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Identification of novel syk-independent functional roles of Fc γ RIIa in platelet outside-in signaling using transgenic mice expressing human Fc γ RIIa

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Abstract

Platelet outside-in signaling regulates important morphological changes such as cell spreading and retraction. A role for Fc γ RIIa in outside-in signaling has been recently described. Using murine platelets expressing human transgenic Fc γ RIIa we expand the evidence for the role of human Fc γ RIIa in platelet outside-in signaling. Transgenic murine platelets spread more on fibrinogen when compared to WT platelets. Importantly, the role of Fc γ RIIa in clot retraction was independent of its association with Syk kinase as evidenced by the kinetics of retraction with the Syk inhibitor OXSI-2. The enhanced platelet spreading in transgenic mice is reflected in increased Syk phosphorylation and activity as downstream targets PLC γ 2 and c-Cbl Y774. Nonetheless, the WT counterparts exhibit phosphorylation of Syk and PLC γ 2 suggesting that murine platelets have evolved an alternative Fc γ RIIa-independent outside-in signaling mechanism.

Platelet outside-in signaling is a complex and dynamic process in which integrin α IIb β 3 transmits signals to the actin cytoskeleton. An important part of the process is the activation of tyrosine kinases from the Src family kinases (SFK) and spleen tyrosine kinase (Syk). Src, which is constitutively bound to β 3 cytoplasmic tail, becomes activated upon integrin clustering (1, 2), and transmits signals which recruit and activate Syk kinase (3).

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Author contribution

LB, designed and performed experiments, analyzed data, wrote the paper
BKM analyzed data and helped to write the paper
PA, provided important reagents, helped to write the paper
SM, provided important reagents, overall direction, helped to write the paper
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Conflict of interest:

Authors have no relevant conflict of interest to disclose.

Syk is a tyrosine kinase that contains two Src-homology 2 domains (SH2). Due to the known interaction of Syk-SH2 domains with immunoreceptor tyrosine-based activation motifs (ITAM's), Syk was thought to be a kinase involved exclusively in classical immunoreceptor signaling. Subsequently, Syk was identified as an important mediator of integrin signaling (4, 5) but the crucial question of how the SH2 domains of Syk were engaged in an integrin signaling context remained elusive. In an elegant study, Woodside *et al* (6) described the direct binding of Syk to the $\beta 3$ tail and proposed a model in which Syk was activated independently of ITAM domains. However, blocking Fc γ RIIa with mAb IV.3 or using platelets from a patient with an autoantibody-induced shedding of GPVI that expressed reduced levels of a crippled Fc γ RIIa receptor, Boylan *et al* (7) proposed a different model that identifies Fc γ RIIa as the ITAM-bearing adaptor required for Syk activation in platelet outside-in signaling.

Our group has identified c-Cbl as an important component of α IIb β 3-mediated signaling. In human platelets, we identified two different tyrosines on c-Cbl (Y700 and Y774) as Syk substrates (8). We observed significantly lower levels of Syk phosphorylation in murine platelets compared to human platelets spread over fibrinogen (8). As murine platelets do not express Fc γ RIIa, this result led us to rationalize that indeed Fc γ RIIa is required for platelet outside-in signaling and drives the observed difference in Syk phosphorylation between human and murine platelets (8). Nonetheless, the precise role of Fc γ RIIa in outside-in signaling remained to be determined, as murine platelets do spread over fibrinogen and some Syk phosphorylation occurs upon fibrinogen binding. Also, a significant difference in platelet spreading over fibrinogen exists between WT and Syk^{-/-} platelets (3). Zhi *et al* (9) published a more detailed description of the role of Fc γ RIIa in thrombus formation in-vitro and in-vivo. The present work extends these observations by demonstrating that, at least in part, the role of Fc γ RIIa in platelet outside-in signaling is Syk-independent and that murine platelets have an outside-in signaling mechanism that differs from human in the requirement for Fc γ RIIa.

