

Distinct Pathways Regulate Syk Protein Activation Downstream of Immune Tyrosine Activation Motif (ITAM) and hemITAM Receptors in Platelets*

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Background: The mechanisms by which the hemITAM receptor activates Syk are not completely understood.

Results: Inhibition of PI3K or Tec family kinases abolished Syk activation downstream of CLEC-2 but not GPVI.

Conclusion: PI3K is upstream of tyrosine kinase Syk in CLEC-2, but not in GPVI, activation.

Significance: Understanding the differences between hemITAM and ITAM signaling helps in designing efficient drugs for thrombosis and hemostasis.

Tyrosine kinase pathways are known to play an important role in the activation of platelets. In particular, the GPVI and CLEC-2 receptors are known to activate Syk upon tyrosine phosphorylation of an immune tyrosine activation motif (ITAM) and hemITAM, respectively. However, unlike GPVI, the CLEC-2 receptor contains only one tyrosine motif in the intracellular domain. The mechanisms by which this receptor activates Syk are not completely understood. In this study, we identified a novel signaling mechanism in CLEC-2-mediated Syk activation. CLEC-2-mediated, but not GPVI-mediated, platelet activation and Syk phosphorylation were abolished by inhibition of PI3K, which demonstrates that PI3K regulates Syk downstream of CLEC-2. Ibrutinib, a Tec family kinase inhibitor, also completely abolished CLEC-2-mediated aggregation and Syk phosphorylation in human and murine platelets. Furthermore, embryos lacking both Btk and Tec exhibited cutaneous edema associated with blood-filled vessels in a typical lymphatic pattern similar to CLEC-2 or Syk-deficient embryos. Thus, our data show, for the first time, that PI3K and Tec family kinases play a crucial role in the regulation of platelet activation and Syk phosphorylation downstream of the CLEC-2 receptor.

Platelets are involved in many processes ranging from fighting microbial infections and triggering inflammation to promoting tumor angiogenesis and metastasis (1–7). Nevertheless, the primary physiological function of platelets is to act as essential mediators in maintaining homeostasis of the circulatory system by forming hemostatic thrombi that prevent blood loss

and maintain vascular integrity (8, 9). When there is vascular damage, exposure of the extracellular matrix recruits and activates platelets thereby leading to aggregation and formation of a fibrin-rich hemostatic plug at the injured site.

Platelets mainly express two different types of agonist receptors, G-protein-coupled receptors and tyrosine kinase pathway receptors, both of which are important for platelet activation. All tyrosine kinase pathway receptors, including GPVI, FcγRIIA, and CLEC-2, are linked to the activation of Syk (10–14). However, there are two different tyrosine kinase-coupled receptors. One is the ITAM³-containing receptors, such as the Fc receptor-γ chain (FcRγ) and FcγRIIA, which has the classic YXX(L/I)X_{6–12}YXX(L/I) with two tyrosine residues in its cytoplasmic domain. The other is a hemITAM-containing receptor, such as CLEC-2, which has only one tyrosine residue (YXX(L/I)) in its cytoplasmic domain (15, 16).

Glycoprotein VI is a platelet collagen receptor that is constitutively associated with the FcRγ (17). Upon activation of GPVI, FcRγ is phosphorylated by Src family kinases on the tyrosine residue of its ITAM (18–20), and spleen tyrosine kinase (Syk) binds to the ITAM through its two SH2 domains and becomes autophosphorylated. Tyrosine phosphorylation of Syk leads to phosphorylation of several adaptor proteins, such as phospholipase C γ2 (PLCγ2), linker for T-cell activation (LAT), and Src homology 2-containing leukocyte protein 76 (SLP76), activation of phosphoinositide 3-kinase (PI3K), and recruitment of Bruton's tyrosine kinase (Btk). Recent studies from our laboratory and others have also shown that PI3Kβ has an important role in GPVI-mediated platelet activation (21, 22).

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³ The abbreviations used are: ITAM, immune tyrosine activation motif; XLA, X-linked agammaglobulinemia; PIP₃, phosphatidylinositol 1,4,5-trisphosphate; SH, Src homology domain; PH, pleckstrin homology; CLL, chronic lymphocytic leukemia; GP, glycoprotein; Btk, Bruton's tyrosine kinase; PLC, phospholipase C; LAT, linker for activator of T-cell; Ab, antibody; SFK, Src family kinase.

Novel Syk Activation Mechanism in Platelets

However, the role of PI3K in CLEC-2 signaling is not clearly understood.

PI3K is also known to regulate the activation of the Tec family tyrosine kinases such as Btk, Tec, and Itk by regulating its translocation to the membrane. Btk and Tec were shown to play an important role in tyrosine kinase pathways in platelets, whereas Itk is known to play a major role in T-cell receptor signaling (23). Tec family kinases consist of a tyrosine kinase domain, SH2 and SH3 domains, and a pleckstrin homology (PH) domain coupled to a distinctive Tec homology domain (24–26). Previous studies have shown that Tec family kinases are regulated through phosphorylation by Src kinases (27) and recruitment to the membrane through interaction of its PH domain with phosphatidylinositol 3,4,5-trisphosphate (28, 29). The importance of Tec family kinases was brought to light when the human immunodeficiency X-linked agammaglobulinemia (XLA) disease was linked to mutations in Btk (30). Patients with XLA do not express functional Btk, and as a result they experience a severe loss of mature B-cells caused by a block during B-cell development (31). Human and murine B-cell lines lacking functional Btk have defective B-cell receptor-dependent tyrosine phosphorylation of PLC γ 2 and defective influx of calcium that is restored by the expression of wild-type Btk (29, 32–34). Platelets express Btk and Tec, which are tyrosine-phosphorylated in response to collagen and thrombin (23, 35). In addition, the importance of Tec family kinases, especially Btk, downstream of GPVI has been shown by using platelets from patients with XLA and Xid mice, both of which have a defective PH domain in Btk (36). In our study we also used ibrutinib, a pharmacological inhibitor for Tec family kinases. Even though ibrutinib is known as a highly potent small molecule inhibitor of Btk, it is in phase 3 clinical trials to treat CML. Recent studies in T-cells showed that it also inhibits other Tec family kinases (37). Ibrutinib selectively binds to cysteine residues in the allosteric inhibitory segment of Tec family kinases and irreversibly blocks the enzymatic activity by inhibiting the autophosphorylation of Tec family kinases (37). However, the role of Tec family kinases downstream of CLEC-2 has not been elucidated in platelets.

C-type lectin-like receptor-2 (CLEC-2) is highly expressed on platelets as noncovalent homodimers and at lower levels in neutrophils and dendritic cells (38). CLEC-2 can be activated by podoplanin, a physiological agonist that is expressed on the surface of a wide variety of cells, including lung type I alveolar cells, kidney podocytes, and lymphatic endothelial cells (39). The activation of CLEC-2 by podoplanin is essential for the separation and maintenance of the lymphatic system and blood vasculature (40, 41). In addition, podoplanin expressed on the surface of certain tumors is implicated in the process of tumor metastasis through CLEC-2 receptor activation on the platelets (42). Previous studies in human and murine platelets showed that, unlike GPVI, CLEC-2-mediated platelet activation is regulated by both SFKs and Syk. An interesting aspect of the CLEC-2 hemITAM is the presence of a single tyrosine motif in its cytoplasmic domain, which, upon phosphorylation by SFKs, activates Syk. However, the mechanism of SFKs regulating Syk activation through a single tyrosine-containing CLEC-2 hemITAM was not elucidated, as it is well established that two

SH2 domains of Syk have to bind to phosphotyrosine to make it active (15).

In this study, we have uncovered the molecular events mediating Syk activation downstream of the CLEC-2 receptor in platelets. Our study shows that novel signaling events mediated by PI3K and Tec family kinases regulate Syk activation downstream of the CLEC-2 receptor.

