Received: 23 February 2022 | Accepted: 8 March 2022

DOI: 10.1002/rth2.12710

rpth research & practice in thrombosis & haemostasis

ORIGINAL ARTICLE

Clustering extent-dependent differential signaling by CLEC-2 receptors in platelets

Carol Dangelmaier B.S. | Hymavathi Reddy Vari PhD | Monica Wright | John C. Kostyak PhD | Satya P. Kunapuli PhD

Sol Sherry Thrombosis Research Center, Lewis Katz School of Medicine, Temple University, Philadelphia, Pennsylvania, USA

Correspondence
Satya P. Kunapuli, Sol Sherry Thrombosis Research Center, Lewis Katz School of Medicine, Temple University, Philadelphia PA 19140, USA.
Email: spk@temple.edu

Funding information
This work was supported by grants HL155694, HL137721, HL137207, HL132171, and HL93231 from the National Institutes of Health (to SPK), and 17SDG33370020 from the American Heart Association (to JCK). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

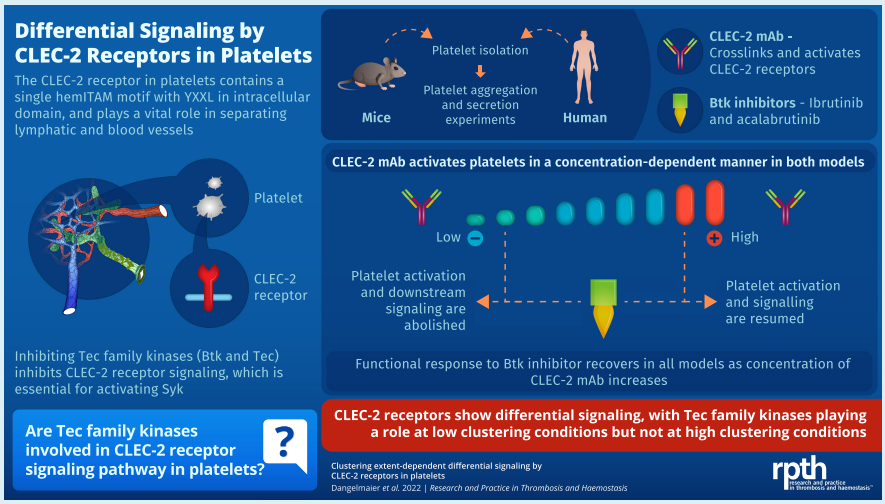
Handling Editor: Prof. Yotis Senis

Abstract

Background: C-type lectin receptor family members play a role in many cells including platelets, where they are crucial in the separation of lymphatic and blood vessels during development. The C-type lectin-like receptor 2 (CLEC-2) receptor contains the canonical intracellular hemITAM motif through which it signals to activate Syk.

Objectives: One proposed hypothesis for signaling cascade is that Syk bridges two receptors through phosphorylated hemITAM motifs. We demonstrated that the phosphorylated hemITAM stimulates PI3 kinase/Btk pathways to activate Syk. To address this controversy, we used a CLEC-2 selective agonist and studied the role of Btk in platelet activation.

Results and Conclusions: Platelet activation and downstream signaling were abolished in murine and human platelets in the presence of the Btk inhibitors ibrutinib or acalabrutinib when a low concentration of a CLEC-2 antibody was used to crosslink CLEC-2 receptors. This inhibition was overcome by increasing concentrations of the CLEC-2 antibody. Similar results were obtained in X-linked immunodeficient mouse platelets, with an inactivating mutation in Btk or in Lyn null platelets. We conclude that at low crosslinking conditions of CLEC-2, Btk plays an important role in the activation of Syk, but at higher crosslinking conditions their role becomes less important and other mechanisms take over to activate Syk.



KEYWORDS
aggregation, hemostasis, platelet, signal transduction, thrombosis

Essentials

- C-type lectin receptor family members play a role in many cells including platelets.
- To address the controversies in C-type lectin-like receptor 2 (CLEC-2) signaling, we used a CLEC-2 selective agonist and studied the role of Btk in platelet activation.
- We conclude that at low crosslinking conditions of CLEC-2, Btk plays an important role in the activation of Syk.
- However, at higher crosslinking conditions, the role of Btk becomes less important and other mechanisms take over to activate Syk.

1 | INTRODUCTION

The C-type lectin proteins belong to a large family containing C-type lectin-like domains.[1] These proteins activate receptors that are both cytoplasmic and transmembrane in a variety of cells.[2] C-type lectin receptors (CLRs) are functionally diverse and play an essential role in several processes including phagocytosis, endocytosis, pathogen recognition, and complement activation.[1,3] These CLRs are mainly expressed in immune cells but are also expressed on platelets.[4] The immunoreceptor tyrosine-based activation motif (ITAM) contains two canonical YXXL motifs separated by a defined distance. Other C-type lectin receptors contain a single ITAM-like motif (hemITAM) with either YXXL or YXXXL in the intracellular domain through which they signal.[5]

CLEC-2 is highly expressed in platelets and is known to play a crucial role in the separation of lymphatic and blood vessels during development.[6,7] CLEC-2 is activated by podoplanin expressed on the endothelial cells at the junction of lymphatic and blood vessels causing platelet activation and plug formation.[6,7] The lack of CLEC-2 receptors results in the blood flowing into the lymphatic vessels.[6,7] CLEC-2 receptors can also be activated by the snake venom protein rhodocytin or by crosslinking the receptors with an antibody or by double crosslinking the antibody bound to CLEC-2 receptors with a secondary antibody.[5] Such double crosslinking of the primary antibody by secondary antibodies has been used for clustering FcRIIA receptors in platelets.[8] CLEC-2 receptors contain a single YXXL motif in the intracellular domain (hemITAM) that is phosphorylated upon agonist interaction by Src family kinases.[5] Such phosphorylated hemITAM was proposed to bind to Syk by bridging two receptor molecules to one Syk molecule and activate Syk.[9,10] Once Syk is activated, it triggers a signaling cascade similar to ITAM signaling involving the linker for activation of T cells (LAT) signalosome, leading to phospholipase C gamma-2 (PLCγ2) activation.[5] In this model, there is activation of PI3 kinase downstream of Syk that activates Tec family kinases that phosphorylate PLCγ2 in a redundant pathway.[11] Thus, inhibition of PI3 kinases or Tec kinases only leads to partial inhibition of PLCγ2 activation. While there was no direct evidence for such interaction, this model was based on the peptide interaction studies.[9,10]

We studied CLEC-2 signaling in platelets and proposed a different model for Syk activation by phosphorylated hemITAMs.[12] In this model, PI3 kinase is activated by binding to the phosphorylated hemITAM and generates phosphatidylinositol triphosphate (PIP3). Tec family kinases translocate to the membrane by binding to PIP3 and get activated, possibly involving phosphorylation by Src family kinases (SFKs). In support of our model, activated Tec family kinases (Btk and Tec) were shown to phosphorylate and activate Syk that is bound to the phosphorylated hemITAM.[10] Hence, inhibition of either PI3 kinase or Tec family kinases leads to complete inhibition of hemITAM signaling. Recently, a study was published that showed no

inhibition of rhodocytin-induced CLEC-2 signaling by the inhibition of Tec family kinases.[13] Hence, we initiated our study to resolve the differences between these studies.

Using multiple pharmacological and molecular genetic approaches, we show that low-level activation of CLEC-2 receptors is influenced by the PI3 kinase/Tec family kinase/Syk activation pathway while increased crosslinking of the CLEC-2 receptors switches from this signaling to a mechanism that is primarily independent of Tec family kinases in the activation of Syk.

2 | MATERIALS AND METHODS

2.1 | Antibodies and reagents

All reagents were purchased from Thermo Fischer Scientific (Waltham, MA, USA) unless otherwise stated. Chronolume, used for the detection of secreted ATP, was purchased from Chrono-log Corporation (Havertown, PA, USA). The CLEC-2 activating antibody for human was purchased from R & D Systems (Minneapolis, MN, USA). The CLEC-2 activating antibody (clone 17D9) for mouse was purchased from BioLegend (San Diego, CA, USA) and the donkey anti-rat (DAR) IgG was purchased from Novex (Wadsworth, OH, USA). Anti-pSyk Y352 (Y346 in mouse) and anti-pSyk Y348 (Y342 in mice) were purchased from Abcam (Cambridge, England). Anti-Syk and anti-PLCγ2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-pSyk Y525/526 (mouse Y519/520), anti-pPLCγ2 Y1217, and anti-pLAT Y191 were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-LAT was purchased from EMD Millipore (Burlington, MA, USA). Anti-human CD32 antibody, clone IV.3 was obtained from STEMCELL Technologies (Vancouver, BC, Canada). Odyssey blocking buffer and secondary antibodies IRDye 800CW goat anti-rabbit and IRDye 680LT goat anti-mouse were purchased from Li-Cor (Lincoln, NE, USA).

