii) area under the curve (AUC) of traces in eptifibatide-treated groups compared with the vehicle (phosphate-buffered saline) control ($n = 3$). Mean ± SD $^{*}P < .05$, $^{**}P < .01$, by paired Student's *t*-test. C, Gal-9 stimulates activation of glycoprotein (GP)IIb/IIIa in human platelets. Ci, Representative histogram of human platelets activated by collagen-related peptide (CRP; 10 μg/ml) and Gal-9 (300 nM), respectively. Cii, Frequency and (Ciii) medium fluorescent intensity (MFI) of Gal-9 or CRP-stimulated platelets labelled by fluorescein isothiocyanate-conjugated human CD41/CD61 antibody (PAC-1) compared to the unstimulated platelets ($n = 3$). Mean ± SD $^{**}P < .01$, by one-way analysis of variance (ANOVA) with Dunnett's post hoc test. D, Gal-9 (300 nM) triggers P-selectin expression in human platelets. Di, Representative histogram of human platelets stimulated by CRP (3 μg/ml) and Gal-9 (300 nM), respectively. Comparison between Gal-9 or CRP-stimulated and unstimulated platelets in terms of percentages (Dii) and MFI (Diii) of phycoeyrthrin-conjugated human CD62P antibody labelling ($n = 3$). Mean ± SD $^{*}P < .05$, $^{***}P < .001$, by one-way ANOVA with Dunnett's post hoc test

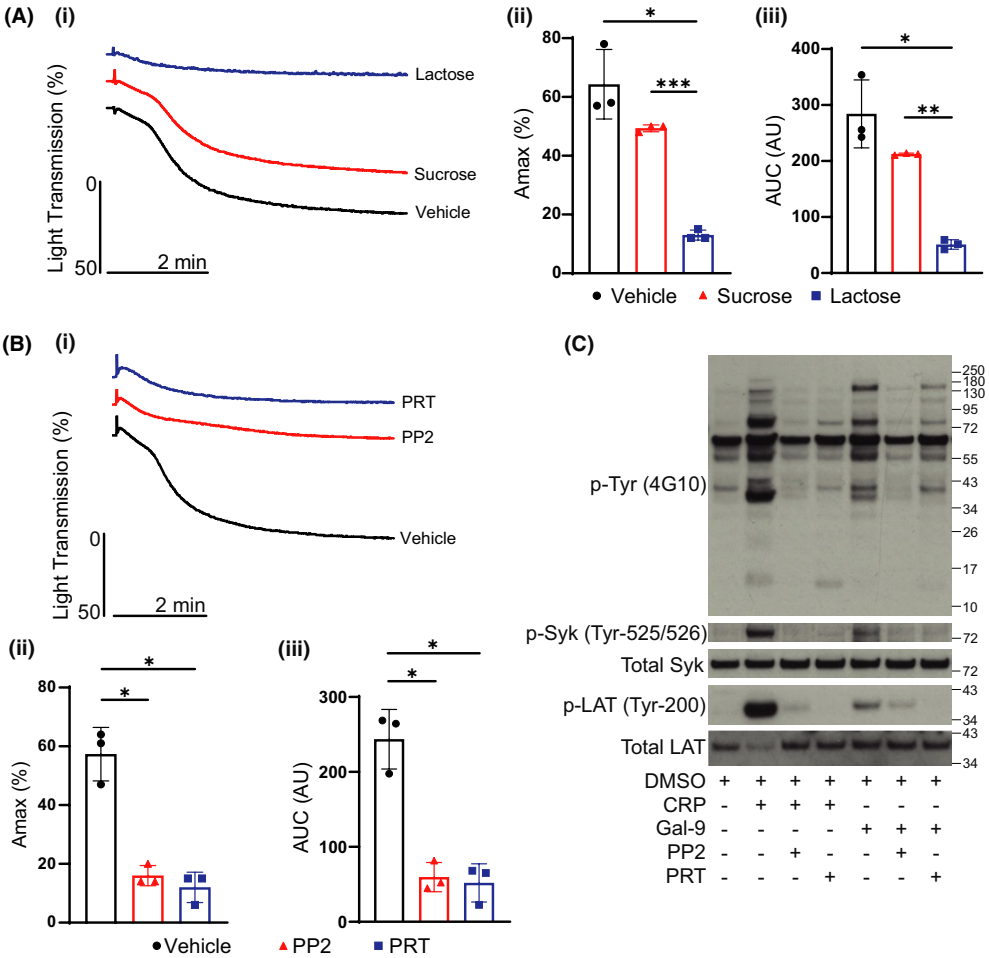


FIGURE 2 Characterization of binding and signalling of galectin-9 (Gal-9)–induced platelet activation. A, Platelet aggregation to Gal-9 (300 nM) in the presence of sucrose (25 mM) or lactose (25 mM). Ai, Representative aggregation traces. Aii, Maximal amplitude (Amax) and (Aiii) area under the curve (AUC) of traces in the vehicle (modified Tyrode's buffer), sucrose, and lactose group were quantified for comparison ($n = 3$). Mean ± standard deviation (SD) $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, by one-way analysis of variance (ANOVA) with Tukey's post hoc test. B, Gal-9 (300 nM)–mediated human platelet aggregation under PP2 (20 μM) and PRT-060318 (10 μM) treatment. Bi, Representative aggregation traces. Bii, Amax and (Biii) AUC of the traces in vehicle (1% DMSO), PP2 and PRT-060318 treated groups were compared ($n = 3$). Mean ± SD $^{*}P < .05$, by one-way ANOVA with Dunnett's post hoc test. C, Protein phosphorylation induced by Gal-9 (300 nM) with or without PP2 (20 μM) and PRT-060318 (10 μM) pre-treatment. The data are representative of three experiments