EXPERIMENTAL PROCEDURES

Mice—Mice used were between 8 and 12 weeks of age, and all animal experiments were performed in compliance with the Institutional Animal Care and Use Committee (IACUC) at Temple University (Philadelphia, PA). Tec/Btk double-deficient mice were previously described (32). The mice used in this study were of a mixed 129/Sv \times C57BL/6 background.

Materials—Apyrase (type VII) was obtained from Sigma. FURA-2 AM was from Molecular Probes (Eugene, OR). Convolvulin was purified according to the method described previously (43). Rhodocytin was isolated as described previously (44). LY294002, wortmannin, PP3, and PP2 were purchased from Biomol (Plymouth Meeting, PA). TGX-221 was purchased from Cayman Chemical (Ann Arbor, MI). PIK75, AS 252424, and IC87114 were from the laboratory of Shaun Jackson (Monash University, Victoria, Australia). Ibrutinib (PCI-32765) was from Selleckchem (Houston, TX). OXSI-2 was from Calbiochem. Whatman protein nitrocellulose transfer membrane was obtained from Fisher; LI-COR Odyssey blocking buffer was purchased from LI-COR Biosciences (Lincoln, NE). Antibodies to Syk (Tyr-525/526), PLC γ 2 (Tyr-759), LAT (Tyr-191), Btk (Tyr-223), Akt (Ser-473), Total Btk, β -actin, and β_3 -integrin were bought from Cell Signaling Technology (Beverly, MA), Total Syk, PLC γ 2, and Akt antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of Human Platelets—Blood was collected from informed healthy volunteers into one-sixth volume of acid/citrate/dextrose (2.5 g of sodium citrate, 2 g of glucose, and 1.5 g of citric acid in 100 ml of de-ionized water). Platelet-rich plasma was obtained by centrifugation at 230 \times g for 20 min at ambient temperature and incubated with 1 mM aspirin for 30 min at 37 $^{\circ}$ C. Platelets were isolated from plasma by centrifugation at 980 \times g for 10 min at ambient temperature and resuspended in Tyrode's buffer, pH 6.5 (138 mM NaCl, 2.7 mM KCl, 2 mM MgCl $_2$, 0.42 mM NaH $_2$ PO $_4$, 5 mM glucose, 10 mM PIPES, pH 6.5, containing 20 nM PGE1, 500 μ M EGTA, and 0.2 units/ml apyrase). Platelets were isolated from Tyrode's buffer, pH 6.5, by centrifugation at 980 \times g for 10 min and resuspended in Tyrode's buffer, pH 7.4 (138 mM NaCl, 2.7 mM KCl, 2 mM MgCl $_2$, 0.42 mM NaH $_2$ PO $_4$, 5 mM glucose, 10 mM HEPES, and 0.2 units/ml apyrase, pH 7.4). The platelet count was adjusted to 2–2.5 \times 10 8 /ml. Approval was obtained from the Institutional Review Board of Temple University for these studies. Informed consent was provided prior to blood donation.

Preparation of Murine Platelets—Washed murine platelets were prepared as described previously (45). Platelet counts were determined using a Hemavet 950FS blood cell counter (Drew Scientific Inc., Dallas). The platelet count was adjusted to 2 \times 10 8 cells/ml for membrane preparation.

Platelet Aggregation—Platelet aggregation was measured using a lumi-aggregometer (Chrono-Log, Havertown, PA) at 37 °C under stirring conditions. A 0.5-ml (for human platelets) or 0.25-ml (for murine platelets) sample of washed platelets was stimulated with different agonists, and the change in light transmission was measured. Platelets were preincubated with different inhibitors where noted before agonist stimulation. The chart recorder (Kipp and Zonen, Bohemia, NY) was set for 0.2 mm/s.

Measurement of Intracellular Ca^{2+} Mobilization—Platelet-rich plasma was incubated with 5 μ M FURA-2 AM for 45 min at 37 °C. Platelets were prepared as described above, and fluorescence was measured in a PerkinElmer Life Sciences apparatus with excitation set at 340 nm and emission set at 510 nm. Ca^{2+} concentration was calculated using a KaleidaGraph.

Syk Kinase Assay—Washed human platelets (1×10^9 cells/ml) were stimulated with the agonists in the presence and absence of the inhibitors. The reaction was stopped by the addition of an equal volume of cold 2 \times Nonidet P-40 lysis buffer (2 \times Lysis Buffer: 50 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM EGTA, 2% Nonidet P-40 plus Halt protease and phosphatase inhibitors), and the samples were rocked at 4 °C for 30 min. Samples were centrifuged at 12,000 $\times g$ at 4 °C for 10 min. Supernatants were transferred to clean tubes, and 2 μ g of anti-Syk (Santa Cruz Biotechnology (4D10) catalog no. sc4210m) was added. Samples were rocked for an hour at 4 °C, and 50 μ l of washed TrueBlot[®] anti-mouse IgG IP beads (Rockland) were added and rocked for an additional hour at 4 °C. Beads were washed three times with 1 \times lysis buffer and one time with Kinase buffer (50 mM MOPS, pH 7.4, 5 mM $MgCl_2$, 5 mM $MnCl_2$, and 1 mM DTT). Kinase buffer (45 μ l) containing 5 μ g of tubulin was added to the beads, and the reaction was started by addition of 5 μ l of 25 μ M ATP and incubated at room temperature for 10 min. Reactions were terminated by addition of 20 mM EDTA. Beads were pelleted by centrifugation, and 30 μ l of supernatant was mixed with 10 μ l of 4 \times sample buffer for measurement of tubulin tyrosine phosphorylation. Beads were washed one time with PBS, and 50 μ l of 2 \times sample buffer was added to the beads to assess the phosphorylation state of Syk. All samples were boiled for 10 min. The samples were run on SDS-8% PAGE.

Preparation of Platelet Membrane Fractions—Platelets (2×10^9 cells/ml) were stimulated with agonists in the presence of inhibitors or antagonists/vehicle, and the reaction was stopped by the addition of 2 \times Halt protease and phosphatase mixture solution (Pierce) in Tyrode's buffer and flash frozen. Platelets were lysed by four freeze/thaw cycles and then centrifuged at 1500 $\times g$ for 10 min at 4 °C to pellet unlysed cells. Supernatants were centrifuged at 100,000 $\times g$ for 30 min at 4 °C. The supernatant is a cytosolic fraction, and the pellet is a membrane and cytoskeleton fraction. The pellet was carefully rinsed with 0.9% saline and resuspended in 100 ml of 1% Triton X-100. Samples were then centrifuged at 15,000 $\times g$ for 10 min at 4 °C to pellet the cytoskeleton. The membrane-rich supernatant was collected, and an equal volume of 2 \times sample buffer was added. Protein estimation was performed using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). 10 μ g of protein were run on an SDS-polyacrylamide gel for separa-

tion and transferred to nitrocellulose membranes for specific immunoblotting.

Western Blot Analysis—Platelets were stimulated with agonists in the presence of inhibitors or vehicles for the appropriate time under stirring conditions at 37 °C, and the reaction was stopped by the addition of 0.1 volume of 6.6 N $HClO_4$. The resulting protein precipitate was collected by centrifugation at 13,000 $\times g$ for 4 min followed by a wash in 0.5 ml of deionized water. The protein was again pelleted by centrifugation at 13,000 $\times g$ for 4 min. The protein pellets were solubilized in sample buffer containing 0.1 M Tris base, 2% SDS, 1% (v/v) glycerol, 0.1% bromphenol blue, and 100 mM DTT and then boiled for 10 min. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Whatman Protran). Membranes were blocked with Odyssey blocking buffer for 1 h at ambient temperature, incubated overnight at 4 °C with the desired primary antibody, and then washed four times with TBS-T. Membranes were then incubated with appropriate secondary infrared dye-labeled antibody for 60 min at room temperature and washed four times with TBS-T. Membranes were examined with a LI-COR Odyssey infrared imaging system.

Statistical Analysis—Each experiment was repeated at least three times. Results are expressed as means \pm S.E. with number of observations (n). Data were analyzed using KaleidoGraph software. Significant differences were determined using Student's t test. Differences were considered significant at $p \leq 0.05$.

