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Peptide LSARLAF induces integrin $\beta 3$ dependent outside-in signaling in platelets

Haixia Niu¹, Zhenlu Xu¹, Ding Li¹, Lin Zhang¹, Kemin Wang¹, Donald B. Taylor², Junling Liu^{1,*}, and T. Kent Gartner^{3,*}

¹Department of Biochemistry and Molecular Cell Biology, Shanghai Jiao Tong University School of Medicine, Shanghai, China

²Department of Biological Sciences, Benedictine University, Lisle, IL, USA

³Department of Biological Sciences, University of Memphis, Memphis, TN, USA

Abstract

Introduction—Peptide LSARLAF (LSA) can bind and activate integrin $\alpha \text{IIb}\beta 3$ in the absence of ‘inside-out’ signal. The active $\alpha \text{IIb}\beta 3$ mediates ‘outside-in’ signaling that elicits platelet aggregation, granule secretion and TxA₂ production. Here we identify the membrane glycoproteins which mediate LSA-induced platelet activation other than $\alpha \text{IIb}\beta 3$, and determine the roles of Src, PLC $\gamma 2$, FcR γ -chain, and SLP-76 in LSA-induced platelet activation.

Method—Ligand-receptor binding assay was performed to study the effect of peptide LSA or its control peptide FRALASL (FRA) on integrins binding to their ligands. Spreading of CHO cells expressing $\alpha \text{IIb}\beta 3$ or $\alpha \text{V}\beta 3$ on immobilized fibrinogen was measured in the presence of LSA or FRA. Washed $\beta 3$, Src, FcR γ -chain, LAT and SLP-76 deficient platelets aggregation and secretion were tested in response to LSA.

Results—Ligand-receptor binding assay indicated that LSA promoted the binding of multiple ligands to $\alpha \text{IIb}\beta 3$ or $\alpha \text{V}\beta 3$. LSA also enhanced CHO cells with $\alpha \text{IIb}\beta 3$ or $\alpha \text{V}\beta 3$ expression spreading on immobilized fibrinogen. $\beta 3$ deficient platelets failed to aggregate and secrete in response to LSA. The phosphorylation of PLC $\gamma 2$ and Syk was also $\beta 3$ dependent. Src, FcR γ -chain, LAT and SLP-76 deficient platelets did not aggregate, secrete ATP or produce TxA₂ in response to LSA.

Conclusion—LSA-induced platelet activation is $\beta 3$ dependent, and signaling molecules Src, FcR γ -chain, SLP-76 and LAT play crucial roles in LSA-induced $\beta 3$ mediated signaling.

Keywords

LSARLAF; integrin $\alpha \text{IIb}\beta 3$; outside-in signaling; Src; FcR γ -chain; SLP-76; PLC $\gamma 2$

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*Corresponding author: T. K. Gartner, Department of Biological Sciences, University of Memphis, Memphis, TN 38152, USA. Tel.: +1-901-6782977, Fax: +1-901-6784457, tgartner@memphis.edu; Junling Liu, Department of Biochemistry and Molecular Cell Biology, Institute of Medical Science, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China, Tel.: +86-21-63846590-776345, Fax: +86-21-54660872, liujl@shsmu.edu.cn.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

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Introduction

Platelet function is partially dependent on the activation of integrin $\alpha\text{IIb}\beta 3$ which plays a pivotal, complex role in platelet physiology, such as adhesion, aggregation, clot formation and retraction [1, 2]. Despite the importance of $\alpha\text{IIb}\beta 3$ -mediated outside-in signaling during platelet activation, quite few aspects of the related signaling pathways are characterized. A series of studies designed on the basis of platelet adhering to immobilized ligands have provided important insights into the $\alpha\text{IIb}\beta 3$ outside-in signaling cascade [3–6], but it is difficult to dissect the $\alpha\text{IIb}\beta 3$ -elicited signaling from agonists-initiated inside-out signaling in suspension [7–9].