with the results of human platelets, aggregation was not completely blocked by GPVI deficiency (Figure 7Aii–iii), which implies that GPVI is not the only receptor involved in the activation of murine platelets mediated by Gal-9. We therefore investigated platelet activation in mice double deficient in GPVI and the second ITAM receptor, CLEC-2. Murine *Gp6*$^{-/-}$/*Clec-2*$^{-/-}$platelets exhibited an augmented

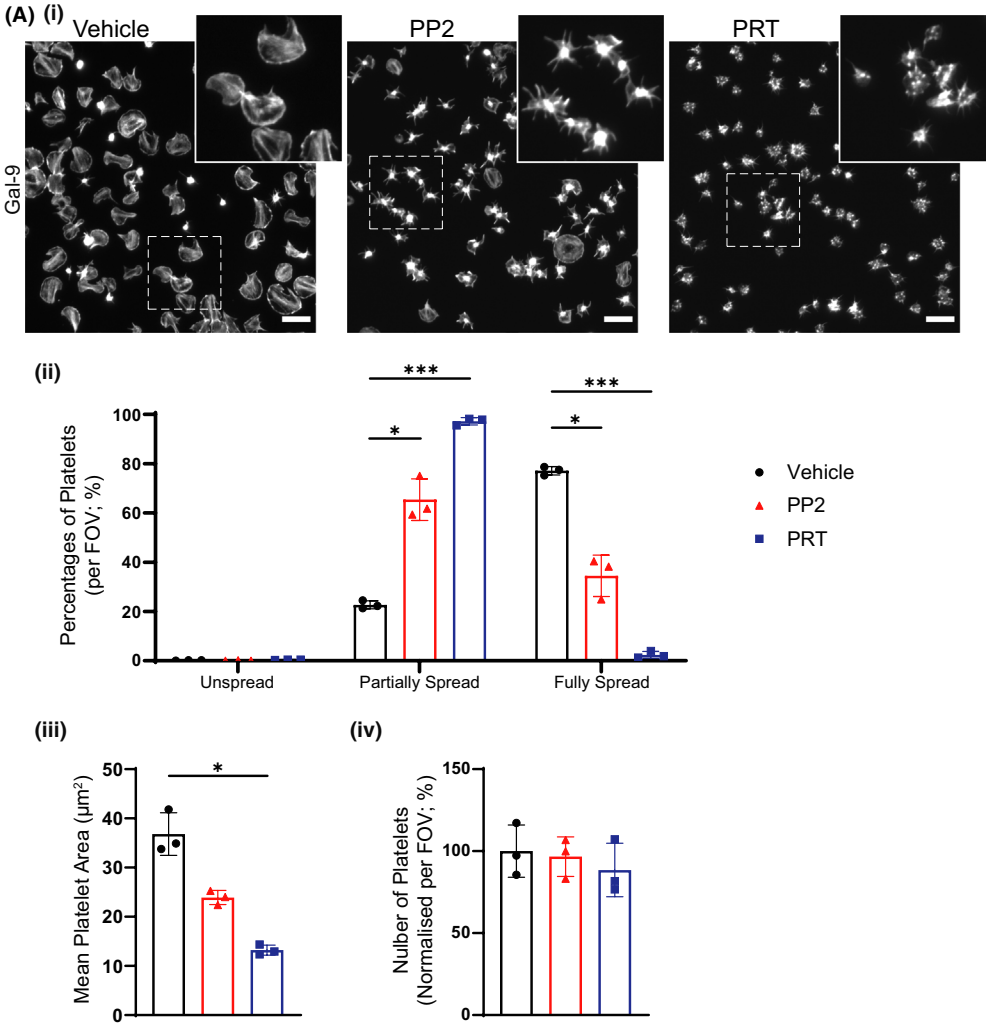


FIGURE 3 Human platelet spreading on immobilized galectin-9 (Gal-9). A, Influence of vehicle (1% DMSO), PP2 (20 μM), and PRT (10 μM) on human platelet spreading mediated by immobilized Gal-9. Ai, Representative images of phalloidin-Alexa488 stained platelets for each treatment. Scale bar = 10 μm. Aii, Quantitation of platelet types (un-spread, partially, and fully spread) in each treatment. Aiii, Mean platelet area and (Aiv) number of adherent platelets in each group were also compared. Mean ± standard deviation $^{*}P < .05$, $^{***}P < .001$, by one-way analysis of variance with Dunnett's post hoc test, five fields of view (FOV) from $n = 3$ experiments were analyzed