RESULTS

PI3K Is Essential for Platelet Activation by CLEC-2 Agonists—Previous studies with pan-PI3K inhibitors and knock-in murine platelets have shown that PI3K plays an important role in mediating platelet activation by GPVI agonists (21, 22, 46). However, the role of PI3K in CLEC-2-mediated platelet activation has not been elucidated. The functional role of PI3K in CLEC-2-mediated platelet activation was investigated by pretreating both human and murine platelets with two structurally different pan-PI3K inhibitors (22), wortmannin and LY294002, prior to activation with three separate CLEC-2 agonists (rhodocytin, human or mouse CLEC-2 Ab, and fucoidan). Pretreatment of human and murine platelets with wortmannin and LY294002 abolished platelet aggregation induced by all CLEC-2 agonists as shown in Fig. 1, similar to platelets treated with PP2, a pan-SFK inhibitor, which abolishes all tyrosine kinase-mediated platelet activation (22, 45, 47). To better understand the role of individual PI3K isoforms, we utilized different isoform-specific PI3K inhibitors and evaluated their effect on CLEC-2-mediated platelet aggregation in human and murine platelets. Rhodocytin-induced platelet aggregation was abolished in platelets treated with the PI3K β inhibitor, TGX-221^{***}, ($p \leq 0.05$) (Fig. 2) (48–50), but not by other isoform-selective inhibitors. These data suggest that PI3K β plays an essential role downstream of CLEC-2.

PI3K Regulates Syk Activation Downstream of the CLEC-2 Receptor—To assess the role of PI3K in CLEC-2 receptor signaling, we pretreated platelets with pan-PI3K inhibitors, induced activation with rhodocytin, and measured protein phosphorylation of the downstream signaling molecules Syk,

Novel Syk Activation Mechanism in Platelets

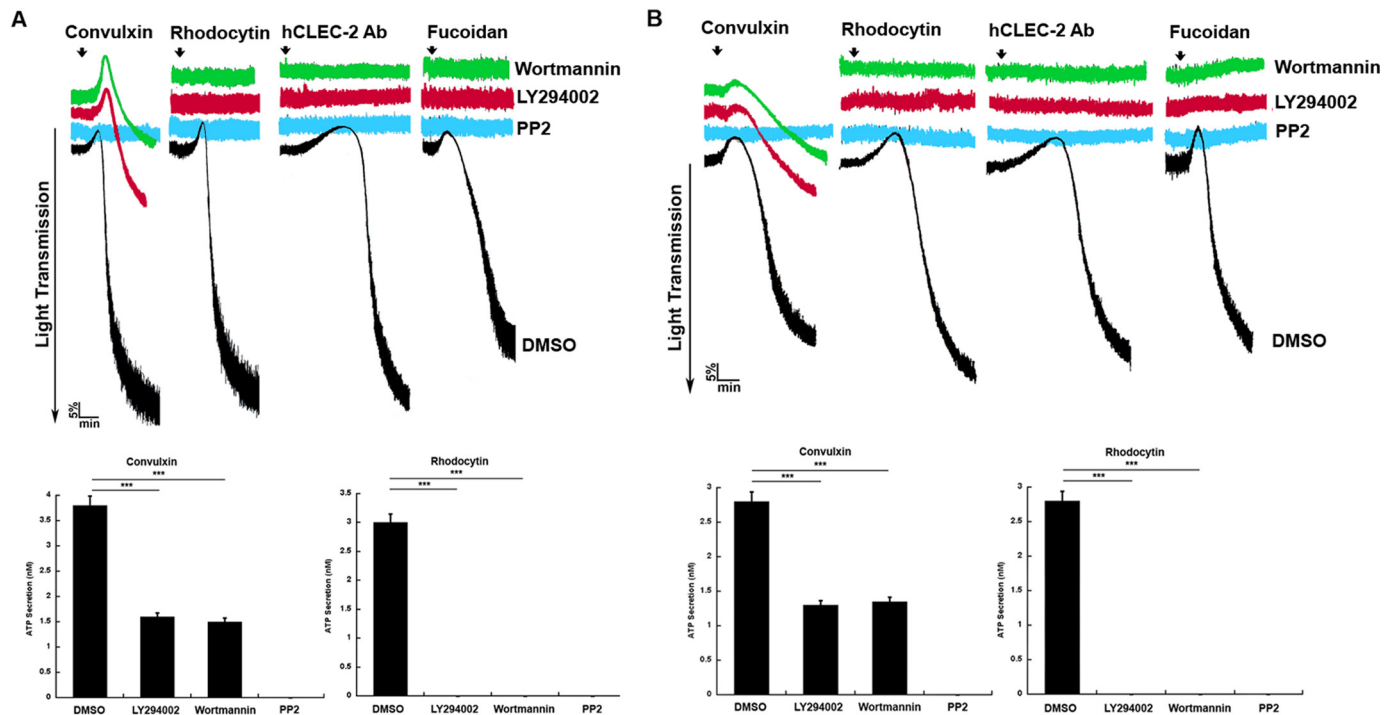


FIGURE 1. Effect of pan-PI3K inhibitors on CLEC-2-mediated platelet aggregation and secretion in human and murine platelets. Washed human (A) and murine (B) platelets were pretreated with the PP2 (10 μ M), LY294002 (25 μ M), or wortmannin (100 nM) at 37 °C for 5 min followed by stimulation with convulxin (100 ng/ml) or rhodocytin (30 or 100 nM) or hCLEC-2 Ab (10 μ g/ml) or fucoidan (50 μ g/ml) under stirred conditions. Platelet aggregation was measured by aggregometry. The tracings are representative of data from at least three independent experiments. Dense granule secretion was assessed by measuring ATP with lumichrome. Graphs represent mean \pm S.E. from at least three different experiments (***, $p \leq 0.05$).

PLC γ 2, LAT, and Akt, as well as calcium mobilization. We compared these results with GPVI signaling. Unlike GPVI, CLEC-2-mediated phosphorylation events were abolished in platelets treated with pan-PI3K inhibitors (Fig. 3A). Similar results were obtained with other CLEC-2 agonists (data not shown). We also evaluated the kinase activity of Syk from platelets pretreated with LY294002 that are stimulated with rhodocytin by using an *in vitro* kinase assay. Syk, immunoprecipitated from LY294002-treated and rhodocytin-activated platelets, did not phosphorylate tubulin *in vitro* as shown in Fig. 3B. These results contrast immunoprecipitated Syk from LY294002-treated platelets stimulated with the GPVI agonist convulxin, where there was no inhibition of Syk kinase activity. These results further demonstrate that PI3K plays a crucial role in Syk activation downstream of CLEC-2 and establishes that PI3K has an important role in CLEC-2 receptor activation.

SFKs Regulate PI3K Activation Downstream of CLEC-2 Receptor—We investigated the mechanism of activation of PI3K by the CLEC-2 receptor and compared it with GPVI. Previous studies have shown that SFKs, especially Fyn and Lyn, regulate PI3K activation by phosphorylation of the p85 subunit (51–53). We evaluated the role of SFKs and Syk in the activation of PI3K downstream of CLEC-2 by using a pan-Src family kinase inhibitor (PP2) and two structurally different Syk inhibitors (OXSI2 and Go6976). We measured Akt phosphorylation as a readout of PI3K activity. As shown in the Fig. 3C, both GPVI- and CLEC-2-induced phosphorylation of Akt was abolished in PP2-treated platelets. Interestingly, Syk inhibitors, OXSI-2 (54) or Go6976 (55), did not abolish Akt phosphorylation downstream of CLEC-2 in contrast to GPVI (Fig. 3, C and

D). These results show that PI3K activation occurs downstream of CLEC-2 receptor in a SFK-dependent and Syk-independent manner.