Integrin outside-in signaling regulates functional responses such as cell spreading and clot retraction. Figure 1-A1 shows an image of platelets spreading over fibrinogen from WT vs. Fc γ RIIa transgenic mice (10). Quantitative analysis demonstrates a significant difference (*P 0.05) in the area of spread platelets expressing human Fc γ RIIa vs. WT at 45 min. Additionally; clot retraction analysis identified a novel role for Fc γ RIIa in the retraction process. Platelets from Fc γ RIIa transgenic mice retract faster than their WT counterparts (Figure 1-A2) demonstrating enhanced retraction kinetics that is visible at 45 and 90 minutes. Using pharmacological inhibitors, our previous work has identified a role for Syk kinase in platelet spreading but not in clot retraction in human platelets (8). In agreement with this, platelets from Fc γ RIIa transgenic mouse treated with the Syk inhibitor OXSI-2 (Figure 1-A2) retracts with the same kinetics as control platelets. In control experiments, we have ascertained that OXSI-2 inhibits Syk using PLC γ 2 phosphorylation as a read out in transgenic platelets spreading on fibrinogen (not shown). Together, we conclude that Fc γ RIIa aids in clot retraction independently of its association with Syk.

The results shown in Figure 1-A1 and A2 demonstrate that Fc γ RIIa is playing a pivotal role in platelet outside-in signaling. Indeed, the enhanced functional responses are reflected in

the phosphorylation state of Syk Y525/526 (activation marker). Figure 1-B1 shows a representative western blot analysis of Syk phosphorylation at 20 and 45 minutes of platelet spreading over a fibrinogen-coated surface. Densitometry of three different experiments indicates that outside-in signaling-mediated Syk phosphorylation is higher in Fc γ RIIa transgenic platelets vs. WT (*P 0.05).

To further investigate the signaling events mediated by Fc γ RIIa upon fibrinogen binding, we analyzed downstream substrates of Syk. As seen on Figure 1-B2,B3 PLC γ_2 and c-Cbl Y774 exhibited enhanced phosphorylation (*P 0.05) in Fc γ RIIa transgenic mice demonstrating increased Syk kinase activity. Importantly, both Syk and PLC γ_2 phosphorylation, although enhanced in Fc γ RIIa transgenic mice, are also occurring in the WT counterparts indicating that murine platelets have an Fc γ RIIa-independent mechanism of α IIB β 3 $\rightarrow$ Syk $\rightarrow$ PLC γ_2 activation.

In conclusion, our study expands the knowledge regarding the role of Fc γ RIIa in platelet outside-in signaling and identifies a novel Syk-independent role for Fc γ RIIa in clot retraction. It also supports the notion that murine platelets have an alternative mechanism of signaling via α IIB β 3 to syk than human platelets. Whether there is another ITAM receptor involved in wild-type murine platelet outside-in signaling needs to be further elucidated.

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Figure 1. Functional analysis of outside-in signaling-mediated events in human FcγRIIa transgenic murine platelets and effects on signaling events downstream of αIIbβ3

A1) Murine platelets were allowed to adhere to a fibrinogen-coated cover slip for the indicated time points. After washing 3 times with PBS, adherent cells were fixed with 3.7% paraformaldehyde and analyzed with DIC imaging. Representative surface area quantitation of at least 3 separate experiments (n=3). Graph represents mean ± S.E.M of platelet area (arbitrary units). Student t-test performed (*P 0.05), (WT vs. FcγRIIa). **A2)** Clot retraction analysis of WT vs. FcγRIIa platelets. Where stated platelets were treated with the Syk inhibitor OXSI-2 (2 μM) 10 minutes prior clot retraction analysis. Thrombin (0.2 U/ml) was used to stimulate platelets. Each image is representative of at least 3 different experiments.

B) Murine platelets (3×10^8 platelets/ml) were plated over fibrinogen (100 μg/ml) coated

plates for the indicated time points. Reaction was stopped with cold lysis buffer. Lysates were examined for **B1)** Syk phosphorylation using phosphor-specific antibodies for residues 525/526 as indicated. Equal protein loading was detected with anti-Syk antibody. WT 45 min set to 100%. **B2)** Phosphorylation and densitometry analysis of Syk-downstream target PLC γ_2 Y-759. **B3)** Phosphorylation and densitometry analysis of Syk-downstream target c-Cbl Y774. Densitometry analysis of at least 3 independent experiments. Bars represent mean $\pm$ S.E.M. of the percentage of phosphorylated protein (*P 0.05).