reduction in aggregation to Gal-9, compared to platelets from *Gp6*$^{-/-}$ mice (Figure 7Aii–iii). Furthermore, the capability of mouse platelets to develop lamellipodial sheets and actin stress fibers on immobilized Gal-9 was significantly impaired by GPVI deficiency; however, we did not observe any further difference between *Gp6*$^{-/-}$ and *Gp6*$^{-/-}$/*Clec-2*$^{-/-}$ groups (Figure 7Bi–ii). Significant proportions of *Gp6*$^{-/-}$ and *Gp6*$^{-/-}$/*Clec-2*$^{-/-}$ platelets partially spread on Gal-9–coated surfaces showing filopodia and actin nodes (Figure 7Bii). Mean platelet coverage area of *Gp6*$^{-/-}$ and *Gp6*$^{-/-}$/*Clec-2*$^{-/-}$ groups substantially decreased, while GPVI and CLEC-2 double deficiency failed to further amplify this reduction (Figure 7Biii). Intriguingly, we did not observe significant differences in the numbers of platelets adhered between the three genotypes (Figure 7Biv), which indicates that additional receptor types may be involved in platelet adhesion on immobilized Gal-9 under static conditions, while platelet spreading on Gal-9 is mainly supported by GPVI. A similar result was also found with collagen whereby GPVI deficiency inhibited platelet spreading, but adhesion remained similar between WT and *Gp6*$^{-/-}$ (Figure S10 in supporting information). These results suggest that activation of mouse platelets by Gal-9 is predominantly by GPVI with a secondary role of CLEC-2.

4 | DISCUSSION

Here, we show a novel function for Gal-9 as a platelet agonist that activates various platelet functions via interaction with the platelet ITAM receptor GPVI and CLEC-2. Gal-9 was observed to promote human and murine platelet aggregation in a dose-dependent manner. The substantially blocked aggregation to Gal-9 by eptifibatide and detection of fibrinogen-binding site exposure on GPIIb/IIIa upon the stimulation of Gal-9 confirmed the response as metabolically active aggregation, rather than passive agglutination. Administration of apyrase and indomethacin further corroborated the participation

