

# Roles of Platelet STIM1 and Orai1 in Glycoprotein VI- and Thrombin-dependent Procoagulant Activity and Thrombus Formation<sup>\*[5]</sup>

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In platelets, STIM1 has been recognized as the key regulatory protein in store-operated  $\text{Ca}^{2+}$  entry (SOCE) with Orai1 as principal  $\text{Ca}^{2+}$  entry channel. Both proteins contribute to collagen-dependent arterial thrombosis in mice *in vivo*. It is unclear whether STIM2 is involved. A key platelet response relying on  $\text{Ca}^{2+}$  entry is the surface exposure of phosphatidylserine (PS), which accomplishes platelet procoagulant activity. We studied this response in mouse platelets deficient in STIM1, STIM2, or Orai1. Upon high shear flow of blood over collagen, *Stim1*<sup>-/-</sup> and *Orai1*<sup>-/-</sup> platelets had greatly impaired glycoprotein (GP) VI-dependent  $\text{Ca}^{2+}$  signals, and they were deficient in PS exposure and thrombus formation. In contrast, *Stim2*<sup>-/-</sup> platelets reacted normally. Upon blood flow in the presence of thrombin generation and coagulation,  $\text{Ca}^{2+}$  signals of *Stim1*<sup>-/-</sup> and *Orai1*<sup>-/-</sup> platelets were partly reduced, whereas the PS exposure and formation of fibrin-rich thrombi were normalized. Washed *Stim1*<sup>-/-</sup> and *Orai1*<sup>-/-</sup> platelets were deficient in GPVI-induced PS exposure and prothrombinase activity, but not when thrombin was present as co-agonist. Markedly, SKF96365, a blocker of (receptor-operated)  $\text{Ca}^{2+}$  entry, inhibited  $\text{Ca}^{2+}$  and procoagulant responses even in *Stim1*<sup>-/-</sup> and *Orai1*<sup>-/-</sup> platelets. These data show for the first time that: (i) STIM1 and Orai1 jointly contribute to GPVI-induced SOCE, procoagulant activity, and thrombus formation; (ii) a compensating  $\text{Ca}^{2+}$  entry pathway is effective in the additional presence of thrombin; (iii) platelets contain two mechanisms of  $\text{Ca}^{2+}$  entry and PS exposure, only one relying on STIM1-Orai1 interaction.

In platelets, elevation in cytosolic  $[\text{Ca}^{2+}]_i$  is imperative to almost all functional responses. Moderate and transient rises in  $[\text{Ca}^{2+}]_i$  mediate shape change, integrin  $\alpha_{\text{IIb}}\beta_3$  activation, thromboxane formation, and secretion of granule contents, whereas high and prolonged  $[\text{Ca}^{2+}]_i$  rises are required for the procoagulant response (1, 2). The latter is achieved by a  $\text{Ca}^{2+}$ -activated scramblase mechanism disturbing the normal phospholipid asymmetry in the plasma membrane, with, as a result, the exposure of phosphatidylserine (PS)<sup>5</sup> at the outer membrane surface (3, 4). Exposed PS provides high affinity binding sites for key coagulation factors and, thereby, facilitates the assembly of tenase and prothrombinase complexes, which are responsible for the formation of factor Xa and thrombin, respectively (3). Because thrombin is one of the most potent platelet agonists, the procoagulant platelet response triggers a potent positive feedback loop of platelet and coagulation activation. Recent *in vivo* studies have indicated that PS exposure and ensuing thrombin generation are key regulatory events in murine arterial thrombus formation (5, 6).

Whereas stored platelets may expose procoagulant PS in a  $\text{Ca}^{2+}$ -independent way, PS exposure in activated platelets relies on a high and prolonged rise in cytosolic  $[\text{Ca}^{2+}]_i$  (7). Platelet stimulation with single G protein-coupled agonists, like thrombin and ADP, results in limited PS exposure (8, 9), but stimulation of the tyrosine kinase-linked collagen receptor glycoprotein VI (GPVI), with ligands such as collagen-related peptide (CRP) or convulxin, results in appreciable procoagulant activity (10, 11). Combined stimulation of the collagen and thrombin receptors though results in high PS exposure, likely because these agonists use different signaling pathways for mobilizing cytosolic  $\text{Ca}^{2+}$  (1). Although thrombin transiently activates  $G_q\alpha$  and phospholipase  $C\beta_2/\beta_3$  isoforms, activation of GPVI causes a more persistent activation of the phospholipase  $C\gamma_2$  isoform (2, 12). For PS exposure, entry of extracellular  $\text{Ca}^{2+}$  is required, complementing the  $\text{Ca}^{2+}$ -mobilizing effect of phospholipase C stimulation, to reach sufficiently high  $[\text{Ca}^{2+}]_i$  (10, 13, 14).