2.2 | Animal housing and production

Mice were housed in a pathogen-free facility, and all animal procedures were approved by the Temple University Institutional Animal Care and Use Committee (Protocol No. 4864). X-linked immunodeficient (Xid) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA; Stock No. 001011; CBA/CaHN-Btk^{xid}/J). Recommended control mice were also purchased from Jackson Laboratory (000654 CBA/CaJ). Lyn null mice were previously described.[14]

2.3 | Preparation of mouse platelets

Mouse blood was collected and platelets were isolated as previously described.[15] The resulting platelets were counted using a Hemavet 950FS blood cell analyzer (Drew Scientific, Miami Lakes, FL, USA). Platelet counts were adjusted to a final concentration of 1.5×10^8 cells/mL in Tyrode's solution (137 mM sodium chloride, 2.7 mM potassium chloride, 2 mM magnesium chloride, 0.42 mM sodium phosphate monobasic, 10 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid and 0.1% dextrose adjusted to pH 7.4) containing 0.2 U/mL apyrase.

2.4 | Isolation of human platelets

Blood was drawn from informed healthy volunteers according to a protocol approved by the Institutional Review Board of Temple University in accordance with the Declaration of Helsinki into one-sixth volume of acid-citrate-dextrose (85 mM sodium citrate, 111 mM glucose, 71.4 mM citric acid). Platelet-rich plasma (PRP) was isolated by centrifugation at 230 *g* for 20 minutes. Platelets were obtained by centrifugation of the PRP for 10 min at 980 *g* and resuspended in Tyrode's buffer as described above. Platelet counts were adjusted to 2×10^8 platelets/mL.

2.5 | Platelet aggregation and ATP secretion

All platelet aggregation and secretion experiments were carried out using a lumi-aggregometer (Chrono-log Corporation) at 37°C under stirring conditions. Platelet aggregation was measured using light transmission, and ATP secretion was measured using Chrono-lume (a luciferin/luciferase reagent).

2.6 | Western blotting

Western blotting procedures were performed as described previously.[15] Briefly, platelets were stimulated for the indicated time points in a Lumi-aggregometer with a CLEC-2 agonist. The reaction was stopped by precipitating the platelet proteins using a final concentration of 0.6 N $HClO_4$. The protein pellet was washed with water before the addition of sample loading buffer. Platelet protein samples were then boiled for 5 minutes before resolution by SDS-PAGE and transfer to nitrocellulose membranes. The membranes were then blocked using Odyssey blocking buffer and incubated overnight with primary antibodies against the indicated protein. The membranes were then washed with Tris-buffered saline containing 0.1% Tween-20 before incubation with appropriate secondary antibodies for 1 hour at room temperature. The membranes were washed again and imaged using a Li-Cor Odyssey infrared imaging system.

2.7 | Statistics

All statistical analysis was performed using Kaleidagraph (Synergy Software, Reston, VA), and data were analyzed using a Student's *t* test where $P < .05$ was considered statistically significant. All data are presented as means ± SD of at least three independent experiments.

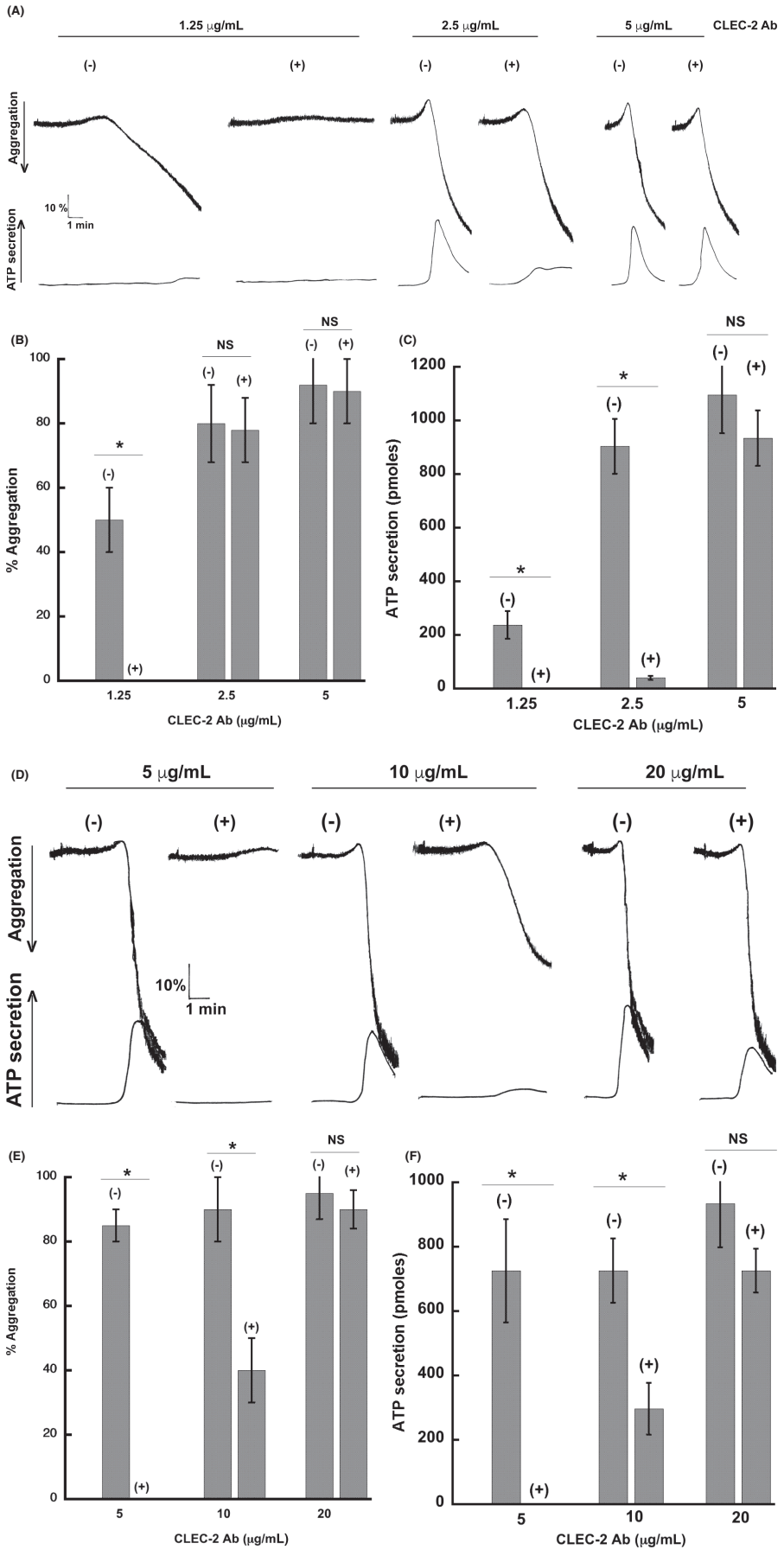


FIGURE 1 Ibrutinib abolishes platelet aggregation and secretion at low concentrations of CLEC-2 Ab. Isolated mouse (A–C) or human (D–F) platelets were preincubated with vehicle (0.2% DMSO) or 20 nM Ibrutinib for 5 minutes at 37°C. Platelets were subsequently activated with various concentrations of CLEC-2 Ab as indicated. Aggregation tracings and ATP secretion (A, D) are representative of four independent experiments. Platelet aggregation (B, E) and ATP secretion (C, F) are calculated as mean ± standard deviation. *$P < .05$. Ab, antibody; CLEC-2, C-type lectin-like receptor 2

3 | RESULTS

3.1 | Effect of ibrutinib or acalabrutinib on CLEC-2–mediated platelet activation

The CLEC-2 receptor can be activated by a number of agonists that cluster the receptor, including a monoclonal antibody against CLEC-2, the snake venom protein rhodocytin, and podoplanin, CLEC-2's endogenous ligand expressed on cells.[5,16-18] Of these agonists, the monoclonal antibody is highly selective, can be added to platelets in a soluble form, and clusters two CLEC-2 molecules to initiate signaling. Hence, we used a CLEC-2 monoclonal antibody (mAb) to activate platelets and evaluated the effect of the Tec family kinase inhibitor ibrutinib. As shown in Figure 1A, the CLEC-2 mAb activated mouse platelets in a concentration-dependent manner, and ibrutinib (20 nM) abolished platelet aggregation and secretion induced by low concentrations of the CLEC-2 mAb, inhibited at moderate concentrations of CLEC-2 mAb but was without effect at high concentrations. These experiments were slightly more difficult to perform in human platelets, as human platelets express FcγRIIA receptors that can be activated by the CLEC-2 mAb. We used an antibody to CD32 (FcγRIIA), clone IV.3, to block these receptors and then performed the experiment with an antibody to human CLEC-2. Unlike mouse platelets, the human platelets required higher concentrations of a CLEC-2 mAb to elicit a response, possibly because human platelets express far less CLEC-2 receptors (≈2000 copies)[19] compared to mouse platelets (about 40 000 copies).[20] As shown in Figure 1D, ibrutinib abolished human platelet aggregation and secretion when lower concentrations of the anti-human CLEC-2 mAb were used, but not when higher mAb was used. These data indicate that at low clustering signaling conditions, CLEC-2 depends on Tec family kinases but at higher clustering, CLEC-2 signals independently of Tec family kinases.