A peptide agonist LSARLAF (LSA) has been designed to directly activate $\alpha\text{IIb}\beta 3$ in the absence of inside-out signaling, which has been proved by using immobilized and solution phase purified receptor complexes in vitro [8–11]. Irreversible aggregation in response to a low level of LSA requires TxA_2 receptors, $\text{P2Y}_1/\text{G}\alpha_q$, protein kinase C and $\text{P2Y}_{12}/\text{Gi}$ signaling [9]. Pearce et al. have reported that LSA activates platelets through a $\text{PLC}\gamma 2$ dependent pathway that lies downstream of Src kinase involving $\text{FcR}\gamma$ -chain (Fc receptor γ -chain), LAT (Linker for activation of T cells) and lipid rafts [12]. They concluded that $\text{PLC}\gamma 2$ mediated platelet aggregation is independent of integrin $\alpha\text{IIb}\beta 3$ based on the observation that $\text{PLC}\gamma 2$ was still phosphorylated in αIIb deficient platelets stimulated by LSA, implying that more than one surface glycoprotein mediate this response. Here, we attempted to investigate the LSA's potential target(s) other than $\alpha\text{IIb}\beta 3$ and the roles of Src family kinase(s), $\text{FcR}\gamma$ -chain, LAT, SLP-76 and $\text{PLC}\gamma 2$ in LSA-triggered signaling using gene deficient mouse platelets.

Material and Methods

Reagents

Apyrase, PGE1, Fibrinogen (Fg), and Fibronectin (Fn) were purchased from Sigma-Aldrich (St. Louis, MO). Collagen type I (CGI) was purchased from CHRONO-LOG (Havertown, PA). Vitronectin (Vn) was a generous gift from Dr. Deane F. Mosher, the University of Wisconsin Madison. $\text{PLC}\gamma 2$ and Syk antibodies were purchased from Santa Cruz (CA, USA). 4G10 antibody was purchased from Millipore (Billerica, MA). PAC-1, an activation dependent monoclonal antibody for $\alpha\text{IIb}\beta 3$, was purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA). Horseradish peroxidase conjugated donkey anti-mouse antibody and anti-biotin-antibody were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). PP2 was purchased from Calbiochem (Darmstadt, Germany). Purified $\alpha\text{IIb}\beta 3$, $\alpha 5\beta 1$, $\alpha 1\beta 1$ or $\alpha \text{V}\beta 3$ were prepared as described method [13, 14]. The peptides LSARLAF (LSA) and FRALASL (FRA) were synthesized and purified as described [10].

Binding of PAC-1 to $\alpha\text{IIb}\beta 3$

The binding of PAC-1 to purified $\alpha\text{IIb}\beta 3$ -coated wells was measured. 100 μL of purified $\alpha\text{IIb}\beta 3$ (5 $\mu\text{g}/\text{mL}$) were immobilized on XENOBIND™ covalent binding micro-well plates as described [10], which were post-coated with a solution of BSA (35 mg/mL). After washed with PBS, 0.5 μL PAC-1 (200 $\mu\text{g}/\text{mL}$) was added to wells in the presence of LSA or FRA peptide at the final concentration of 250 μM . After incubation for 4 hours, the wells were washed with PBS and incubated with a final concentration of 0.2 $\mu\text{g}/\text{mL}$ horseradish peroxidase-conjugated donkey anti-mouse antibody in PBS containing 3% BSA at 37°C for 1 hour. After washed 6 times with PBS, the plates were developed according to the manufacturer's instructions and the absorbance was measured at 405 nm. Nonspecific

binding was determined by amount of binding of antibody to BSA-coated wells. Bars in graph represent the means $\pm$ SD.

Ligand-receptor binding assay

Fg, Vn, Fn binding to their respective integrin receptors α IIb β 3, α 5 β 1, α 1 β 1 or α V β 3 were measured. In the presence of 250 μ M of LSA or FRA peptide, biotinylated Fg, Vn, Fn, vWF (0.5 μ g/well) were added to XENOBIND™ covalent binding micro-well plates immobilized with α IIb β 3, α 5 β 1, α 1 β 1 or α V β 3. After incubation for 2 hours and afterward PBS washing, anti-biotin-antibody in PBS containing 3% BSA was added and plate was developed as standard ELISA, and the absorbance was measured at 405 nm. In experiments, specificity binding was determined in the presence of 0.1 mM of RGDS peptide [15]. Bars in graph represent the means $\pm$ SD.