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<sup>5</sup> The abbreviations used are: PS, phosphatidylserine; BSA, bovine serum albumin; CRP, collagen-related peptide; FITC, fluorescein isothiocyanate; GPVI, glycoprotein VI; PPACK, H-Phe-Pro-Arg chloromethyl ketone; PRP, platelet-rich plasma; SOCE, store-operated  $\text{Ca}^{2+}$  entry; STIM, stromal-interacting molecule; Z-GGR-AMC, Z-Gly-Gly-Arg aminomethyl coumarin.

## Roles of *STIM1* and *Orai1* in Platelet Procoagulant Activity

In platelets like other cells,  $\text{Ca}^{2+}$  entry can be triggered by receptor stimulation as well as by  $\text{Ca}^{2+}$  mobilization from stores via the processes of receptor-operated  $\text{Ca}^{2+}$  entry and store-operated  $\text{Ca}^{2+}$  entry (SOCE), respectively (15). For long, not only the responsible  $\text{Ca}^{2+}$  entry channels, but also the coupling mechanisms of receptor activation and  $\text{Ca}^{2+}$  store depletion to channel opening have remained elusive. In earlier work with platelets, roles of the TRPC1 and TRPC6 channel proteins in  $\text{Ca}^{2+}$  entry have been proposed (16, 17). Recent studies, however, have shown the importance of the Orai class of plasma membrane  $\text{Ca}^{2+}$  channels. The channel Orai1 (also called CRACM1) oligomerizes and opens, following depletion of the  $\text{Ca}^{2+}$  stores, by interacting with  $\text{Ca}^{2+}$  sensing STIM1, which is a transmembrane protein located in the endoplasmic reticulum (18–20). The homologous protein STIM2 can have a similar regulatory role in  $\text{Ca}^{2+}$  entry (21). Both Orai1 and STIM1 have been implicated in the physiological activation of T cells and mast cells (22, 23). Recent studies using genetically modified mice have established that STIM1 and Orai1 account for the large majority of SOCE in platelets. The importance of this SOCE pathway appeared from the finding that platelet deficiency in either Orai1 or STIM1 protects against collagen-dependent arterial thrombus formation and brain infarction *in vivo* (24, 25). In confirmation, others have provided evidence that a functional R93W mutation in Orai1 leads to impaired GPVI-induced platelet activation (26).

In the present paper, we investigated whether the STIM isoforms and Orai1 provide the main  $\text{Ca}^{2+}$  entry mechanism responsible for PS exposure and procoagulant activity in platelets stimulated by the collagen and thrombin receptors. The studies were carried out using mice with *Stim1*<sup>-/-</sup>, *Stim2*<sup>-/-</sup>, or *Orai1*<sup>-/-</sup> platelets.

### EXPERIMENTAL PROCEDURES

**Mouse Strains**—Animal studies were approved by the local animal care and use committees. Mice homozygously deficient in *Stim1* or *Orai1* were generated from embryonic stem cell clones and germ line transmission, as described (24, 25). Because these animals suffered from early lethality and growth retardation, bone marrow chimeras were created which had normal viability. Female, 5–6-week-old C57BL/6 mice were irradiated with a single dose of 10 Gy and injected intravenously with bone marrow cells from donor *Stim1*<sup>-/-</sup>, *Orai1*<sup>-/-</sup>, or wild type mice ( $4 \times 10^6$  cells/animal). The recipient mice received acidified water containing 2 g/liter neomycin sulfate for 6 weeks after transplantation. Blood was taken from the chimeras after >6 weeks. Mice homozygously deficient in *Stim2* had a mixed genetic background and were compared with wild types of the same background (27). Blood cell counts of all mice were in the normal range. Purified platelets from (bone marrow-transplanted) mice were subjected to Western blotting to confirm knock-out of STIM proteins. Deficiency in Orai1 transcripts was confirmed by reverse transcription-PCR analysis (25).