To evaluate the effects of ibrutinib on downstream signaling events, we measured phosphorylation of LAT and PLCγ2 under the conditions of low and high clustering of CLEC-2 in both mouse and human platelets. As shown in Figure 2, phosphorylation of Syk at 519/520, 346, and 342 were dramatically inhibited by ibrutinib at low CLEC-2 clustering conditions, but this inhibition was not significant at high clustering conditions. LAT tyrosine residue 191 was dramatically

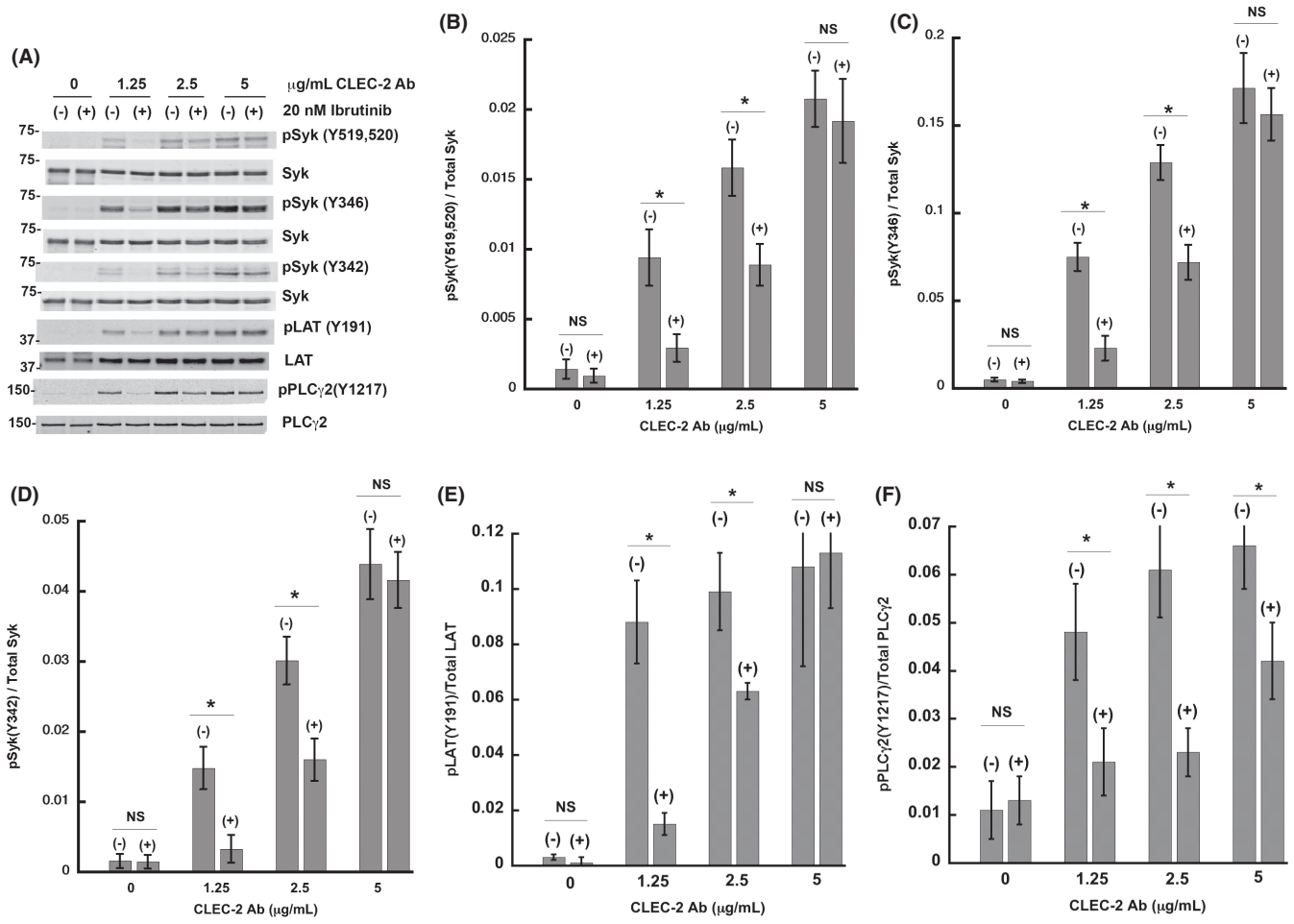


FIGURE 2 Ibrutinib dramatically inhibits Syk, LAT, and PLCγ2 phosphorylation at low concentrations of CLEC-2 Ab in mouse platelets. Isolated mouse platelets were treated as in Figure 1. Reactions were stopped by the addition of perchloric acid as described in Experimental Methods. Platelet proteins were separated by SDS-PAGE, western blotted, and probed for phospho-Syk 519/520, phospho-Syk 346, phospho-Syk 342, phospho-LAT (Y191) or phospho-PLCγ2 (Y1217) (A). Ratios were calculated by dividing the phosphorylated protein by the total protein. Phosphorylations (B-F) are graphed as mean ± SD from four independent experiments. *$P < .05$. Ab, antibody; CLEC-2, C-type lectin-like receptor 2; LAT, linker for activation of T cells; PLCγ2, phospholipase C gamma-2

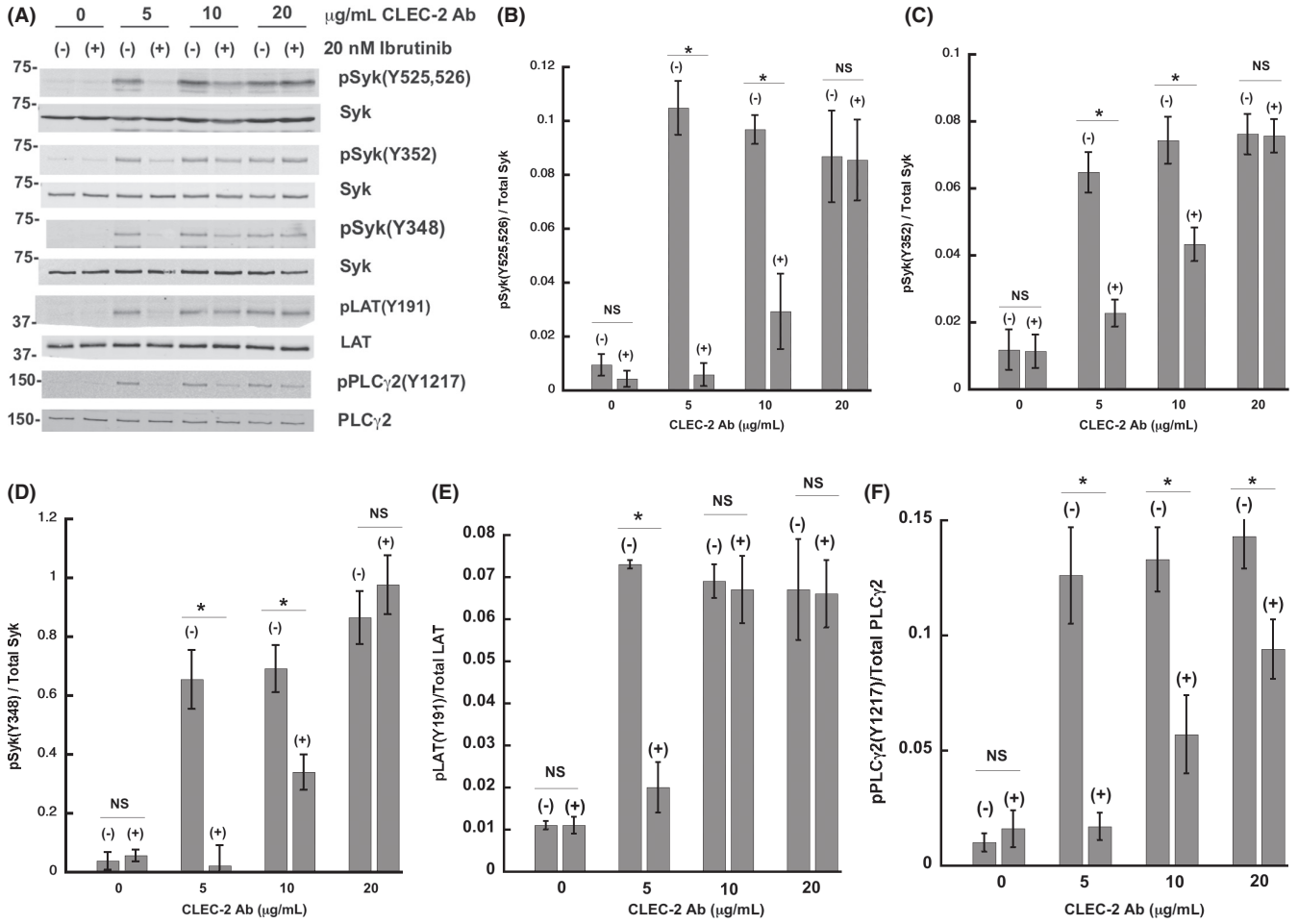


FIGURE 3 Ibrutinib dramatically inhibits Syk, LAT, and PLCγ2 phosphorylation at low concentrations of CLEC-2 Ab in human platelets. Isolated human platelets were treated as in Figure 1. Reactions were stopped by the addition of perchloric acid as described in Experimental Methods. Platelet proteins were separated by SDS-PAGE, western blotted, and probed for phospho-Syk 519/520, phospho-Syk 346, phospho-Syk 342, phospho-LAT (Y191) or phospho-PLCγ2 (Y1217) (A). Ratios were calculated by dividing the phosphorylated protein by the total protein. Phosphorylations (B-F) are graphed as mean ± standard deviation from 4 independent experiments. *$P < .05$. Ab, antibody; CLEC-2, C-type lectin-like receptor 2; LAT, linker for activation of T cells; PLCγ2, phospholipase C gamma-2

inhibited by ibrutinib in the low clustering conditions of CLEC-2 in mouse platelets. However, at higher clustering conditions, these phosphorylations were unaffected by ibrutinib (Figure 2E). Similar effects were observed on PLCγ2 tyrosine 1217 phosphorylation (Figure 2E), except that even at the high clustering conditions, PLCγ2 phosphorylation was significantly inhibited. This could be because of an additional role for PI3K/Btk downstream of Syk in the activation of PLCγ2.[21] As shown in Figure 3, identical results of Syk, LAT, and PLCγ2 phosphorylations were obtained when human platelets were used with varied CLEC-2 clustering. Similar results were obtained when the second-generation Btk inhibitor acalabrutinib was used (Figures 4,5).