Tec Family Kinases Regulate Syk Activity Downstream of the CLEC-2 Receptor in Human Platelets—Because PI3K is a lipid kinase and not a tyrosine kinase, there must be an intermediate molecule that regulates Syk activation downstream of CLEC-2. Previous studies in platelets and B-cells have shown that PI3K plays a key role in activation of Tec family kinases (28, 35, 56). In platelets, Tec family kinases, especially Btk and Tec, are known to regulate the phosphorylation of PLC γ 2 downstream of GPVI (23, 57, 58), but little is known about the role of these Tec family kinases in CLEC-2 signaling. In this study, we evaluated the role of Tec family kinases in CLEC-2-mediated platelet activation by using ibuprofen, a Tec family kinase inhibitor (24, 59, 60). Strikingly, in contrast to GPVI, ibuprofen abolished not only CLEC-2-induced platelet aggregation and secretion (Fig. 4A) but also the tyrosine phosphorylation of Syk, LAT, and PLC γ 2 (Fig. 4B). Furthermore, ibuprofen-treated platelets stimulated with rhodocytin abolished Syk kinase activity in an *in vitro* kinase assay, demonstrating that Tec family kinases have an important role in CLEC-2 signaling (Fig. 4C).

Role of PI3K and Syk in Tec Family Kinase Translocation Downstream of GPVI and CLEC-2—Previous studies have shown that PI3K plays an important role in the activation of Tec family kinases by helping in their membrane translocation (35, 61). PIP $_3$ serves as a docking site for the PH domain of Btk, where it is phosphorylated and activated by SFKs (62). We evaluated the effect of the PI3K inhibitor LY294002 on the translocation of Btk upon stimulation of platelets with rhodocytin. As

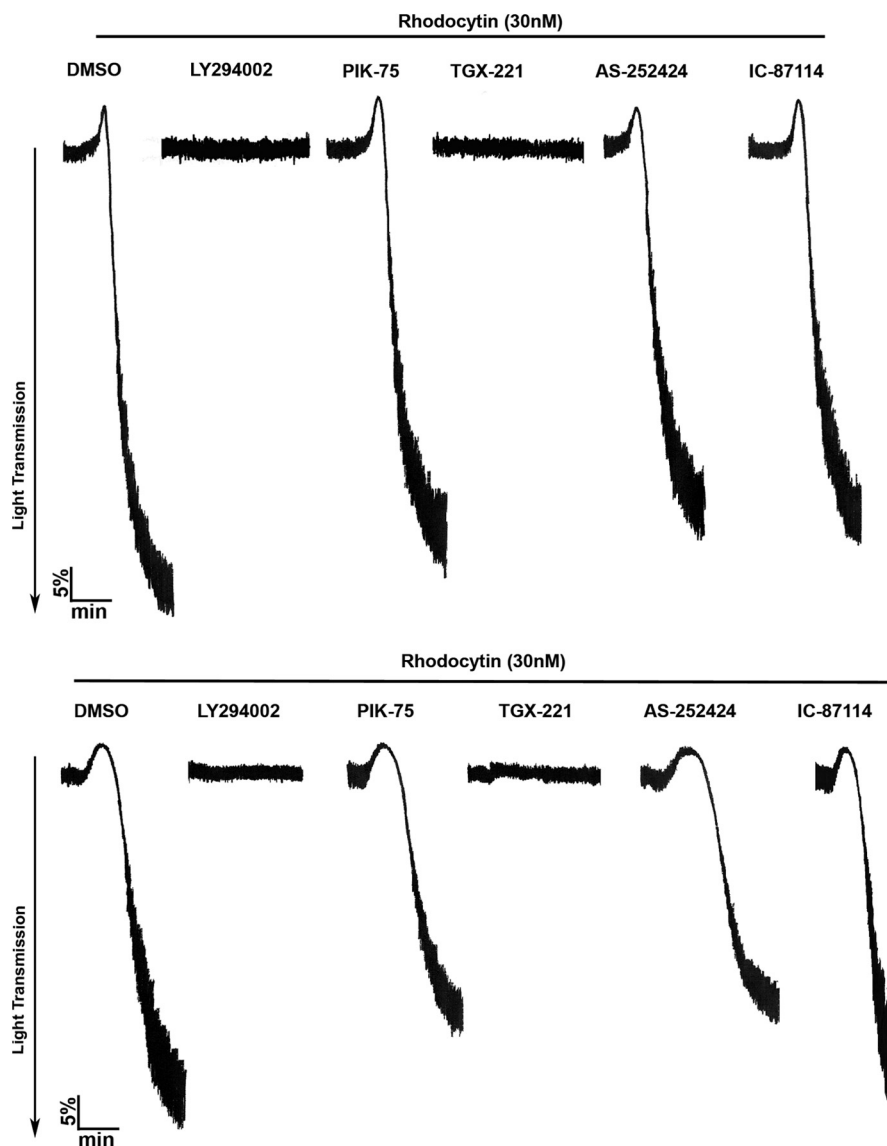


FIGURE 2. **Effect of specific PI3K isoform inhibitors on CLEC-2-mediated platelet aggregation in human and murine platelets.** Washed human (A) and murine (B) platelets were incubated with 25 μM LY294002 (pan-PI3K inhibitor), 100 nM PIK75 (PI3K α inhibitor), 500 nM TGX-221 (PI3K β inhibitor), 2 μM AS-252424 (PI3K γ inhibitor), or 1 μM IC87114 (PI3K δ inhibitor) and stimulated at 37 $^{\circ}\text{C}$ with rhodocytin (30 nM) under stirred conditions. Platelet aggregation was measured by aggregometry. The tracings are representative of data from at least three independent experiments.

shown in Fig. 5, translocation of Btk was abolished in LY294002-treated platelets stimulated with rhodocytin. However, the Syk inhibitor, OXSI-2, did not affect Btk translocation (Fig. 5). These results show that Btk is activated in a Syk-independent and PI3K-dependent manner downstream of CLEC-2 and that Btk regulates Syk activation downstream of CLEC-2.

PI3K and Tec Family Kinases Are Crucial for CLEC-2 Activation Even at Higher Concentration of Agonists—In this study, the role of PI3K and Tec family kinases in regulation of Syk was further analyzed by activation of human platelets with higher concentrations of rhodocytin. Both LY294002 and ibrutinib abolished platelet aggregation induced by intermediate and higher concentrations of rhodocytin (Fig. 6, A and B). This was associated with inhibition of tyrosine phosphorylation of Syk as well as the downstream protein PLC γ 2 (Fig. 6B). These data confirm that PI3K and Tec family kinases are essential mediators of platelet activation by CLEC-2.

Tec Family Kinases Play a Crucial Role in CLEC-2 Receptor Activation in Murine Platelets—We confirmed the crucial role of Tec family kinases in CLEC-2 signaling in murine platelets by using pharmacological and genetic approaches. As shown in Fig. 7A, murine platelets treated with ibrutinib abolished platelet functional responses and phosphorylation of proteins downstream of CLEC-2 (Fig. 7B), similar to what was observed in human platelets with different CLEC-2 agonists. However, CLEC-2-mediated platelet activation is only inhibited but not abolished in *Xid* mice (Btk-defective), which suggests that other Tec family kinases may also have a role in CLEC-2-mediated platelet activation. Ibrutinib, a Tec family kinase inhibitor, was used in *Xid* (Btk-deficient) murine platelets to show the role of other Tec kinases in the absence of Btk. As shown in the Fig. 7C, platelet aggregation induced by the CLEC-2 monoclonal mouse antibody was abolished in *Xid* platelets similar to wild-type platelets treated with ibrutinib, which suggests that, apart from