CHO cells spreading on Fibrinogen

CHO cells expressing α IIb β 3 or α V β 3 were incubated with peptide LSA or control peptide FRA at the concentration of 500 μ M (the concentration was determined by preliminary experiment) in chamber slides coated with 100 μ g/mL fibrinogen for 10 minutes or 20 minutes. Adherent cells were rinsed with PBS and fixed with 4% paraformaldehyde. Then cells were washed and incubated with Rhodamine-phalloidin in labeling buffer (0.5% BSA, 0.5% Triton X-100 in PBS) for 30 minutes for F-actin staining. The chamber slides were washed with PBS twice before fluorescence microscopy survey.

Mice

Fc receptor γ -chain knockout (*FcR γ -chain*^{-/-}) and control mice were purchased from Taconic (Germantown, NY). Mice deficient in *LAT* (linker for activation of T cells) were generated as described [16]. Drs. Barbi Judd and Gary A. Koretzky at University of Pennsylvania gave us access to *SLP-76* deficient platelets in the early stages of this study. Dr. Steven Teitelbaum at Washington University supplied β 3 deficient and littermates control mice. Breeding stocks to produce the *Src* deficient mice were obtained from Jackson Laboratories (Bar Harbor, ME). Unless otherwise stated, wild type littermate siblings were used as controls.

Mouse platelet aggregation

Blood was collected from the abdominal aorta of isoflurane-anesthetized mice into syringes containing 100 μ L/mL White anticoagulant (2.94% sodium citrate, 136 mM glucose, pH 6.4), 0.1 μ g/mL PGE1, and 1 U/mL apyrase. Washed platelets were prepared from platelet-rich plasma (PRP) by differential centrifugation of the PRP containing 5 mM EDTA at 1100g for 10 minutes. Platelets were resuspended in modified Tyrode solution (12 mM NaHCO₃, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, 2 mM MgCl₂, 0.42 mM NaH₂PO₄, 10 mM HEPES, pH 7.4). Aggregation was measured in a lumiaggregometer (Chrono-Log, Havertown, PA) using washed platelets (300 μ L) adjusted to approximately 10⁶ platelets per microliter. Inhibitors were incubated with the platelets for 3 minutes prior to 375 μ M LSA stimulation (the valid LSA concentration was determined by preliminary experiment).

Measurement of ATP secretion

ATP secretion was measured using CHORONO-LUME reagent (Chrono-Log, Havertown, PA) according to the manufacturer's protocol. ATP secretion data were obtained from at least three tests. Bars in graph represent the means $\pm$ SD.

Measurement of TxA2 production

After a 5-minute aggregation period, platelets were removed by centrifugation in the presence of 5 mM EDTA. The platelet-free supernatant fraction was diluted at 1:50 with the assay buffer supplied in the TxB2 EIA kit (Assay Designs, Ann Arbor, MI). TxB2, a stable metabolite of TxA2, was measured according to the manufacturer's protocol. TxB2 production data were obtained from at least three tests. The terms "TxA2" and "TxB2" are used interchangeably throughout the text. Bars in graph represent the means $\pm$ SD.

Immunoprecipitation and Western Blot analysis

For the detection of tyrosine-phosphorylated Syk and PLC γ 2, aggregated platelet samples were added to the same volume of lysis buffer (100 mM Tris-HCl, pH 7.4; 2% NP-40; 300 mM NaCl; 2 mM EDTA; 2 mM PMSF; 2 μ g/mL aprotinin, leupeptin, and pepstatin; 2 mM Na₃VO₄; 2 mM NaF; and a Complete Mini Protease Inhibitor Cocktail Tablet). Next, the samples were incubated on ice for 30 minutes, and then 2 μ g/mL 4G10 was added and the samples were incubated overnight at 4°C. Then, 50 μ L Protein A/G PLUS-Agarose was added to each sample prior to incubation for 2 hours at 4°C. The beads were harvested by centrifugation at 3000g for 2 minutes and washed 3 times with 500 μ L lysis buffer and twice with PBS solution. Proteins were boiled in sample buffer and resolved on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to PVDF membrane. Western blots were performed using anti-Syk or anti-PLC γ 2 antibody at a 1:1000 dilution, followed by incubation with horseradish peroxidase-conjugated goat anti rabbit antibody at a 1:5000 dilution. Blots were developed using Supersignal chemiluminescent substrate (Pierce, Rockford, IL).