3.2 | CLEC-2–induced platelet activation in Xid mouse platelets

A genetically altered mouse with an inactivating mutation in the Tec kinase Btk is available and has been used in our previous study with rhodocytin. To evaluate the role of Btk in CLEC-2 clustering-dependent platelet activation, we stimulated Xid mouse platelets with different concentrations of the CLEC-2 mAb. These data were compared to control mouse platelets as recommended by Jackson Laboratory. As can be seen in Figure 6, when low concentrations of CLEC-2 mAb were used, platelet activation was dramatically inhibited in Xid mouse platelets compared to control mouse platelets. However, as the concentration of mAb was increased, this inhibition of platelet activation was less apparent in Xid mouse platelets. Consistently, LAT and PLCγ2 phosphorylations were dramatically inhibited in Xid mouse platelets with low CLEC-2 clustering conditions, while LAT was unaffected with high clustering of CLEC-2 (Figure 6C-E). Phosphorylation of PLCγ2 on tyrosine 1217 was still inhibited at high clustering conditions, indicating that Btk may contribute to its phosphorylation (Figure 6E). These data suggest an essential role for Btk in low clustering conditions of CLEC-2, while Btk appears to be dispensable when CLEC-2 receptor clustering is very high. The reason for the lack of abolishment of platelet responses in low clustering conditions in Xid mice is that, although they lack a functional Btk, they still express Tec kinase, which might have a redundant function as Btk, similar to glycoprotein VI (GPVI) activation.[22]

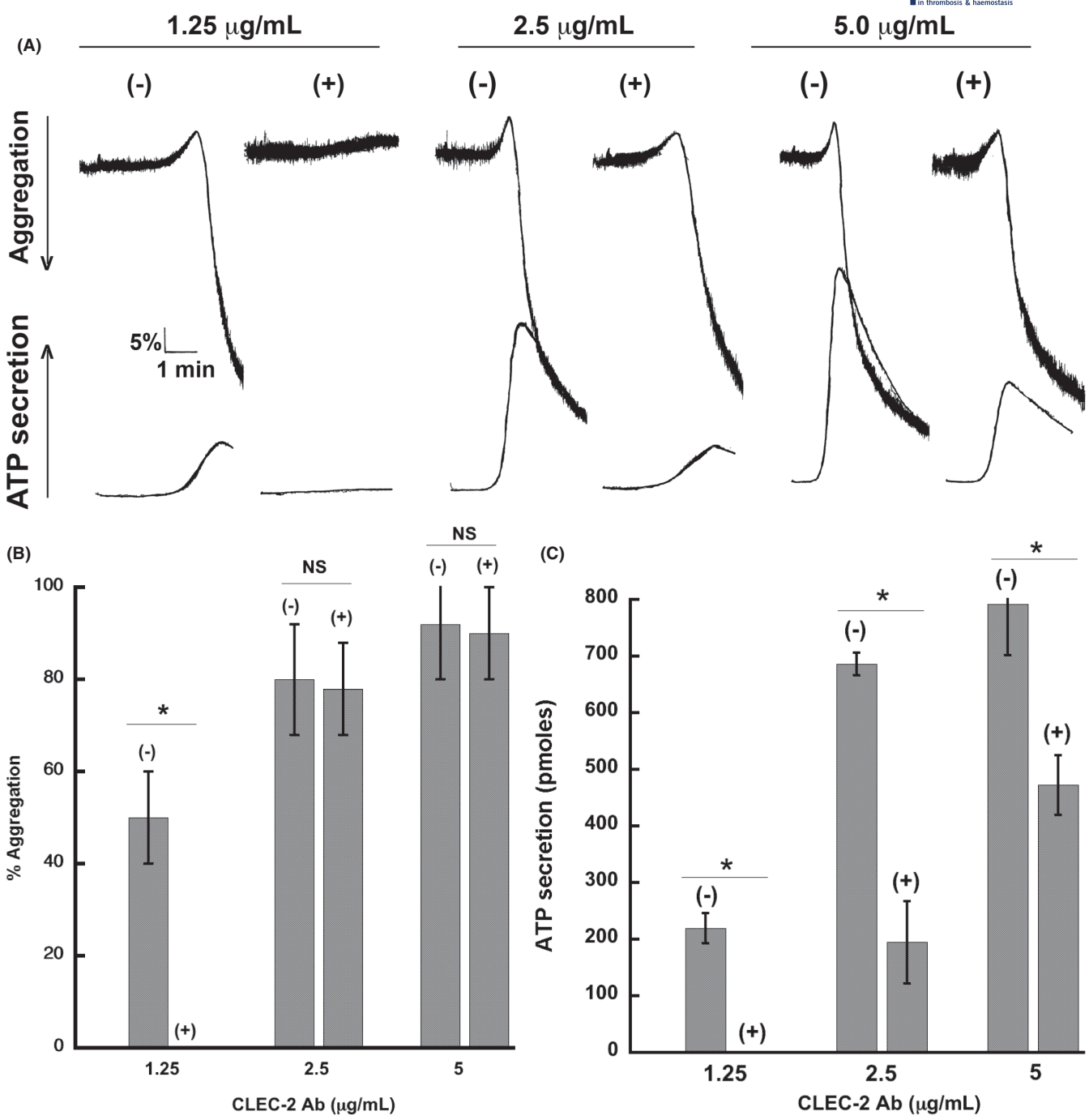


FIGURE 4 Acalabrutinib abolishes platelet aggregation and secretion at low concentrations of CLEC-2 Ab. Isolated mouse platelets were preincubated with vehicle (0.2% DMSO) or 1 μM acalabrutinib for 5 minutes at 37°C. Platelets were subsequently activated with various concentrations of CLEC-2 Ab as indicated. Aggregation tracings and ATP secretion (A) are representative of four independent experiments. Aggregation (B) and ATP secretion (C) is calculated as mean ± standard deviation. *$P < .05$. Ab, antibody; CLEC-2, C-type lectin-like receptor 2; LAT, linker for activation of T cells; PLCγ2, phospholipase C gamma-2

3.3 | CLEC-2 activation of Src family kinase–deficient mouse platelets

Severin et al[23] have shown that aggregation was dramatically delayed in Lyn null mouse platelets when the platelets were activated with CLEC-2 mAb but not in Fyn or Src null platelets. However, they used a single concentration of CLEC-2 mAb. We investigated the effect of different concentrations of CLEC-2 mAb on the functional responses in Lyn null mouse platelets, to evaluate the role of Lyn under different clustering conditions of CLEC-2. As shown in Figure 7, platelet aggregation and secretion were abolished when a low concentration of the CLEC-2 mAb was used in Lyn null mice, but the functional responses began to recover as the concentration of CLEC-2 mAb was increased. Consistently, PLCγ2 and LAT phosphorylations were also