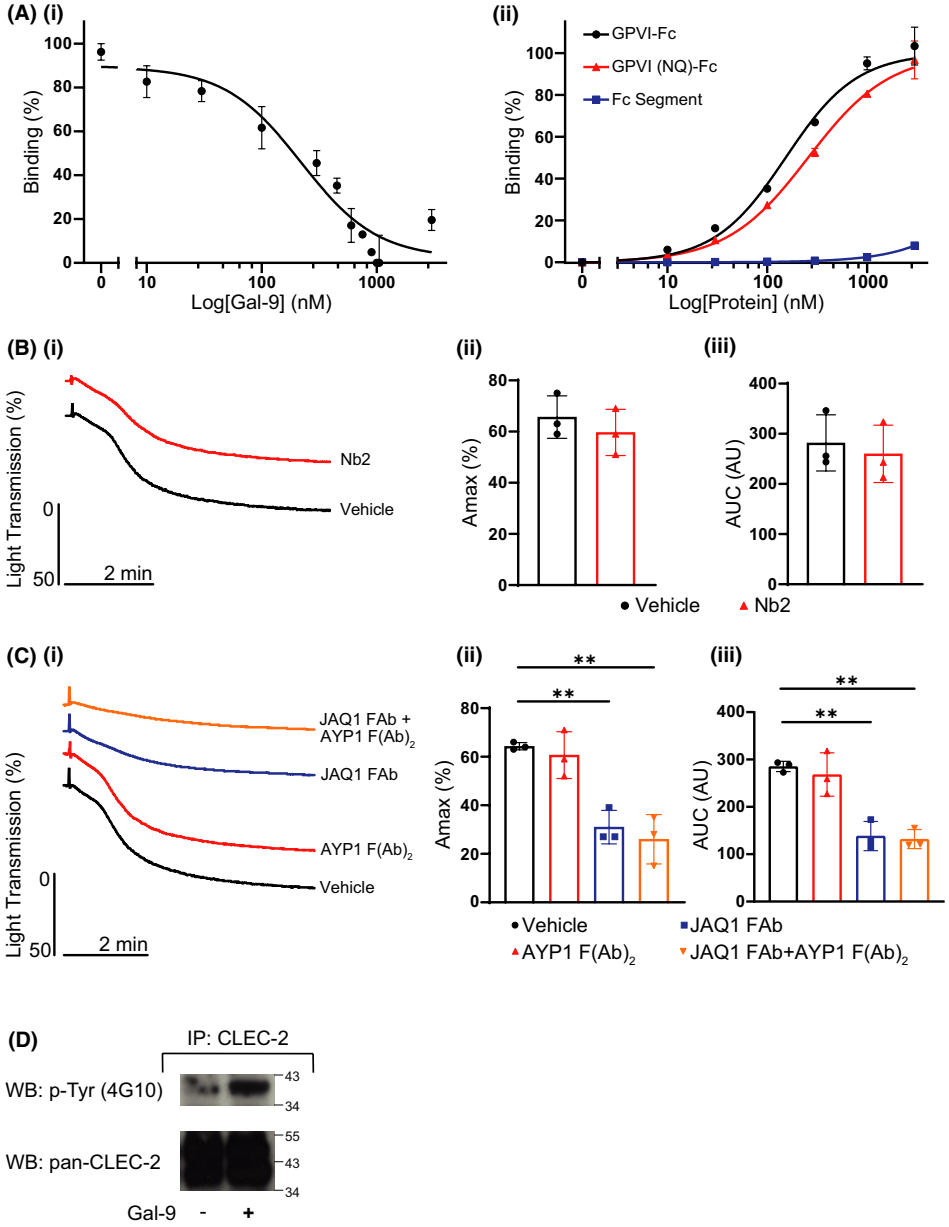


FIGURE 4 Galectin-9 (Gal-9) activates glycoprotein VI (GPVI) and C-type lectin-like receptor 2 (CLEC-2) on human platelets. Ai, The competitive binding assay shows recombinant human GPVI-Fc dimer (25 nM) is displaced from a collagen-coated surface (coated in the presence of collagen [10 nM]) by increasing concentrations of Gal-9 (IC_{50} ≈ 220 nM; $n = 3$ experiments). Mean ± standard error of the mean. Aii, The dose-binding curves of GPVI-Fc (EC_{50} ≈ 153 nM), GPVI (NQ)-Fc (EC_{50} ≈ 257 nM), and Fc segment on Gal-9-coated surface (coated in the presence of 10 nM Gal-9). The data of one experiment in triplicate are presented as mean ± standard deviation (SD). B, The effect of nanobody 2 (Nb2) on Gal-9 (300 nM)-mediated platelet aggregation. Bi, Representative aggregation traces. Bii; Biii, Traces of Nb2 (100 nM) and vehicle (phosphate-buffered saline [PBS]) treatment were quantified and compared ($n = 3$). Mean ± SD by paired Student's *t*-test. C, The effect of 10 μg/ml JAQ1 Fab and/or 10 μg/ml AYP1 Fab_2 on human platelet aggregation induced by 300 nM Gal-9. Ci, Representative aggregation traces. Multiple comparisons in (Cii) maximal amplitude (Amax) and (Ciii) area under the curve (AUC) among vehicle (PBS), JAQ1 Fab, AYP1 Fab_2, and JAQ1 Fab + AYP1 Fab_2-treated groups ($n = 3$). Mean ± SD $^{**}P < .01$, by one-way analysis of variance with Tukey's post hoc test. D, Gal-9 induced CLEC-2 phosphorylation in platelet lysates. The data are representative of three experiments

of secondary mediators, ADP and thromboxane A_2 in Gal-9-induced aggregation in human platelets, which is consistent with most classic platelet agonists.[49,50] Immobilized Gal-9 also supported human and murine platelet adhesion and spreading under static conditions. P-selectin expression on both human and murine washed platelets was induced by Gal-9, which is consistent with similar results reported with Gal-1, -3, and -8.[10–12] Altogether, our results support a novel role of Gal-9 that mediates platelet aggregation, adhesion, spreading, and secretion.

In the past decade, the role of galectins in platelet biology has been expanding.[10–12,51–54] Gal-1 was first reported to interact with the GPIIb subunit of GPIIb/IIIa initiating outside-in signals