Novel Syk Activation Mechanism in Platelets

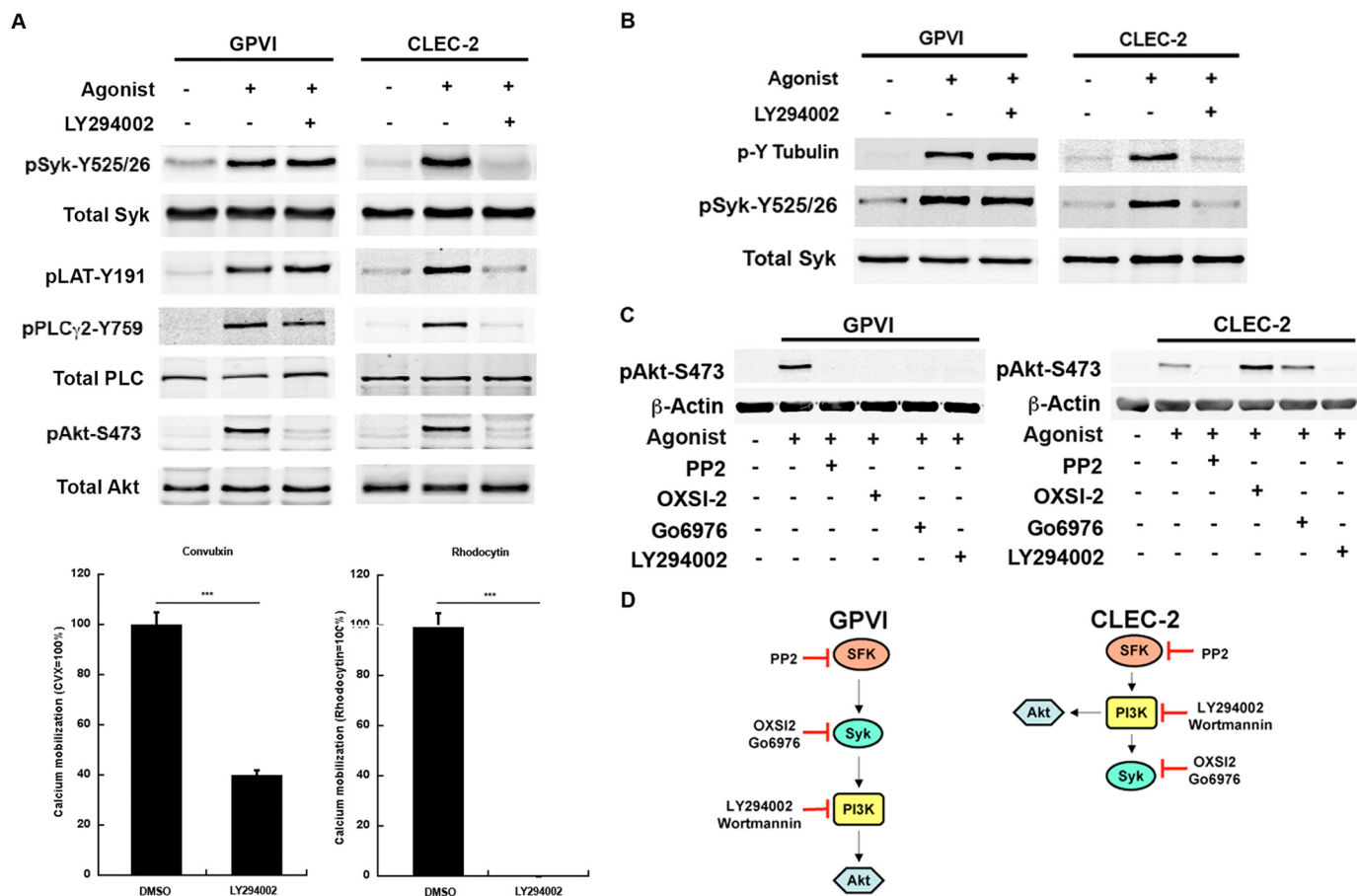


FIGURE 3. Role of PI3K in CLEC-2 signaling. **A**, washed human platelets were incubated with 25 μ M LY294002 for 5 min followed by stimulation with convulxin (100 ng/ml) or rhodocytin (30 nM) under stirred conditions. Platelet proteins were separated by SDS-PAGE, Western-blotted, and probed for phospho-Syk (Tyr-525/526), LAT (Tyr-191), PLC γ 2 (Tyr-759), and Akt (Ser-473). The Western analysis shown is a representative of three experiments. Calcium mobilization was measured by using FURA-2. Data are mean \pm S.E. ($n = 3$); ***, $p < 0.05$ compared with agonist without inhibitor. **B**, washed human platelets were incubated with 25 μ M LY294002 for 5 min followed by stimulation with convulxin (100 ng/ml) or rhodocytin (30 nM) under stirred conditions. Platelets were lysed in Nonidet P-40 lysis buffer and immunoprecipitated for Syk. Syk kinase assay was performed to measure the activity of Syk by measuring tubulin phosphorylation. **C**, washed human platelets were incubated with PP2 (10 μ M), OXSI-2 (1 μ M), Go6976 (1 μ M), or LY294002 (25 μ M) followed by stimulation with convulxin (100 ng/ml) or rhodocytin (30 nM) under stirred conditions. Platelet proteins were separated by SDS-PAGE, Western-blotted, and probed for phospho-Akt (Ser-473). The results shown are representative of data from at least three independent experiments. **D**, model representation of role of SFK and Syk in PI3K activation.

Btk, other Tec kinases also regulate CLEC-2-mediated platelet activation. Similar results were obtained with rhodocytin (data not shown). These results suggest that Tec family kinases are essential in murine platelet activation downstream of CLEC-2.

Tec and Btk Are Required for CLEC-2 Function in Vivo—Loss of platelet CLEC-2 function results in blood-filled lymphatics due to loss of lympho-venous hemostasis in the developing embryo and post-natal animal (40, 63, 64). Embryos lacking CLEC-2 or the essential CLEC-2 signaling effectors Syk, SLP-76, or PLC γ 2 exhibit a characteristic pattern of blood-filled lymphatic vessels in mid-gestation that includes those in the skin and the developing mesentery and intestine (40). To test the role of Tec family kinases downstream of CLEC-2 *in vivo*, we examined mice lacking this class of tyrosine kinases. Animals lacking Btk or Tec alone exhibited no vascular phenotype, but Btk^{-/-}Tec^{-/-} double deficient embryos exhibited cutaneous edema associated with blood-filled vessels in a typical lymphatic pattern (Fig. 8, A and B). Histologic examination of LYVE1+ lymphatic vessels confirmed the presence of blood in cutaneous and mesenteric lymphatic vessels of Btk^{-/-}Tec^{-/-}

doubly deficient embryos (Fig. 8, C and D). These studies demonstrate that Btk and Tec play crucial roles in CLEC-2 signaling in murine platelets *in vivo*.

DISCUSSION

CLEC-2 is the first member of the C-type lectin family of receptors identified to regulate platelets through sequential activation of Src and Syk family tyrosine kinases, thereby initiating a signaling cascade that leads to platelet activation. Syk is shown to be activated downstream of both the GPVI and CLEC-2 receptors. Syk activation requires binding of its two SH2 domains to two phosphorylated tyrosine residues. Upon GPVI activation in platelets, Syk is recruited to the membrane via its two SH2 domains that bind two phospho-ITAM motifs in the cytoplasmic domain of Fc γ R that are phosphorylated by SFKs. It was also established by using cell lines that mutation of any one of the SH2 domains of Syk or lack of any one of the two phospho-ITAMs completely abolished Syk activation. However, unlike GPVI, the CLEC-2 hemITAM contains only one tyrosine motif in the intracellular domain. The mechanism by