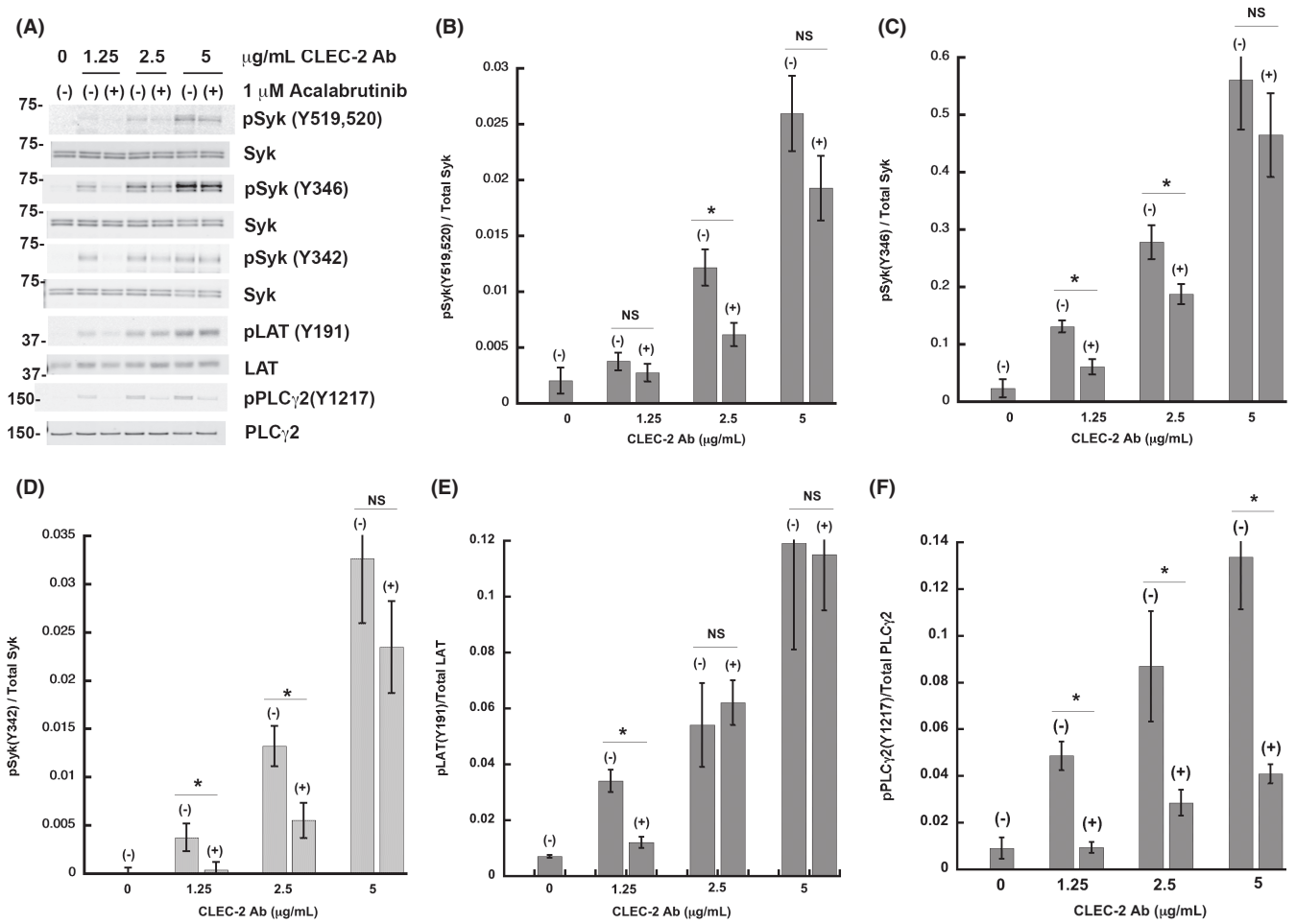


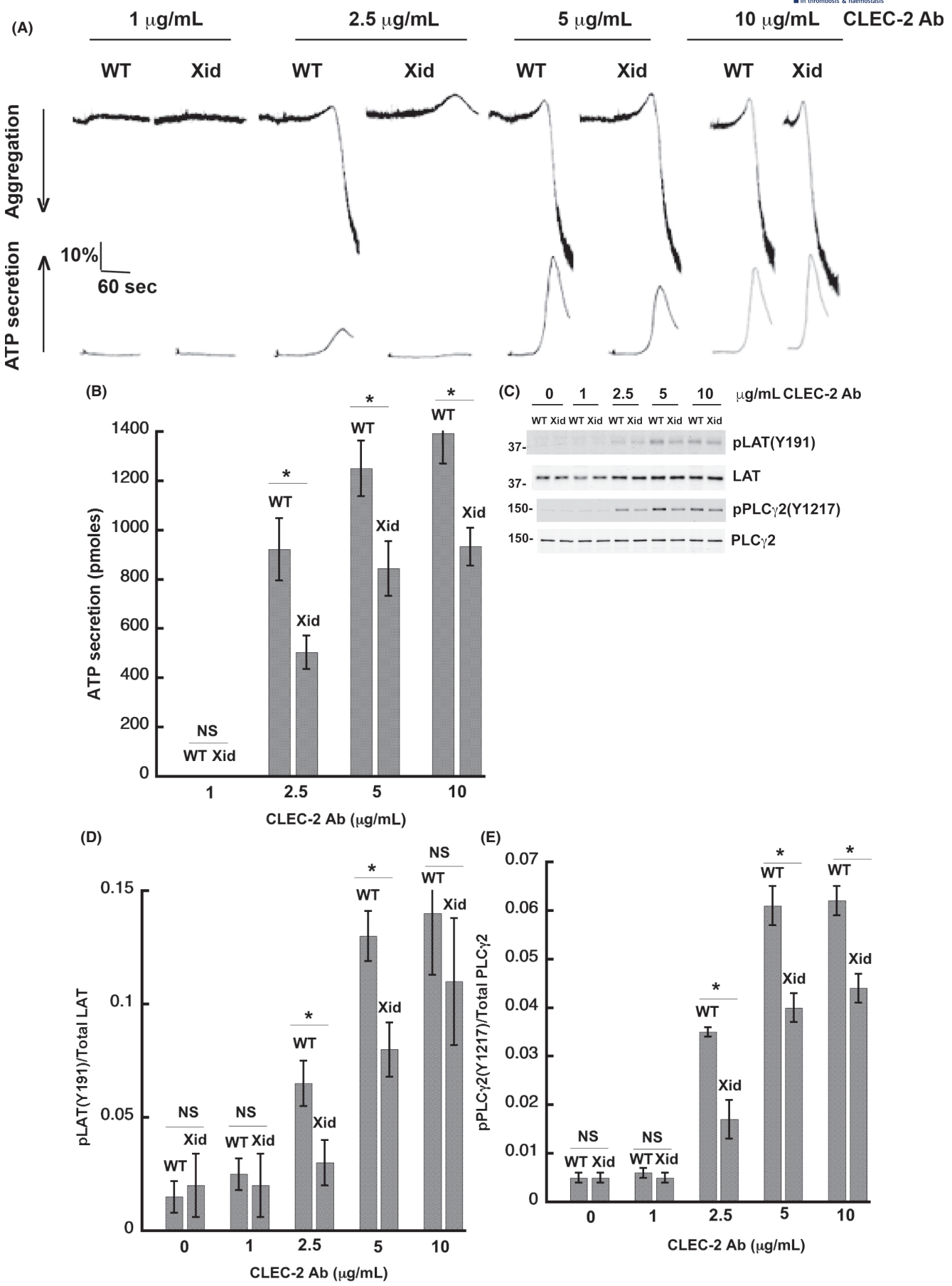
FIGURE 5 Acalabrutinib dramatically inhibits LAT and PLCγ2 phosphorylation at low concentrations of CLEC-2 Ab. Isolated mouse platelets were treated as in Figure 4. Reactions were stopped by the addition of perchloric acid as described in Experimental Methods. Platelet proteins were separated by SDS-PAGE, Western blotted, and probed for phospho-Syk 519/520, phospho-Syk 346, phosphor-Syk 342, phospho-LAT (Y191) or phospho-PLCγ2 (Y1217) (A). Ratios were calculated by dividing the phosphorylated protein by the total protein. Phosphorylations (B–F) are graphed as mean ± standard deviation from four independent experiments. *$P < .05$. Ab, antibody; CLEC-2, C-type lectin-like receptor 2; LAT, linker for activation of T cells; PLCγ2, phospholipase C gamma-2

abolished when low CLEC-2 mAb was used. When high mAb was used, PLCγ2 phosphorylation completely recovered (Figure 7E), although LAT phosphorylation recovered partially (Figure 7D).

4 | DISCUSSION

CLRs, such as Dectin-1 and CLEC-2, have been known to signal through phosphorylation of a hemITAM motif in the cytoplasmic tail. The collagen receptor, which is not a CLR, also associates with the FcRγ chain and signals through ITAM motifs. In both cases, these ITAM and hemITAM motifs are phosphorylated by SFKs upon receptor clustering. The phosphorylated ITAM, containing two YXXL motifs separated by a specific distance, can bind to the two Src homology 2 (SH2) domains of Syk and activate Syk possibly by inducing a transition from autoinhibited to the active conformation and subsequent phosphorylation events. However, in the case of the hemITAM, there is only one YXXI/L motif that can be phosphorylated and only one SH2 of Syk that can bind to it. Hence, extending the model of ITAM binding, a model was proposed for hemITAM signaling in which each SH2 domain of Syk binds to one phosphorylated hemITAM on each receptor. This was a widely accepted model, although

FIGURE 6 Btk plays an essential role under low clustering conditions of CLEC-2. Isolated platelets from control or Xid mice were activated with varying concentrations of the CLEC-2 Ab as indicated. Aggregation and ATP secretion were monitored in a lumi-aggregometer (A). Reactions were stopped with perchloric acid, proteins separated by SDS-PAGE, Western blotted and probed for the indicated proteins (C). ATP secretion (B) and phosphorylated protein ratios (D, E) are graphed as means ± SD from 3 independent experiments. *$P < .05$. Ab, antibody; CLEC-2, C-type lectin-like receptor 2; LAT, linker for activation of T cells; PLCγ2, phospholipase C gamma-2; WT, wild-type; Xid, X-linked immunodeficient