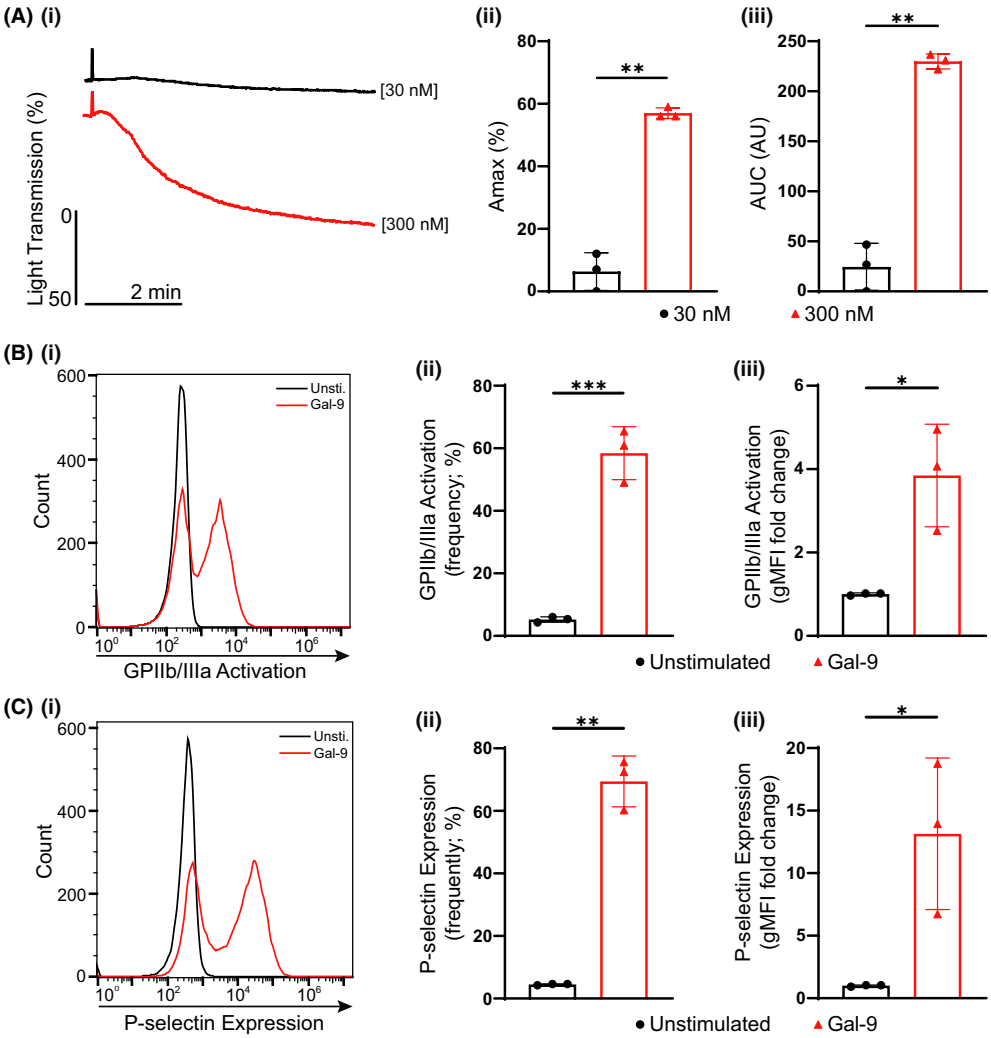


FIGURE 5 Galectin-9 (Gal-9) stimulates mouse platelet activation. A, Gal-9 in murine platelet aggregation. Comparisons between aggregation induced by 30 nM and 300 nM Gal-9 based on (Aii) maximal amplitude (Amax) and (Aiii) area under the curve (AUC) of each trace ($n = 3$ mice per group). Mean ± standard deviation (SD) ***$P < .001$, by paired Student's *t*-test. B, Conformational change of glycoprotein (GP)IIb/IIIa on murine platelets mediated by Gal-9 (300 nM). Bi, Representative histogram of GPIIb/IIIa activation. Significant differences between Gal-9–stimulated and -unstimulated groups in the (Bii) frequency and (Biii) geometric mean fluorescence intensity (gMFI) of murine platelets labelled with phycoeyrthrin-conjugated mouse CD41/CD61 antibody (JON/A; n = 3 mice). Mean ± SD *$P < .05$, ***$P < .001$, by paired Student's *t*-test. C, P-selectin expression on murine platelets induced by Gal-9 (300 nM). Ci, Representative histogram of P-selectin expression. Comparison between Gal-9-stimulated and unstimulated groups in terms of the (Cii) frequency and gMFI of murine platelets labelled by fluorescein isothiocyanate-conjugated mouse CD62P antibody ($n = 3$ mice). Mean ± SD **$P < .01$, by paired Student's *t*-test

and activating human platelets in both soluble and immobilized forms,[10,52] while GPIb was proposed to be the essential counter-receptor for exogenous Gal-8 signalling in human platelet activation *in vitro*.[11] Endogenous Gal-8 expressed on megakaryocyte surface was demonstrated to support factor V endocytosis.[55] Furthermore, cancer cell–derived Gal-3 has been found to be a novel ligand for platelet GPVI facilitating tumor metastasis,[12,56] although whether Gal-3-GPVI interaction could contribute to hemostasis or thrombosis remains unknown.

The effect of Gal-9 on platelet aggregation and spreading implies its potential contribution to thrombus formation. In addition, the interplay between P-selectin expressed on platelets in response to Gal-9 and P-selectin glycoprotein ligand-1 expressed on leukocytes potentially facilitates platelet–leukocyte heterotypic aggregation playing a central role in platelet-mediated leukocyte recruitment and inflammation.[3,57] Thus, apart from directly mediating leukocyte chemoattraction and trafficking,[58] Gal-9 could enhance leukocyte recruitment via activating platelets, which is a novel mode of action for this protein.

Gal-9 has been shown to be widely expressed by endothelial cells in a variety of tissues[38,39] and overexpressed by endothelial cells following activation by pro-inflammatory cytokines such as INF-γ,[41,42] which could provide a potential substrate for platelets. A recent study reported the elevation of plasma Gal-9 in COVID-19 patients, up to 60 times more than in healthy controls,