Results

LSA, not FRA, enhanced the binding of multiple ligands to their specific integrin receptors

Several studies have showed that LSA directly activates integrin α IIb β 3 in the absence of inside-out signaling [9–12]. Pearce et al. published results indicated that LSA activates platelets through more than one platelet surface glycoprotein [12]. In this study, a series of ligand-receptor binding experiments were developed to investigate the effect of LSA on multiple ligands to their specific integrin receptors. First of all, we found LSA, not FRA, enhanced α IIb β 3 binding to its RGD motif containing ligands Fg, Fn, and Vn [17], which was blocked by 0.1mM RGDS peptide specifically (Figure 1A). We also found that LSA, not FRA, enhanced α IIb β 3 binding to PAC-1, an active state-dependent monoclonal antibody against α IIb β 3 (Figure 1B). Further experiment revealed that LSA also enhanced α V β 3 binding to its ligand Fg, but had no effect on α 5 β 1 binding to its ligand Fn or α 1 β 1 binding to its ligand CGI (Figure 1C). These results indicated that LSA enhanced both integrin α IIb β 3 and α V β 3 binding to their ligands.

LSA enhanced the spreading of α IIb β 3 or α V β 3-expressing CHO cells on immobilized fibrinogen

CHO cells expressing α IIb β 3 or α V β 3 were used to verify LSA enhances α IIb β 3 or α V β 3 activation. CHO cells were let spread on immobilized fibrinogen for 10 minutes or 20 minutes in the presence of LSA or FRA (Figure 2). The spreading of CHO cells with LSA treatment was remarkably enhanced at 20 minutes compared with control peptide FRA treatment (Figure 2). These results indicated that LSA enhanced α IIb β 3 or α V β 3 mediated outside-in signaling which causes CHO cells spreading on fibrinogen.

LSA induced platelet aggregation and secretion is $\beta 3$ dependent

To elucidate the important role of $\beta 3$ in the signaling induced by LSA, washed platelets from $\beta 3$ deficient mice and control mice were stimulated with LSA. $\beta 3$ deficient platelets did not aggregate whereas control platelets reached almost full aggregation (Figure 3A). Moreover, ATP secretion and TxA₂ production were almost completely abolished in $\beta 3$ deficient platelets compared to control platelets (Figure 3B, C). Syk and PLC $\gamma 2$ were reported to be phosphorylated in platelets stimulated by LSA [12]. It is necessary to measure whether the phosphorylation of Syk and PLC $\gamma 2$ is $\beta 3$ dependent. As Figure 3D shows, PLC $\gamma 2$ and Syk were phosphorylated in wild type platelets stimulated by LSA but not FRA. However, the phosphorylation of PLC $\gamma 2$ and Syk were dismissed in $\beta 3$ deficient platelets stimulated by LSA. These observations support our hypothesis that LSA induced platelet activation is $\beta 3$ dependent.

Src family kinases are required for LSA induced platelet aggregation and secretion

Integrins interacting with Src kinase are involved in tumor progression, immune response and thrombosis [18–20]. To investigate the function of Src family kinases in LSA induced platelet activation, the Src family kinases selective inhibitor PP2 was used. We found that PP2 prevented LSA induced aggregation and secretion of wild type mouse platelets (Figure 4A, 4B and 4C). Murine platelets lacking Src were also treated with LSA to test Src's function in LSA induced platelet activation. In contrast to the *Src* heterozygous control platelets that aggregated irreversibly and secreted a high level of ATP and TxA₂, the *Src* deficient platelets did not aggregate, secrete ATP or produce TxA₂ in response to LSA (Figure 4D, E and F). These results indicated that Src family kinase(s) play an important role in LSA induced platelet activation.

FcR γ -chain is required for platelet aggregation and secretion induced by LSA

ITAM (Immunoreceptor Tyrosine-based Activation Motif) signaling is induced by cell-cell and cell-extracellular matrix (ECM) interactions that are mediated by integrins [21–24]. Fc γ RII α , as an ITAM-bearing receptor, mediates α IIb $\beta 3$ outside-in integrin signaling in human platelets [25–27]. The role of FcR γ -chain in LSA induced platelet activation was investigated using *FcR γ -chain* deficient platelets. In contrast to the platelets from wild type littermates, the *FcR γ -chain* deficient platelets did not aggregate, secrete ATP or produce TxA₂ in response to LSA (Figure 5A, 5B and 5C), which demonstrated platelet activation is FcR γ -chain dependent.