(A)
1 μg/mL
2.5 μg/mL
5 μg/mL
10 μg/mL
CLEC-2 Ab
WT
Xid
Aggregation
ATP secretion
10%
60 sec
(B)
ATP secretion (pmoles)
NS
WT Xid
CLEC-2 Ab (μg/mL)
(C)
0 1 2.5 5 10 μg/mL CLEC-2 Ab
WT Xid WT Xid WT Xid WT Xid WT Xid
37-
pLAT(Y191)
LAT
150-
pPLCγ2(Y1217)
PLCγ2
(D)
pLAT(Y191)/Total LAT
(E)
pPLCγ2(Y1217)/Total PLCγ2

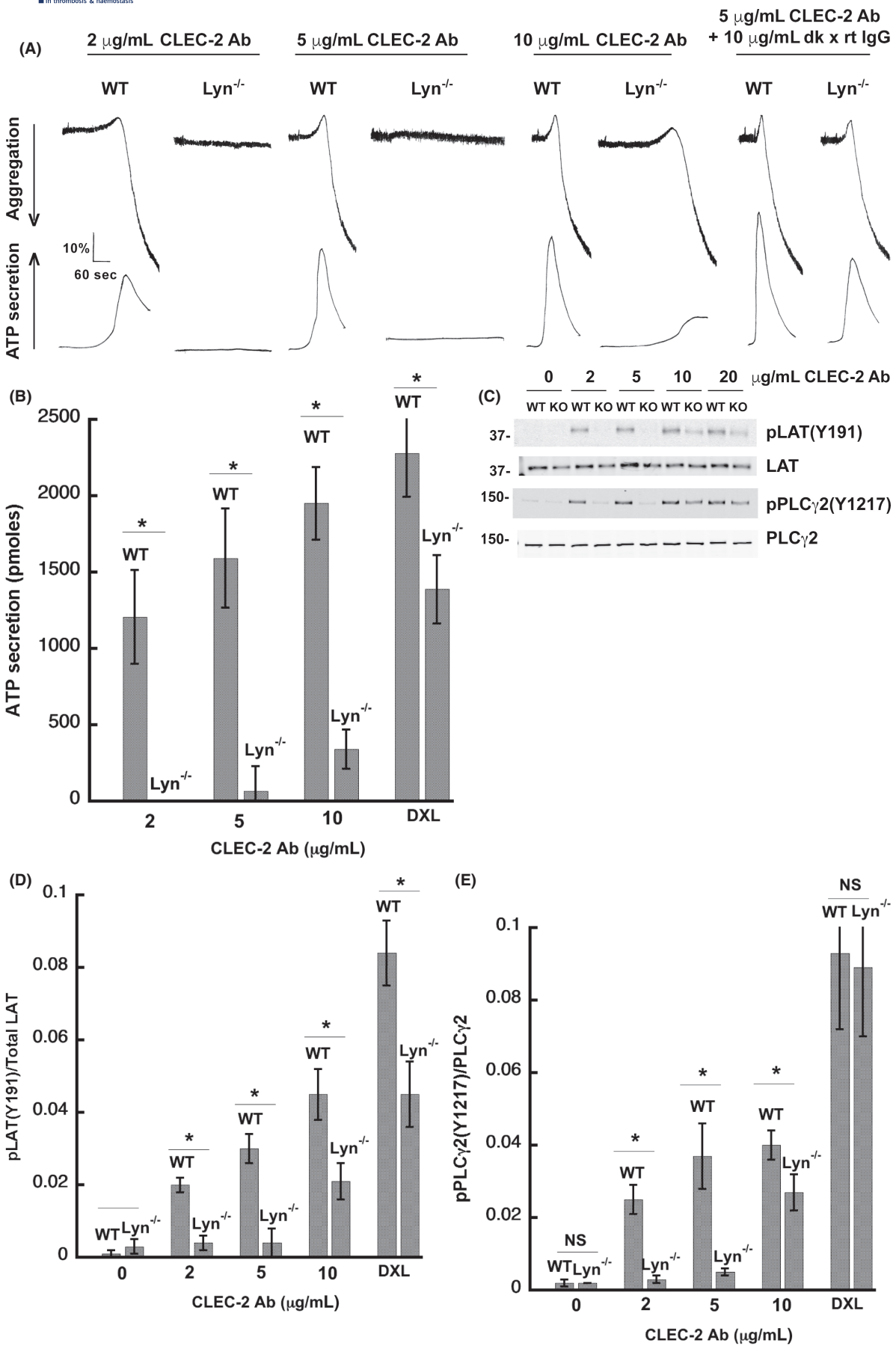

(A)
2 μg/mL CLEC-2 Ab
5 μg/mL CLEC-2 Ab
10 μg/mL CLEC-2 Ab
5 μg/mL CLEC-2 Ab + 10 μg/mL dk x rt IgG
WT
Lyn-/-
Aggregation
ATP secretion
10%
60 sec
(B)
ATP secretion (pmoles)
CLEC-2 Ab (μg/mL)
DXL
(C)
0 2 5 10 20 μg/mL CLEC-2 Ab
WT KO WT KO WT KO WT KO WT KO
pLAT(Y191)
LAT
pPLCγ2(Y1217)
PLCγ2
(D)
pLAT(Y191)/Total LAT
(E)
pPLCγ2(Y1217)/PLCγ2
NS

FIGURE 7 Lyn plays an essential role under low clustering conditions of CLEC-2. Isolated platelets from control or $Lyn^{-/-}$ mice were activated with varying concentrations of the CLEC-2 Ab as indicated. Aggregation and ATP secretion were monitored in a lumi-aggregometer (A). Reactions were stopped with perchloric acid, proteins separated by SDS-PAGE, western blotted, and probed for the indicated proteins (C). ATP secretion (B) and phosphorylated protein ratios (D, E) are graphed as means ± standard deviation from three independent experiments. *$P < .05$. DAR, double crosslinked (5 μg/mL CLEC-2 Ab + 10 μg/mL dk × rt IgG). Ab, antibody; CLEC-2, C-type lectin-like receptor 2; LAT, linker for activation of T cells; PLCγ2, phospholipase C gamma-2; WT, wild-type; Xid, X-linked immunodeficient

there is no direct evidence to support this model. Indirect evidence comes from the use of peptides from hemITAM that can bind to Syk and vice versa.

We have published that CLEC-2/hemITAM signaling involves PI3 kinase and Tec kinase activation that is essential for the stimulation of Syk.[12] In this model, we provided evidence that inhibition of either PI3 kinase or Tec kinases leads to abolished CLEC-2–mediated phosphorylation of Syk as well as its in vitro kinase activity.[12] We demonstrated that this was not the case for the ITAM-linked GPVI-mediated Syk activation.[12] However, a recent study indicated that ibrutinib, a Tec family kinase inhibitor, failed to inhibit 300 nM rhodocytin-induced platelet activation.[13] Hence, we investigated the discrepancies between our studies.

While there are several agonists that can activate CLEC-2, the mAb against CLEC-2 provides the lowest activation as it can cluster two CLEC-2 molecules. The strength of CLEC-2 clustering can be enhanced by crosslinking the mAb with anti-IgG secondary antibodies in which four CLEC-2 receptors could be activated, as has been shown for the FcRIIA receptor on platelets.[8] Rhodocytin is a snake venom protein and can crosslink 8 CLEC-2 molecules,[24] but its effects on other platelet receptors cannot be ruled out.[25] Finally, podoplanin, a physiological agonist, can only be used when it is expressed on cells, and its avidity depends on the expression levels and cannot be regulated accurately. CLEC-2 receptors have been shown to exist as monomers, oligomers, and oligomers of higher order on the platelet surface.[9] These different structures of CLEC-2 might have varied affinity for the ligands and might proceed with activation of monomers to higher-order oligomers as the ligand concentrations increase.[26] This aspect is further complicated by the fact that human platelets express far fewer CLEC-2 receptors (≈2000 copies)[19] compared to mouse platelets (≈40 000 copies).[20] Thus, work with mouse platelets, as they are more sensitive, requires much more careful design of the ligand concentrations.

We initiated our studies using a mAb against CLEC-2 to cluster under the lowest activation conditions using the lowest concentration of the mAb. Under these conditions, the signal is low but sufficient to activate platelets. Our data show that, under these conditions, ibrutinib abolished platelet activation induced by the CLEC-2 mAb. When the concentrations of mAb were increased, the inhibition by ibrutinib became less apparent. Thus, as the mAb concentration is increased, more CLEC-2 receptors are engaged in signaling, and this results in the decreased effect of ibrutinib. This effect was demonstrated in both human and mouse platelets (Figures 1-3). Consistently, ibrutinib abolished phosphorylations of LAT and PLCγ2 when a low concentration of the CLEC-2 mAb was used but not when higher mAb concentrations were used.