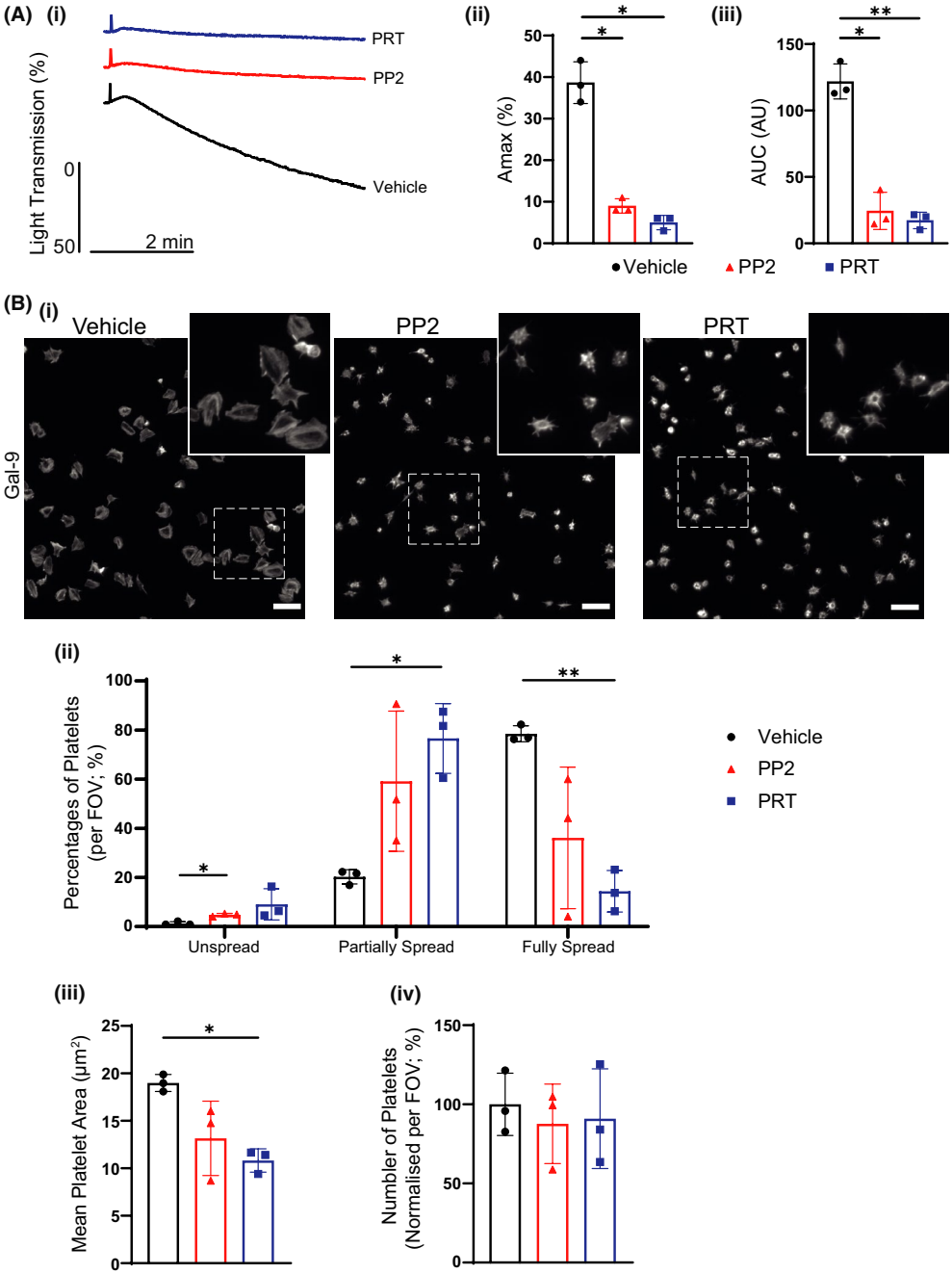


FIGURE 6 Mouse platelet spreading on galectin-9 (Gal-9). A, The influence of PP2 (20 μM) and PRT (10 μM) on mouse platelet aggregation and spreading induced by Gal-9 (300 nM). Ai, Example aggregation traces. Aii, Maximal amplitude (Amax) and (Aiii) area under the curve (AUC) of vehicle (1% DMSO), PP2, and PRT-treated groups were compared ($n = 3$ mice). Mean ± standard deviation (SD) $^{*}P < .05$, $^{**}P < .01$, one-way analysis of variance (ANOVA) with Tukey's post hoc test. B, Murine platelet spreading on immobilized Gal-9 in the presence of PP2 (20 μM) and PRT (10 μM). Bi, Representative images of phalloidin-Alexa488 stained platelets. Scale bar =10 μm. Bii, Percentages of un-spread, partially (vehicle vs. PRT, $P = .21$), and fully spread (vehicle vs. PRT, $P = .18$) platelets in vehicle (1% DMSO), PP2, and PRT treated groups were compared, respectively. Multiple comparisons based on mean platelet area (Biii) and normalized number of platelets per field of view (FOV; Biv) were also carried out. Mean ± SD $^{*}P < .05$, $^{**}P < .01$, RM one-way ANOVA with Dunnett's post hoc test. Five FOV from $n = 3$ experiments were analyzed

with blood-derived immune cells including neutrophils and mononuclear cells as a putative source.[59] Nonetheless, endotheliopathy caused by SARS-CoV-2 infection could also be a non-negligible factor to consider in leading to Gal-9 overexpression.[60] The substantial elevation of Gal-9 expression and its possible local concentration in the injured vasculature during SARS-CoV-2 infection could be a factor promoting the characteristic platelet-rich thrombus formation in multi-organ vasculature, particularly small and medium pulmonary arteries,[61,62] and potential platelet hyperactivity.[60,63]