**Materials**—H-Phe-Pro-Arg chloromethyl ketone (PPACK) was obtained from Calbiochem. Annexin A5 labeled with fluorescein isothiocyanate (FITC), Fura-2 and Fluo-4 acetoxy-methyl esters, and pluronic F-127 were from Invitrogen. Thrombin substrate, Z-Gly-Gly-Arg aminomethyl coumarin (Z-GGR-

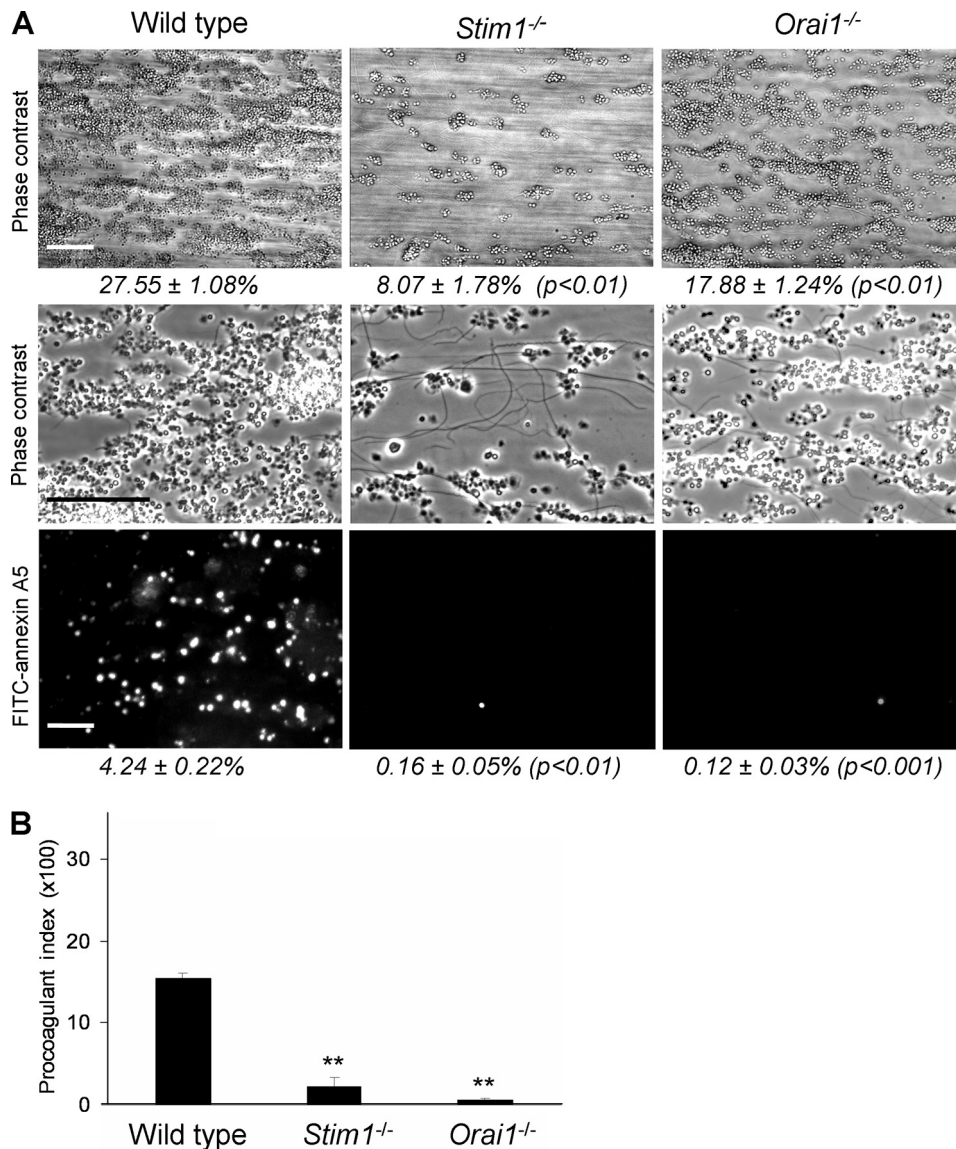
AMC), was from Bachem. Fibrillar type I collagen (Horm) was from Nycomed. Recombinant human tissue factor was from Dade Behring. Apyrase (grade V), bovine serum albumin (BSA), heparin, SKF96365, and thrombin were from Sigma. The GPVI agonist CRP was synthesized and cross-linked by Tana Laboratories. The agonist convulxin was purified as described (11). FITC-labeled anti-mouse P-selectin monoclonal antibody was from Emfret Analytics. Bovine coagulation factors and other materials were from sources indicated before (28).