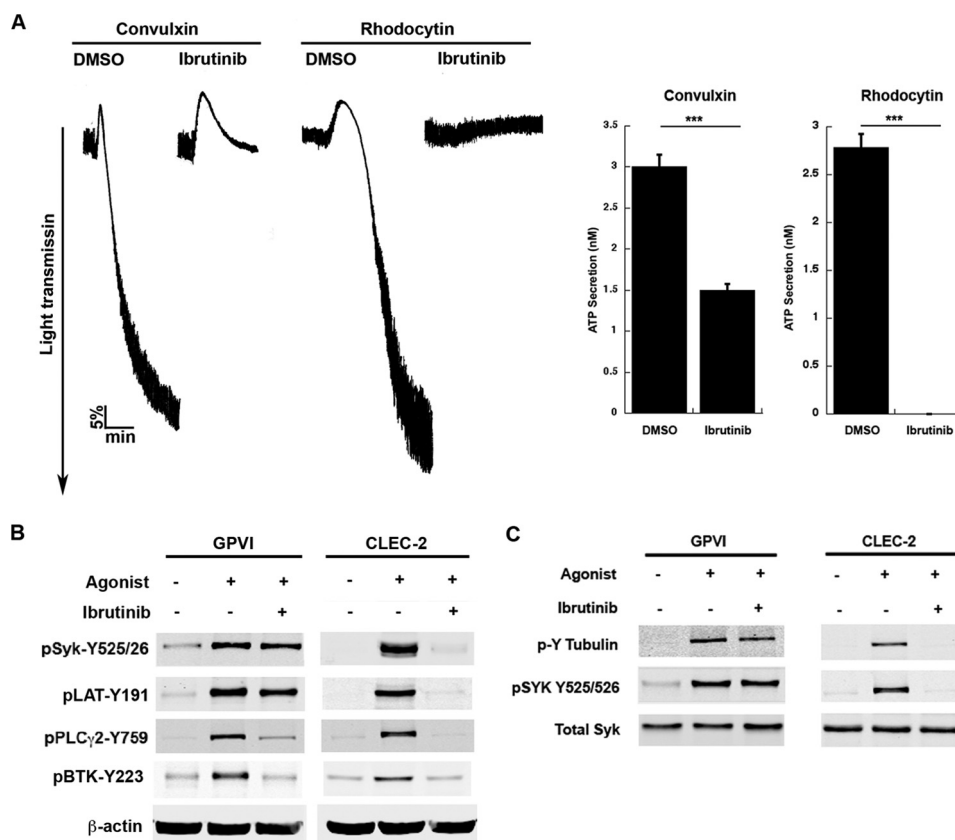


FIGURE 4. Effect of ibrutinib on CLEC-2-mediated platelet activation and signaling in humans. *A*, washed human platelets were incubated with ibrutinib (10 nM) for 5 min followed by stimulation with convulxin (100 ng/ml) or rhodocytin (30 nM) under stirred conditions, and aggregation was measured. *** represents significance of $p < 0.05$. *B*, washed human platelets were incubated with ibrutinib (5 nM) for 5 min followed by stimulation with convulxin (100 ng/ml) or rhodocytin (30 nM) under stirred conditions. Platelet proteins were separated by SDS-PAGE, Western-blotted, and probed for phospho-Syk (Tyr-525/526) and PLCγ2 (Tyr-759). β-Actin was used as a lane loading control. *C*, washed human platelets were incubated with ibrutinib (10 nM) for 5 min followed by stimulation with convulxin (100 ng/ml) or rhodocytin (30 nM) under stirred conditions. Platelets were lysed in Nonidet P-40 lysis buffer and immunoprecipitated for Syk. Syk kinase assay was performed to measure the activity of Syk by measuring tubulin phosphorylation. The results shown are representative of data from at least three independent experiments.

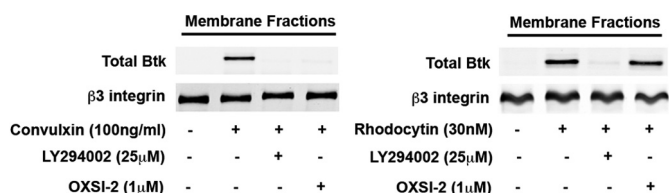


FIGURE 5. Role of PI3K and Syk in membrane translocation of Tec family kinases downstream of CLEC-2. Washed human platelets, incubated with LY294002 (25 μM) or OXSI-2 (1 μM), were stimulated with convulxin (100 ng/ml) or rhodocytin (30 nM), and the translocation of Btk to the membrane was measured. β₃-Integrin was used as lane loading control. All the Western blot analysis shown is a representative of at least three independent experiments.

which a single tyrosine motif containing CLEC-2 hemITAM activates Syk is not completely understood. The results from our study show, for the first time, that a novel PI3K/Btk axis regulates Syk activation downstream of the CLEC-2 receptor in platelets.

Previous studies have shown that PI3K plays an important role in GPVI-mediated platelet activation (21, 22, 46). However, little is known about the role of PI3K in CLEC-2 signaling. It is expected that it will have the same role in both GPVI- and CLEC-2-mediated platelet activation, due to similarity of the pathways. Surprisingly, PI3K inhibition, especially PI3Kβ, abolished CLEC-2-mediated platelet activation in both human and

murine platelets, identifying its essential role in CLEC-2 signaling. A recent study on Dectin-1, a C-type lectin phagocytic receptor for macrophages, also showed that PI3K is essential for the phagocytic function of this receptor (65).

Unlike GPVI, CLEC-2-mediated platelet functional responses were abolished (Fig. 1) in the presence of pan-PI3K inhibitors. However, PI3K is known to regulate calcium mobilization but not Syk activation downstream of GPVI (22). Interestingly, in contrast to GPVI agonists, we found that CLEC-2-mediated Syk and PLCγ2 activation, as well as calcium mobilization, were completely abolished by PI3K inhibitors (Fig. 3A). Furthermore, the crucial role of PI3K for Syk activation was analyzed by an *in vitro* kinase assay (55). Syk activity was abolished in PI3K inhibitor-treated platelets stimulated with rhodocytin but not with convulxin, a GPVI agonist (Fig. 3B). These results further prove that Syk activation is regulated by PI3K downstream of CLEC-2, whereas PI3K is downstream of Syk in GPVI signaling pathways.

While evaluating the possible tyrosine kinases that depend on PI3K activation, we found that Tec family kinases (Btk, Itk, and Tec) translocate to the membrane in a PIP₃-dependent manner (28, 35, 56). It was shown that macrophages deficient in Btk abolished the phagocytic function of the Dectin-1 receptor similar to that of pretreatment with PI3K inhibitors (65, 67).

Novel Syk Activation Mechanism in Platelets

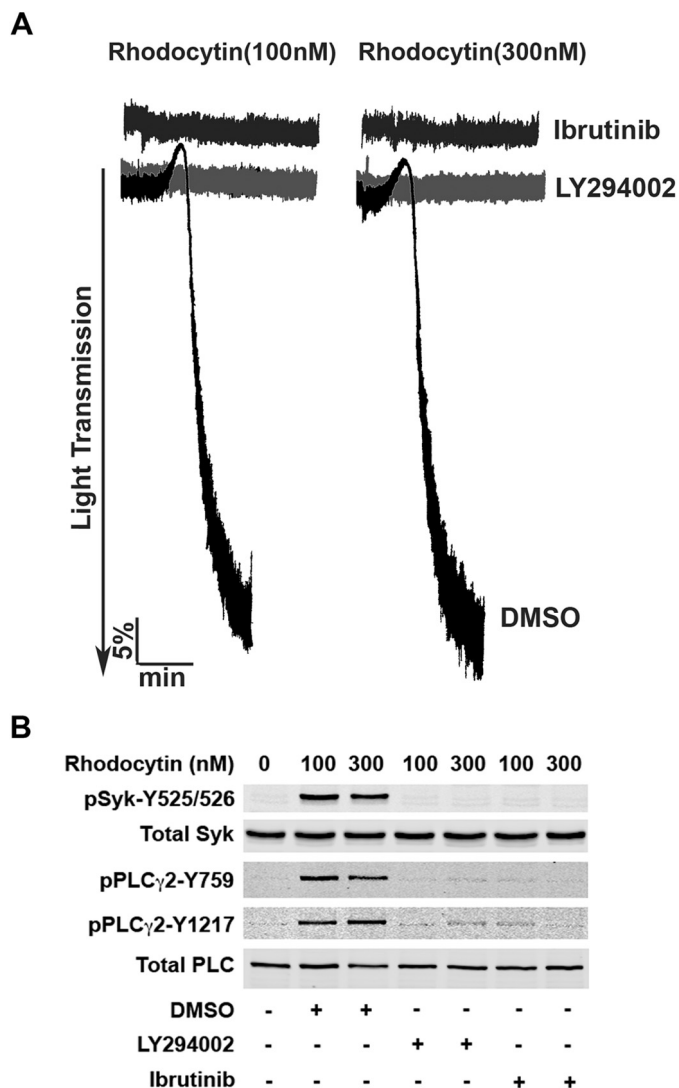


FIGURE 6. Effect of ibrutinib and LY294002 on human platelet activation at high concentrations of CLEC-2 agonist. *A*, washed human platelets, incubated with ibrutinib (10 nM) and LY294002 (25 μ M), were stimulated with 100 and 300 nM rhodocytin. Platelet aggregation was measured by aggregometry. The tracings are representative of data from at least three independent experiments. *B*, platelet proteins were separated by SDS-PAGE, Western-blotted, and probed for phospho-Syk (Tyr-525/526) and PLC γ 2 (Tyr-759 and Tyr-1217). All the Western blot analyses shown are a representative of at least three independent experiments.