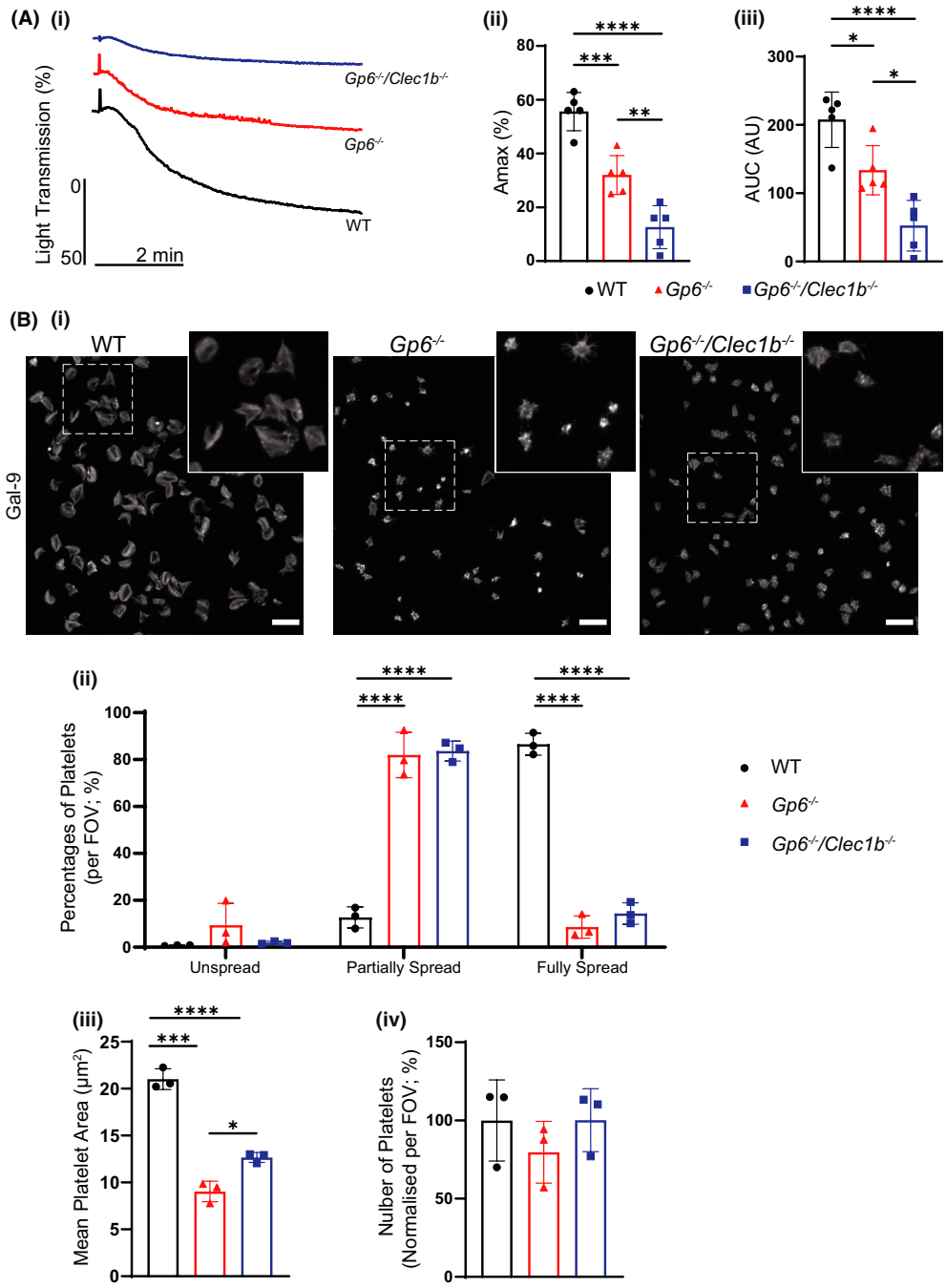


FIGURE 7 Glycoprotein VI and C-type lectin-like receptor 2 deficiency in murine platelet activation induced by galectin-9 (Gal-9). A, The effect of Gal-9 (at 300 nM) on the aggregation of wild-type (WT), *Gp6*$^{-/-}$, and *Gp6*$^{-/-}$/*Clec-2*$^{-/-}$ murine platelets. (AiExample aggregation traces. Multiple comparison of Gal-9–mediated aggregation among WT, *Gp6*$^{-/-}$, and *Gp6*$^{-/-}$/*Clec-2*$^{-/-}$ groups based on the (Aii) maximal amplitude (Amax) and (Aiii) area under the curve (AUC) of original traces ($n = 5$ mice). Mean ± standard deviation (SD) *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$, by one-way analysis of variance (ANOVA) with Tukey's post hoc test. B, Platelet spreading on Gal-9–coated coverslips (coated in the presence of 600 nM Gal-9) in WT, *Gp6*$^{-/-}$, and *Gp6*$^{-/-}$/*Clec-2*$^{-/-}$ groups. Bi, Representative images of Phalloidn-Alexa488 labelled platelets. Scale bar = 10 μm. Bii, Percentages of un-spread, partially, and fully spread platelets in WT, *Gp6*$^{-/-}$, and *Gp6*$^{-/-}$/*Clec-2*$^{-/-}$ groups were compared, respectively. Multiple comparisons based on (Biii) mean platelet area and (Biv) number of platelets per field of view (FOV) among the three groups. Mean ± SD *$P < .05$, ***$P < .001$, ****$P < .0001$, by one-way ANOVA with Tukey's post hoc test. Five FOV from $n = 3$ experiments were analyzed