SLP-76 and LAT are required for LSA induced platelet aggregation and secretion

SLP-76 is required for collagen induced platelet activation [28] and integrin mediated signaling [29]. In response to LSA stimulation, *SLP-76* deficient platelets did not aggregate, secrete ATP or produce TxA₂ in contrast to the control platelets (Figure 5D, E and F). These results suggested that SLP-76 is required for LSA induced platelet activation. LAT, required for collagen induced platelet activation [30, 31], has been studied in LSA induced platelet activation. *LAT* deficient platelets did not aggregate, secrete ATP or produce TxA₂ in contrast to the control platelets (Figure 5G, H and I). Therefore, these results indicated that LSA induced platelet activation is SLP76 and LAT dependent.

Discussion

The integrins are a major class of adhesive and signaling molecules present on most cell types [32]. Heterodimers of α and β subunits are non-covalently associated and generally involved in linking adhesive molecules to the cellular cytoskeleton. Platelets have approximately six different integrins: $\alpha 2\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$, $\alpha L\beta 2$, $\alpha IIb\beta 3$, and $\alpha V\beta 3$.

Integrin $\alpha\text{IIb}\beta 3$ has the capacity to bind fibrinogen (and other ligands) and mediate dual-directions signaling involved in platelet activation.

The peptide LSA was originally developed as a tool to investigate the integrin $\alpha\text{IIb}\beta 3$ mediated outside-in signaling in platelet in suspension. In this study, we found that LSA increased the binding of multiple ligands to $\alpha\text{IIb}\beta 3$ (Figure 1A) and $\alpha\text{IIb}\beta 3$ activation (Figure 1B), which confirmed the results of previous observations [9-12]. Interestingly, LSA also specifically enhanced the binding of fibrinogen to integrin $\alpha\text{V}\beta 3$, but not $\alpha 1\beta 1$ or $\alpha 5\beta 1$ binding to their specific ligands (Figure 1C). These results were further confirmed using spreading experiments. LSA was able to enhance CHO cells, expressing $\alpha\text{IIb}\beta 3$ or $\alpha\text{V}\beta 3$, spreading on immobilized fibrinogen (Figure 2). These results suggested that $\alpha\text{IIb}\beta 3$ is not the only effector of LSA, and $\alpha\text{V}\beta 3$ is another one. To verify these, $\beta 3$ deficient platelets were stimulated with LSA. $\beta 3$ deficient platelets failed to aggregate, secrete ATP and produce TxA₂ in response to low (Figure 3) or high level of LSA (Supplemental Figure 1A, B, C). And the molecules, PLC $\gamma 2$ and Syk, which were phosphorylated in the LSA induced signaling, were not phosphorylated in $\beta 3$ deficient platelets treated with LSA. These results demonstrated that $\beta 3$ is crucial in LSA induced platelet activation. Considering other group's observation that PLC $\gamma 2$ was still phosphorylated in αIIb deficient platelets stimulated by LSA, we concluded that integrin $\alpha\text{V}\beta 3$ also mediate LSA induced platelet activation even though $\alpha\text{V}\beta 3$ presents on platelets in rather small amounts [33].

Although several studies demonstrated the requirement for a variety of agonist receptors and G-proteins in LSA induced $\alpha\text{IIb}\beta 3$ mediated outside-in signaling, which kinases, molecular adaptors and effectors involved in this signaling is still uncharacterized. Interaction of Src and integrins involves in tumor progression, immune response and thrombosis [18–20]. Src was also required for regulation of $\alpha\text{IIb}\beta 3$ mediated granule release in response to a low level of γ -thrombin [34]. Src family kinases' function in LSA induced platelet activation was investigated using PP2 and platelets from mice lacking Src (Figure 4). The results obtained with PP2 reflect an absolute requirement of Src family kinases' function in LSA induced signaling. Platelets lacking Src failed to aggregate in response to LSA. This is consistent with the observation that platelet Src is constitutively associated with $\alpha\text{IIb}\beta 3$ and becomes activated during aggregation and upon adhesion of platelets to immobilized fibrinogen [3, 35]. So, $\alpha\text{IIb}\beta 3$ mediated outside-in signaling induced by LSA appears to require Src's function [3]. But it is still not known if other Src family kinases could have substituted for Src function in this situation, further work is required to clarify.