Another study in platelets has shown that a Tec family kinase inhibitor abolished CLEC-2-mediated micro-particle generation (68). These results suggest a crucial role of Tec family kinases in CLEC-2 signaling. We elucidated the role of Tec family kinases in CLEC-2 signaling by using ibrutinib, a Tec family kinase inhibitor (24, 59). Ibrutinib is known as a highly potent small molecule inhibitor of Btk (37). Because of the structural similarity between Btk, Itk, and Tec, ibrutinib is known to affect all these Tec family kinases (60). Ibrutinib abolished platelet aggregation and Syk phosphorylation induced by rhodocytin and other CLEC-2 agonists (Fig. 4, *A* and *B*). Consistent with our results and interpretation, a previous study has shown that CLEC-2-expressing DT40 cells deficient in Tec kinases failed to respond to rhodocytin, demonstrating the crucial role of Tec kinases in CLEC-2 signaling (15).

Interestingly, Btk translocation downstream of CLEC-2 was abolished by PI3K inhibitors but not by Syk inhibitors (Fig. 5). From these data, we suggest that PI3K is activated upstream of Syk and Tec kinases. Previous studies have shown that Btk translocation to the membrane via PIP₃ is necessary for its activation by SFKs (35, 61). Furthermore, the crucial role of Tec kinases for Syk activation was analyzed by an *in vitro* kinase assay. Syk activity was abolished in ibrutinib-treated platelets stimulated with rhodocytin similar to platelets treated with a PI3K inhibitor (Fig. 4C). These results further prove that, downstream of CLEC-2, Syk activation is regulated by Tec family kinases and is dependent on PI3K but not Syk, unlike GPVI signaling pathways. These data also demonstrate that Tec family kinases are activated upstream of Syk. Btk phosphorylation is abolished by PP2, an SFK inhibitor, in both convulxin- and rhodocytin-stimulated platelets (data not shown). This is consistent with the proposal that phosphorylation of Btk is mediated by Src family kinase after translocation to the plasma membrane (26). Src family kinases might also regulate the pathways upstream of PI3K enabling the activation of PI3K upon binding to phosphorylated hemITAM.

Similar to human platelets, CLEC-2-induced murine platelet aggregation and protein phosphorylation of Syk and PLC γ 2 are abolished with treatment of ibrutinib (Fig. 7*A*). However, platelet activation by CLEC-2 agonists is only inhibited but not abolished in *Xid* murine platelets (Btk-defective) similar to GPVI (Fig. 7*B*). It is possible that other Tec kinases could compensate for the lack of Btk as shown in GPVI signaling (69). When murine platelets were treated with a PI3K inhibitor, we observed that CLEC-2-mediated platelet activation was abolished because all Tec family kinases depend on PIP₃ for activation (28, 35, 56). Thus, the lack of PIP₃ prevents activation of all Tec family kinases, and hence, in both human and murine platelets, CLEC-2-induced platelet aggregation is abolished. We supported this hypothesis by stimulating ibrutinib-treated or *Xid* (Btk-defective) murine platelets with CLEC-2 agonists and compared this with GPVI. As shown in the Fig. 7*C*, when *Xid* murine platelets were treated with ibrutinib, CLEC-2-mediated aggregation was abolished similar to wild-type platelets treated with ibrutinib, suggesting that more than one Tec family kinase regulates CLEC-2-mediated platelet activation. We also studied the role of these Tec family kinases downstream of CLEC-2 *in vivo* by using Btk^{-/-}, Tec^{-/-}, and Btk^{-/-}Tec^{-/-} double-deficient mouse embryos. Previous studies have shown that CLEC-2 expressed on platelets regulates blood/lymphatic vessel separation and lymphatic vascular development and also helps in maintaining high endothelial vascular integrity during lymphocyte transmigration from blood vessels to lymph nodes (40, 41, 63, 64, 70–72). Our studies showed that embryos lacking both Btk^{-/-} and Tec^{-/-} exhibited cutaneous edema associated with blood-filled vessels in a typical lymphatic pattern in the skin similar to CLEC-2-deficient or Syk-deficient mice (Fig. 8, *A–D*) (63). This phenotype was not seen in either Btk^{-/-} or Tec^{-/-}-deficient mice, supporting our hypothesis that lack of Btk in platelets can be compensated by other Tec family kinases downstream of the CLEC-2 receptor. It would be interesting to establish whether these signaling events are also used by other hemITAM receptors such as Dectin-1.