According to the screening assay, Gal-9 exhibited affinity for recombinant GPVI. Given the ability of the galectins to recognize and bind glycosylated ligands Ca^{2+}-independently,[9] the identification of GPVI as a binding partner of Gal-9 seems reasonable. In our study, the substantial blockade of Gal-9 by lactose suggests a carbohydrate-dependent action is also involved in aggregation

induced by Gal-9. The formation of supramolecular lattice upon binding to multivalent ligands[64] could potentially lead to clustering and subsequent activation of GPVI.[24,25] The competition between Gal-9 and collagen for binding GPVI in turn suggested potential overlap with the collagenbbinding site of GPVI in this interaction. Apart from one consensus site $N^{72}GS^{74}$ of D1 domain reported to be N-linked glycosylated,[65] D1 and D2 domains of GPVI are not glycosylated.[43] Accordingly, the un-glycosylated variant GPVI (NQ), of which the original N^{72} residue was mutated into glutamine to remove the only N-glycosylated site in D1 domain, was employed to investigate whether the binding of Gal-9 to GPVI was dependent on the glycosylation at N^{72}. However, similar binding levels of Gal-9 to GPVI and GPVI (NQ) were detected on solid-phase binding assay, which indicates a protein–protein interaction between recombinant Gal-9 and the two GPVI constructs. Nanobody 2, a potent GPVI blocker against collagen binding, did not interfere with the aggregation to Gal-9 in human platelets, which not only reflects high selectivity of this biologic, but also implies the binding manners of Gal-9 and collagen to GPVI are not identical. It is important to note that both of these recombinant GPVI-Fc and GPVI (NQ)-Fc fusions used in the binding assays consist of the D1 and D2 portions (residues 1--183) without the stalk domain, which is different from other recombinant constructs like Revacept.[66] Hence, we cannot rule out that the stalk, which is rich in O-linked glycosylation, also plays a role in the interaction between Gal-9 and GPVI.

In the present study, we have demonstrated that the ITAM receptor signalling is essential in platelet aggregation and spreading mediated by Gal-9, as these two functions of human and murine platelets were substantially inhibited by PP2 and PRT. The reduced aggregation in GPVI-deficient mouse platelets and JAQ1 Fab-treated human platelets corroborated the critical role of GPVI signalling in this interaction with Gal-9. Nevertheless, GPVI did not appear to be the only receptor accounting for Gal-9-mediated platelet activation, as the aggregation was not completely abolished by GPVI deficiency or JAQ1. Therefore, we also examined another platelet ITAM receptor CLEC-2, which contains an extracellular lectin-like recognition domain lacking the Ca^{2+} binding site and a short cytosolic tail harboring a single YxxL sequence termed hemITAM. Gal-9 induced phosphorylation of CLEC-2, but AYP1 $F(ab)_2$ failed to alter human platelet aggregation to Gal-9 on its own or in combination with JAQ1 Fab. The aggregation of *Gp6*$^{-/-}$/*Clec-2*$^{-/-}$ murine platelets in response to Gal-9 decreased significantly compared to *Gp6*$^{-/-}$ platelets, which led us to postulate that Gal-9 may bind to a site which is distinct from podoplanin or rhodocytin on CLEC-2. Indeed, as previously reported, AYP1 failed to block human platelet aggregation induced by heme/hemin, which is another novel binding partner of CLEC-2.[36] Interestingly, while spreading was impaired in *Gp6*$^{-/-}$ and *Gp6*$^{-/-}$/*Clec-2*$^{-/-}$ platelets, adhesion remained unaffected. This observation could be in part due to the presence of other receptor(s) involved specifically in adhesion rather than spreading on Gal-9.

In conclusion, we have identified Gal-9 as a novel platelet agonist, which triggers signal transduction of the ITAM receptors GPVI and CLEC-2 in human and mouse platelets and supports multiple platelet functions including aggregation, secretion, and spreading. Given the new aspect of Gal-9 biology illustrated by the present study, we believe this protein should attract more interest in hemostasis and thrombosis research. Therapeutic targeting of Gal-9 could be a potentially novel strategy for treating thrombotic or thrombo-inflammatory diseases.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Z. Zhi, N. J. Jooss, Y. Sun, M. Colicchia, A. Slater, L. A. Moran, H. Y. F. Cheung, Y. Di, and J. Rayes performed experiments. Z. Zhi, N. J. Jooss, and N. S. Poulter acquired the data. Z. Zhi and N. J. Jooss conducted data analysis. S. P. Watson and A. J. Iqbal designed the research, interpreted experiments, and wrote the paper. All authors provided critical revision of the manuscript.

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SUPPORTING INFORMATION

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