Furthermore, we found aggregation, secretion and TxA₂ production were abolished in *FcR γ -chain*, *SLP-76* and *LAT* deficient platelets in response to LSA stimulation. These results unequivocally indicated the importance of FcR γ -chain, SLP-76 and LAT in LSA induced $\beta 3$ dependent outside-in signaling. Since collagen receptor GPVI mediated signaling also requires Src family kinases, FcR γ -chain, SLP-76 and LAT [25, 36–38], and LSA induced platelet activation was GPVI independent (Supplemental Figure 1D), we concluded that both LSA induced $\beta 3$ dependent signaling and collagen receptor GPVI induced signaling appear to use a similar signaling pathway for platelet activation. Our results presented here suggest that LSA is a useful tool to investigate $\beta 3$ mediated outside-in signaling and give a partial explanation of the mechanism of LSA induced platelet activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

FcRγ-chain	Fc receptor, γ -chain
SLP-76	SH2 domain-containing leukocyte protein of 76KDa
PLCγ2	phospholipase C γ 2
LAT	linker for activation of T cells
TxA2	thromboxane A2
Syk	spleen tyrosine kinase
RGDS	Arg-Gly-Asp-Ser
ITAM	immunoreceptor tyrosine-based activation motif
BSA	bovine serum albumin
PBS	phosphate buffered saline

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Figure 1. LSA, not FRA, enhanced binding of αIIbβ3 and αVβ3 to their ligands

(A) LSA, not FRA, enhanced the binding of integrin αIIbβ3 to biotinylated Fg, Vn, and Fn. Specificity of binding was also determined in the presence of 0.1 mM RGDS. (B) LSA, not FRA, enhanced the binding of PAC-1, an activation dependent monoclonal antibody for αIIbβ3, to immobilized αIIbβ3. (C) LSA enhanced αVβ3 binding to biotinylated Fg, but did not enhance α5β1 or α1β1 binding to their biotinylated ligands Fn and CGI. Each bar represented the mean of quadruplicate determinations. The error bar was corresponding to the standard deviations of the data.

Figure 2. LSA enhanced spreading of CHO cells expressing α IIb β 3 or α V β 3 on immobilized fibrinogen

In the presence of LSA (500 μ M) or FRA (500 μ M), CHO cells expressing α IIb β 3 or α V β 3 were allowed to spread on chamber slides coated with 100 μ g/mL fibrinogen for 10 minutes or 20 minutes. At the end of the time point, the chamber slides were rinsed with PBS and the cells were fixed with 4% paraformaldehyde and stained with Rhodamine-phalloidin for F-actin. The images represent three separated experiments.

Figure 3. LSA induced platelet activation is $\beta 3$ dependent

Aggregation (A), ATP secretion (B) and TxA₂ production (C) by $\beta 3$ deficient and wild type platelets stimulated with 375 μ M LSA. (D) The lysate of $\beta 3$ deficient and wild type platelets was immunoprecipitated with monoclonal antibody 4G10, and the tyrosine phosphorylation of Syk and PLC γ 2 was detected. The experiments were repeated three times.

Figure 4. Src family kinases are required for LSA induced platelet aggregation and secretion
Wild type platelets aggregation (A), ATP secretion (B) and TxA₂ production (C) of platelets stimulated by LSA were prevented by 5 μM PP2. In contrast to the control platelets, *Src* deficient platelets did not aggregate (D), secrete ATP (E) or produce TxA₂ (F) in response to 375 μM LSA. Thus, Src family kinases are required in LSA induced platelet activation. The experiments were repeated three times.

Figure 5. FcR γ -chain, SLP-76 and LAT are required for LSA induced platelet aggregation, granule secretion and TxA2 production

Compared with normal platelets, *FcR γ -chain* deficient platelets did not aggregate (A), secrete ATP (B) or produce TxA2 (C) in response to LSA. *SLP-76* deficiency prevented LSA caused platelets aggregation (D), ATP secretion (E) and TxA2 production (F). Similarly, *LAT* deficiency prevented platelets aggregation (G), ATP secretion (H) and TxA2 production (I) in response to LSA. Thus, FcR γ -chain, SLP-76 and LAT are required in LSA induced platelet activation. The experiments were repeated three times.