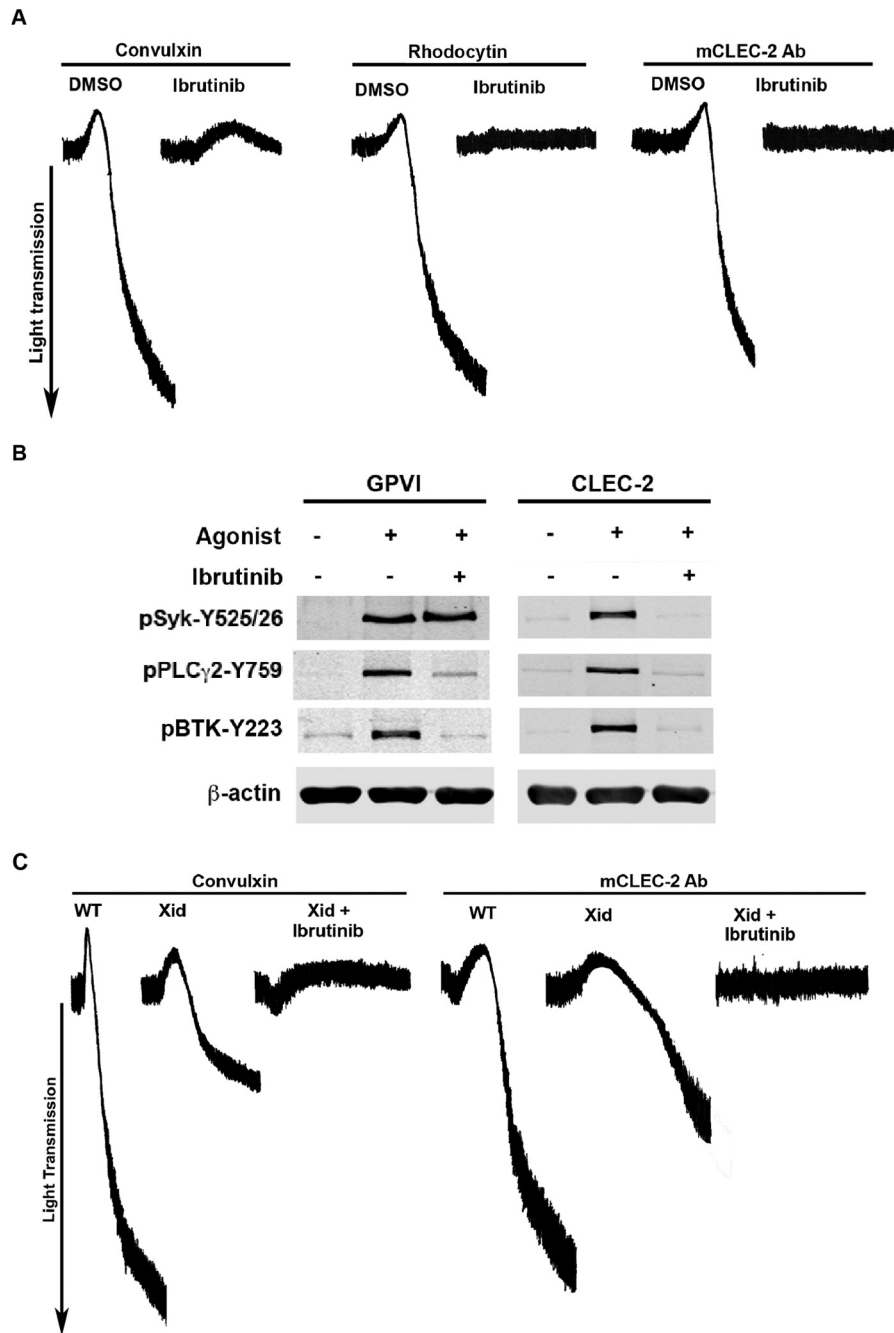


FIGURE 7. Effect of ibrutinib on murine platelet activation downstream of CLEC-2 receptor. *A*, washed murine wild-type platelets were incubated with DMSO or ibrutinib (10 nM) for 5 min followed by stimulation with convulxin (30 ng/ml) or rhodocytin (10 nM) or mouse CLEC-2 ab (3 μg/ml) under stirred conditions. Platelet aggregation was measured by aggregometry. The tracings are representative of data from at least three independent experiments. *B*, platelet proteins were separated by SDS-PAGE, Western-blotted, and probed for phospho-Syk (Tyr-525/526), Btk (Tyr-223), and PLC-γ2 (Tyr-759). β-Actin was used as a lane loading control. *C*, washed *Xid* (Btk-defective) and wild-type murine platelets were incubated with DMSO or ibrutinib (5 nM) for 5 min followed by stimulation with mouse CLEC-2 Ab (3 μg/ml) under stirred conditions. Platelet aggregation was measured by aggregometry. The tracings are representative of data from at least three independent experiments.

It would also be interesting to evaluate the potential affect of ibrutinib on platelet activation in human patients with chronic lymphocytic leukemia (CLL) (24). Recent phase 3 clinical studies using ibrutinib showed that a small percentage of patients have bruises on the skin, a characteristic of a lack of platelet activation (24). It is also shown that CLL patients treated with ibrutinib have defects in collagen and von Willebrand factor-mediated platelet activation (73). It would be another key aspect to take into consideration while prescribing ibrutinib for

a CLL patient. It is possible that treatment of CLL patients with ibrutinib may abolish podoplanin- and CLEC-2-mediated platelet activation *in vivo* that helps to maintain lymphatic system integrity.

In summary, this study demonstrated, for the first time, that a PI3K/Tec kinase axis regulates Syk activation downstream of CLEC-2. We propose a model (Fig. 9) demonstrating the following. 1) Upon activation of CLEC-2 in platelets, SFKs phosphorylate the hemITAM. Previous studies have shown that

Novel Syk Activation Mechanism in Platelets

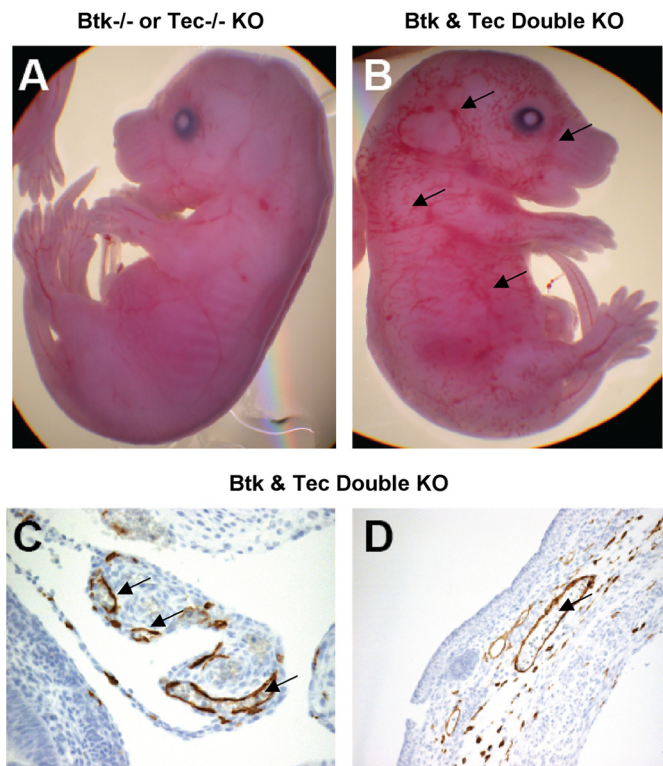


FIGURE 8. Btk and Tec are essential for CLEC-2 function in vivo. A and B, E15.5 embryos lacking both Btk and Tec exhibit cutaneous edema and a pattern of blood-filled cutaneous vessels consistent with lymphatic vessels like those observed in CLEC-2-deficient embryos. C and D, immunostaining for the lymphatic endothelial marker LYVE1 demonstrates blood-filled lymphatic vessels in the mesentery (C) and skin (D). These results are representative of at least three independent experiments.

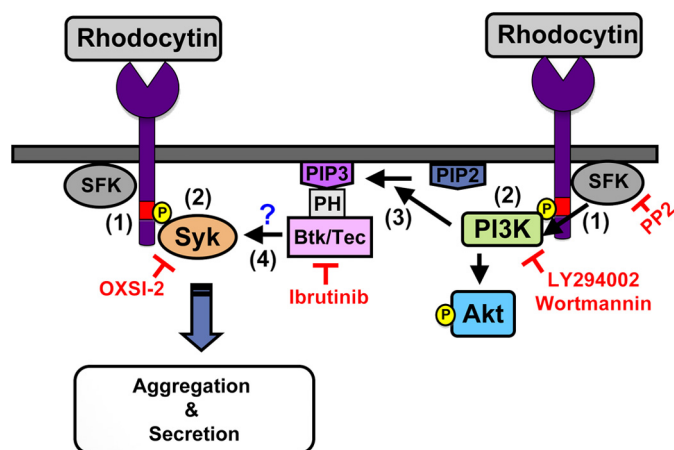


FIGURE 9. Model for PI3K/Btk axis regulating Syk activation downstream of CLEC-2. We propose the following: 1) upon activation of CLEC-2 in platelets, SFKs become activated and phosphorylates the hemITAM. 2) SFK activates PI3K. 3) Once activated, PI3K generates PIP₃ that is important for the recruitment of Tec family kinases to the membrane, where it is phosphorylated and activated by SFKs. 4) Activated Tec family kinases then phosphorylates and activates Syk that is already bound to phosphorylated CLEC-2 hemITAM.

activated PI3K binds to the phosphorylated ITAM of GPVI and DAP10 receptors (66, 74). 2) Phosphotyrosine residue on individual hemITAMs bind to the SH2 domains of PI3K and Syk, thereby bringing it close to SFKs to phosphorylate them. 3) Once activated, PI3K β generates PIP₃ that is important for the recruitment of Tec kinases to the membrane, where it is phos-

phorylated and activated by SFKs. 4) Activated Tec kinases then phosphorylate and activate Syk that is already bound to the phosphorylated hemITAM. We also believe that binding to the phosphorylated hemITAM is important for Syk activation because, downstream of P2Y₁₂, PI3K β is activated, and PIP₃ is generated potentially translocating Btk to the membrane, without resulting in Syk activation (data not shown). Thus, we propose for the first time that distinct pathways regulate Syk activation downstream of ITAMs and hemITAMs in platelets. We also propose a novel mechanism of Syk activation downstream of CLEC-2, which is regulated by PI3K and Tec family kinases.

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