In our experience, platelet preparations play a key role in the responsiveness to the CLEC2 mAb minimal concentrations to activate platelets. We caution that blindly using one concentration of agonist to evaluate the effect of ibrutinib will not accurately represent the state of signaling, and one must do a titration of agonist to determine the lowest concentration to activate platelets. We could correlate the differences in this platelet reactivity to the desensitization of ADP receptors; the less responsive the ADP receptors are, the more mAb needed. The second point we want to emphasize is that the concentration of rhodocytin does not accurately represent the active amount of rhodocytin in the preparation.[13] We have noticed that there is a great batch-to-batch variation of rhodocytin. While some batches are weak and require higher concentrations (up to 40 nM), other batches needed only 3 nM to activate platelets. Hence, one cannot blindly follow the concentration of rhodocytin in the publications and should titer each batch in the laboratory to determine the low and high concentrations required for platelet activation. In this context, we feel that 300 nM of rhodocytin used by Nicolson et al[13] could be high, and that could be the reason they did not see any inhibition by ibrutinib.

The results obtained with ibrutinib were confirmed using Xid mice that lack a functional Btk (Figure 5). In the Xid mice, platelet aggregation and secretion were dramatically inhibited by low-avidity conditions of CLEC-2 clustering compared to wild-type mice. However, at high clustering conditions of CLEC-2 engagement, there was no difference between Xid and wild-type mouse platelet aggregation and secretion. The lack of complete inhibition of platelet aggregation in Xid mice at low clustering conditions is due to Tec kinase, which might be taking over the function of Btk to some extent. Consistently, the phosphorylations of LAT and PLCγ2 were also dramatically inhibited under low CLEC-2 clustering conditions but unaffected at high clustering conditions, compared to wild-type platelets. Consistent with the role of Tec kinases, we have previously shown that separation of lymphatic and blood vessels is disrupted only in the Btk/Tec double-knockout mice but not in single-knockout mice. These data indicate that Btk and Tec can serve redundant functions downstream of CLEC-2 receptors during development.

While the platelet functional studies are carried out with ex vivo platelets and low CLEC-2 clustering conditions require Btk for the functional activation of Syk, one wonders what the relevant physiological condition for CLEC-2 clustering may be. Under physiological conditions, the primary function of platelet CLEC-2 is to interact with endothelial podoplanin and form a microthrombus at the junctions of lymphatic and blood vessels, thereby aiding the lymphatic and blood vessel separation during development. Our data showing

the disruption of lymphatic and blood vessel separation during development in Btk/Tec double-knockout mice demonstrates that the Tec kinases play a role in the CLEC-2 signaling under physiological conditions. If the physiological podoplanin engagement of platelet CLEC-2 were with high clustering then, based on the results where we show that Tec kinases are not important under high clustering conditions, we should not have seen dysfunctional lymphatic and blood vessel separation in Btk/Tec double-knockout mice.

Our studies extend the observations made by Severin et al[23] who reported that CLEC-2 -induced platelet aggregation was delayed in Lyn null mice with CLEC-2 mAb but not with rhodocytin. In our studies, we also show that under low clustering conditions, Lyn is absolutely necessary for platelet activation by CLEC-2 but becomes dispensable as more CLEC-2 receptors are engaged. Furthermore, specific phosphorylations on LAT and PLCγ2 are also abolished when low CLEC-2 mAb is used. Our studies are also consistent with the observations of Severin et al[23] that Lyn plays an important role when low-valency agonists of CLEC-2 are used and multivalent ligand engagement with podoplanin expressing Chinese hamster ovary cells or CLEC-2 IgM antibodies can bypass the Lyn-dependent pathways.

We connect the dependency of CLEC-2 signaling on Lyn and Tec family kinases at low clustering conditions and propose that Lyn initially phosphorylates the hemITAM motif on CLEC-2 under low clustering conditions (model outlined in Figure 8). Subsequently, PI3 kinase binds to the phosphorylated hemITAM and is activated, converting phosphatidylinositol bisphosphate (PIP2) to PIP3. Tec family kinases translocate to the membrane by binding to PIP3 and then phosphorylate and activate Syk, which might be bound to the phosphorylated hemITAM or in the cytosol. Lyn might also be involved in the phosphorylation of Y551 on PIP3-bound Btk, and this phosphorylation might be necessary for the Tec family kinase activation.[27] Hence, under low clustering CLEC-2 conditions, Lyn deficiency or inhibition of Tec family kinases with ibrutinib, Syk activation is abolished resulting in dramatically inhibited platelet functional responses. Interestingly, Fyn and Src, still present in the Lyn null platelets, are not able to phosphorylate CLEC-2 under these conditions, most likely due to the conformational requirements of CLEC-2 for these kinases. As the multivalent ligands are used, under high clustering engagement of CLEC-2, Lyn is dispensable and other SFKs can phosphorylate the hemITAM, and Syk is activated by alternate pathways. Interestingly, Severin et al[23] observed that the CLEC-2 cytoplasmic tail was not phosphorylated in Syk-deficient platelets, suggesting an essential role for Syk in this event. They also observed that pyrazolopyrimidine compound PP2, an inhibitor of SFKs, failed to inhibit CLEC-2 tyrosine phosphorylation, although it blocked CLEC-2–mediated platelet activation. Hence, they concluded that SFKs play an important role in the downstream signaling events. However, it is not clear how Syk is activated downstream of

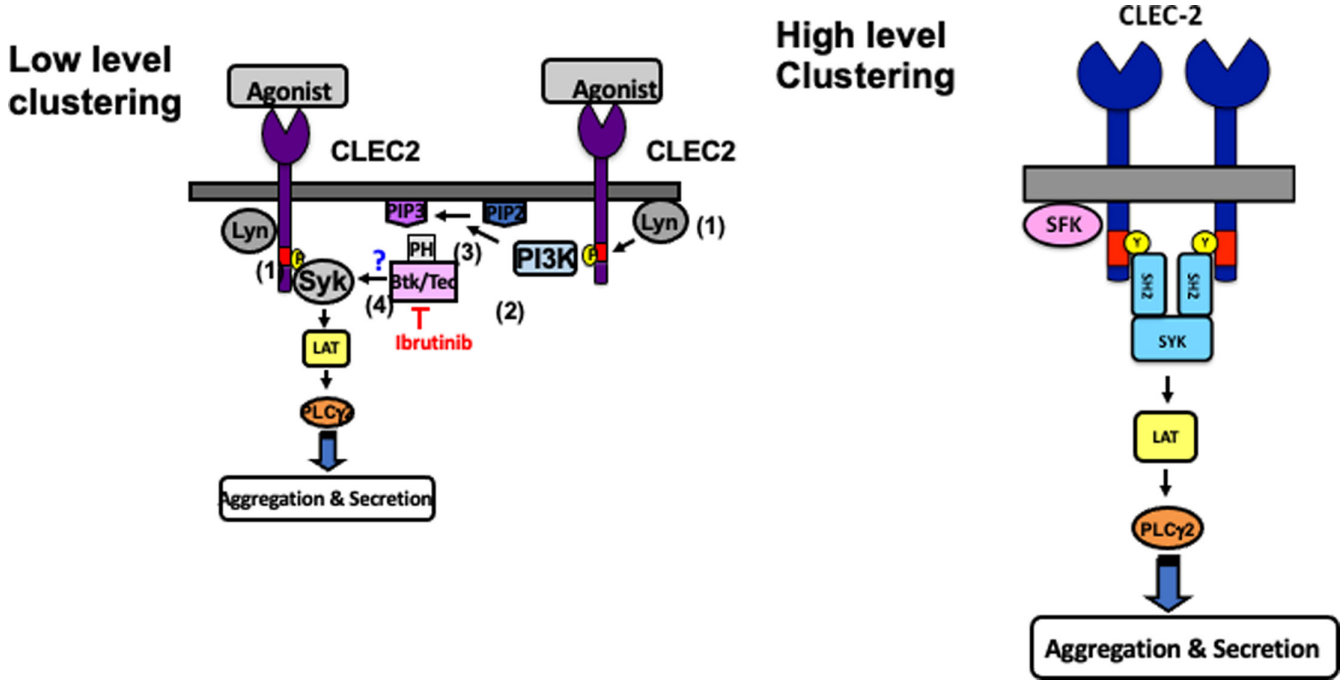


FIGURE 8 Models for differential signaling downstream of CLEC-2. We propose the following: under low clustering conditions, (1) upon activation of CLEC-2 in platelets, Lyn phosphorylates the hemITAM. (2) PI3K binds to phosphorylated hemITAM and gets activated. (3) Once activated, PI3K generates PIP_3 that is important for the recruitment of Tec family kinases to the membrane, where it is phosphorylated and activated. (4) Activated Btk then phosphorylates and activates Syk that is already bound to phosphorylated CLEC-2 hemITAM. The exact site of phosphorylation on Syk by Tec kinase is not known. Under high clustering conditions, as proposed by other investigators, Syk binds to two phosphorylated hemITAM through its SH2 domains and gets activated. There is no role of Tec kinases under these conditions. Whether hemITAM bound Syk gets phosphorylated by SFKs for activation remains to be established. Once activated, Syk causes phosphorylation of LAT and activation of PLCγ2, leading to platelet aggregation and secretion. Ab, antibody; CLEC-2, C-type lectin-like receptor 2; LAT, linker for activation of T cells; PLCγ2, phospholipase C gamma-2; WT, wild-type; Xid, X-linked immunodeficient

CLEC-2 if it must phosphorylate and bind to phosphorylated hemITAM. It is possible that if the hemITAM is phosphorylated, then the classical model of one Syk molecule binding to two CLEC-2 receptors through phosphorylated hemITAM occurs as the clustering increases and the CLEC-2 intracellular domains come closer. An alternative mechanism was proposed in which Syk could be activated by actin-cytoskeleton/ezrin, radixin, moesin proteins, by translocating Syk to the plasma membrane.[28] This has the potential to represent an alternate mode of Syk recruitment to the membrane. Thus, while we have supporting evidence for the involvement of Lyn and Tec family kinases under the conditions of low CLEC-2 clustering, we only have possible models of alternate pathways of Syk engagement and activation under a high CLEC-2 clustering condition, which certainly do not depend on Lyn or Tec kinases.

In conclusion, we provide evidence that CLEC-2 signals differently under low and high clustering conditions. Under low clustering conditions, CLEC-2 signaling depends on Lyn and Tec kinases, while under high clustering conditions, alternate pathways, without the involvement of Lyn and Tec family kinases, take over.

RELATIONSHIP DISCLOSURE

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

CD performed experiments, analyzed the data, and wrote the manuscript; HRV performed experiments; MW performed experiments; JCK performed experiments and analyzed the data including statistics; SPK was involved in conceptualization and overall supervision of the project.

DATA AVAILABILITY STATEMENT

All data concerning this report are available in the article.

ORCID

Carol Dangelmaier https://orcid.org/0000-0001-8941-7002
John C. Kostyak https://orcid.org/0000-0002-8712-1192
Satya P. Kunapuli https://orcid.org/0000-0002-1298-3214

REFERENCES

1. ZelenskyAN,GreadyJE.TheC-typelectin-likedomainsuperfamily.*FEBS J*. 2005;272:6179-6217. doi:10.1111/j.1742-4658.2005.05031.x
2. Drickamer K, Taylor ME. Recent insights into structures and functions of C-type lectins in the immune system. *Curr Opin Struct Biol*. 2015;34:26-34. doi:10.1016/j.sbi.2015.06.003
3. Weis WI, Taylor ME, Drickamer K. The C-type lectin superfamily in the immune system. *Immunol Rev*. 1998;163:19-34. doi:10.1111/j.1600-065x.1998.tb01185.x
4. Chaipan C, Soilleux EJ, Simpson P, et al. DC-SIGN and CLEC-2 mediate human immunodeficiency virus type 1 capture by platelets. *J Virol*. 2006;80:8951-8960. doi:10.1128/JVI.00136-06
5. Suzuki-Inoue K, Fuller GL, Garcia A, et al. A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2. *Blood*. 2006;107:542-549. doi:10.1182/blood-2005-05-1994
6. Suzuki-Inoue K, Inoue O, Ding G, et al. Essential in vivo roles of the C-type lectin receptor CLEC-2: embryonic/neonatal lethality of CLEC-2–deficient mice by blood/lymphatic misconnections and impaired thrombus formation of CLEC-2-deficient platelets. *J Biol Chem*. 2010;285:24494-24507. doi:10.1074/jbc.M110.130575
7. Osada M, Inoue O, Ding G, et al. Platelet activation receptor CLEC-2 regulates blood/lymphatic vessel separation by inhibiting proliferation, migration, and tube formation of lymphatic endothelial cells. *J Biol Chem*. 2012;287:22241-22252. doi:10.1074/jbc.M111.329987
8. Chacko GW, Duchemin AM, Coggeshall KM, Osborne JM, Brandt JT, Anderson CL. Clustering of the platelet Fc gamma receptor induces noncovalent association with the tyrosine kinase p72syk. *J Biol Chem*. 1994;269:32435-32440.
9. Hughes CE, Pollitt AY, Mori J, et al. CLEC-2 activates Syk through dimerization. *Blood*. 2010;115:2947-2955. doi:10.1182/blood-2009-08-237834
10. Watson AA, Christou CM, James JR, et al. The platelet receptor CLEC-2 is active as a dimer. *Biochemistry*. 2009;48:10988-10996. doi:10.1021/bi901427d
11. Watson SP, Asazuma N, Atkinson B, et al. The role of ITAM- and ITIM-coupled receptors in platelet activation by collagen. *Thromb Haemost*. 2001;86:276-288.
12. Manne BK, Badolia R, Dangelmaier C, et al. Distinct pathways regulate Syk protein activation downstream of immune tyrosine activation motif (ITAM) and hemITAM receptors in platelets. *J Biol Chem*. 2015;290:11557-11568. doi:10.1074/jbc.M114.629527
13. Nicolson PLR, Nock SH, Hinds J, et al. Low dose Btk inhibitors selectively block platelet activation by CLEC-2. *Haematologica*. 2020;106(1):208-219. doi:10.3324/haematol.2019.218545
14. Chari R, Kim S, Murugappan S, Sanjay A, Daniel JL, Kunapuli SP. Lyn, PKC-delta, SHIP-1 interactions regulate GPVI-mediated platelet-dense granule secretion. *Blood*. 2009;114:3056-3063. doi:10.1182/blood-2008-11-188516
15. Kostyak JC, Mauri BR, Dangelmaier C, et al. TULA-2 deficiency enhances platelet functional responses to CLEC-2 agonists. *TH Open*. 2018;2:e411-e419. doi:10.1055/s-0038-1676358
16. May F, Hagedorn I, Pleines I, et al. CLEC-2 is an essential platelet-activating receptor in hemostasis and thrombosis. *Blood*. 2009;114:3464-3472. doi:10.1182/blood-2009-05-222273
17. Christou CM, Pearce AC, Watson AA, et al. Renal cells activate the platelet receptor CLEC-2 through podoplanin. *Biochem J*. 2008;411:133-140. doi:10.1042/BJ20071216
18. Suzuki-Inoue K, Kato Y, Inoue O, et al. Involvement of the snake toxin receptor CLEC-2, in podoplanin-mediated platelet activation, by cancer cells. *J Biol Chem*. 2007;282:25993-26001. doi:10.1074/jbc.M702327200
19. Gitz E, Pollitt AY, Gitz-Francois JJ, et al. CLEC-2 expression is maintained on activated platelets and on platelet microparticles. *Blood*. 2014;124:2262-2270. doi:10.1182/blood-2014-05-572818
20. Zeiler M, Moser M, Mann M. Copy number analysis of the murine platelet proteome spanning the complete abundance range. *Mol Cell Proteomics*. 2014;13:3435-3445. doi:10.1074/mcp.M114.038513
21. Watson SP, Auger JM, McCarty OJ, Pearce AC. GPVI and integrin alphaIIb beta3 signaling in platelets. *J Thromb Haemost*. 2005;3:1752-1762.
22. Atkinson BT, Ellmeier W, Watson SP. Tec regulates platelet activation by GPVI in the absence of Btk. *Blood*. 2003;102:3592-3599. doi:10.1182/blood-2003-04-1142
23. Severin S, Pollitt AY, Navarro-Nunez L, et al. Syk-dependent phosphorylation of CLEC-2: a novel mechanism of hemimmunoreceptor tyrosine-based activation motif signaling. *J Biol Chem*. 2011;286:4107-4116. doi:10.1074/jbc.M110.167502
24. Watson AA, O'Callaghan CA. Molecular analysis of the interaction of the snake venom rhodocytin with the platelet receptor CLEC-2. *Toxins*. 2011;3:991-1003. doi:10.3390/toxins3080991

25. Suzuki-Inoue K, Ozaki Y, Kainoh M, et al. Rhodocytin induces platelet aggregation by interacting with glycoprotein Ia/IIa (GPIa/IIa, Integrin alpha 2beta 1). Involvement of GPIa/IIa-associated Src and protein tyrosine phosphorylation. *J Biol Chem*. 2001;276:1643-1652. doi:10.1074/jbc.M006191200
26. Martin EM, Zuidscherwoude M, Moran LA, Di Y, Garcia A, Watson SP. The structure of CLEC-2: mechanisms of dimerization and higher-order clustering. *Platelets*. 2021;32:733-743. doi:10.1080/09537104.2021.1906407
27. Wahl MI, Fluckiger AC, Kato RM, Park H, Witte ON, Rawlings DJ. Phosphorylation of two regulatory tyrosine residues in the activation of Bruton's tyrosine kinase via alternative receptors. *Proc Natl Acad Sci U S A*. 1997;94:11526-11533. doi:10.1073/pnas.94.21.11526
28. Urzainqui A, Serrador JM, Viedma F, et al. ITAM-based interaction of ERM proteins with Syk mediates signaling by the leukocyte adhesion receptor PSGL-1. *Immunity*. 2002;17:401-412. doi:10.1016/s1074-7613(02)00420-x

How to cite this article: Dangelmaier C, Vari HR, Wright M, Kostyak JC, Kunapuli SP. Clustering extent-dependent differential signaling by CLEC-2 receptors in platelets. *Res Pract Thromb Haemost*. 2022;6:e12710. doi:10.1002/rth2.12710