**Blood Collection and Platelet Preparation**—Mouse blood was obtained via orbital puncture under anesthesia. For perfusion studies in the absence of coagulation, blood was collected into a mixture of 40  $\mu\text{M}$  PPACK, 5 units/ml heparin, and 40 units/ml fragmin. For later perfusions with coagulation, blood was collected into 12.9 mM trisodium citrate. Citrate-anticoagulated blood was also used to prepare platelet-rich plasma (PRP), normalized with autologous platelet-poor plasma to a platelet count of  $1 \times 10^8$ /ml. Washed platelets were prepared from PRP by supplementation with ACD solution (85 mM sodium citrate, 78 mM citric acid, and 11 mM D-glucose) (1:25). After centrifugation, platelets were suspended in Hepes buffer, pH 7.45 (136 mM NaCl, 5 mM Hepes, 2.7 mM KCl, 2 mM  $\text{MgCl}_2$ , 0.42 mM  $\text{Na}_2\text{HPO}_4$ , 5 mM glucose, and 0.1% BSA). Cells were counted with a Coulter counter.

**Activation of Suspended Platelets**—Washed mouse platelets were loaded with the ratiometric  $\text{Ca}^{2+}$  probe Fura-2, as described (24). Platelets suspended in the presence or absence of 1 mM  $\text{CaCl}_2$  were preincubated with 100  $\mu\text{M}$  SKF96365 and activated with collagen and/or thrombin receptor agonists, as indicated. Changes in fluorescence were measured with a PerkinElmer Life Sciences 55 fluorometer. Excitation was alternated between 340 and 380 nm, and emission was measured at 509 nm. Calibration parameters of nanomolar  $[\text{Ca}^{2+}]_i$  were obtained by lysis with 1% Triton X-100 and addition of a surplus of EGTA. For flow cytometry, unloaded washed platelets ( $1 \times 10^8$ /ml) were activated in the presence of  $\text{CaCl}_2$  with the indicated agonists for 10 min; stirring was absent to prevent platelet aggregation (29). Surface expression of PS was detected in the presence of  $\text{CaCl}_2$  (2 mM) with FITC-annexin A5 (0.5  $\mu\text{g}/\text{ml}$ ).

**Thrombus Formation on Collagen**—Glass coverslips were coated with fibrillar type I collagen and blocked with BSA-containing Hepes buffer, pH 7.45. The coverslips were mounted in a transparent, 50  $\mu\text{m}$ -deep parallel-plate poly(methyl) methacrylate flow chamber (30). Mouse blood was perfused through the flow device at a defined shear rate under physiological, millimolar concentrations of divalent cations. For experiments in the absence of coagulation, PPACK/heparin-treated blood was flowed over collagen at  $1000 \text{ s}^{-1}$  for 4 min. For experiments in the presence of coagulation, citrate-anticoagulated blood was recalcified directly before entering the flow chamber, using a two-pump system (29). Briefly, 1-ml syringes were filled with citrate-anticoagulated blood or isotonic  $\text{CaCl}_2/\text{MgCl}_2$  solution (110 mM NaCl, 13.3 mM  $\text{CaCl}_2$ , and 6.7 mM  $\text{MgCl}_2$ ). The syringes were connected to the flow chamber via a v-shaped inlet, designed to give optimal fluid mixing. By co-infusing both fluids into the chamber at an equal flow rate (final shear rate,  $1000 \text{ s}^{-1}$ ), coagulation was started by collagen- and factor XII-dependent activation (31). The thrombi on coverslips were





**FIGURE 1. Deficiency in *STIM1* or *Orai1* impedes GPVI-dependent thrombus formation and PS exposure under flow.** PPACK/heparin-anticoagulated blood of C57BL/6 mice transplanted with bone marrow from wild type, *Stim1*<sup>-/-</sup> or *Orai1*<sup>-/-</sup> animals was flowed over collagen at a shear rate of  $1000 \text{ s}^{-1}$ . **A**, representative contrast images after 4 min, captured at low (*top panels*) or high (*middle panels*) magnification. *Bottom panels*, fluorescence images after staining with FITC-annexin A5. Percentages in *italic* indicate area covered with platelets (*scale bars*,  $50 \mu\text{m}$ ). **B**, procoagulant index representing relative number of PS-exposing platelets. Data are percentage fractions of adhered platelets exposing PS. Means  $\pm$  S.E. (*error bars*) are shown.  $n = 6-8$ ; \*\*,  $p < 0.01$  versus wild type.

postlabeled by perfusion with FITC-annexin A5 ( $0.5 \mu\text{g/ml}$ ) in HEPES buffer, pH 7.45, containing  $2 \text{ mM CaCl}_2$  and  $1 \text{ unit/ml}$  heparin. Bright-field and fluorescence images were recorded from at least 10 randomly chosen microscopic fields (32). Images were analyzed with ImagePro software (Media Cybernetics). The procoagulant index of thrombi was determined as the ratio of surface coverage of PS-exposing platelets (FITC-annexin A5) to the coverage of total platelets (33).

**Single-platelet  $\text{Ca}^{2+}$  Fluxes under Flow**—Mouse platelets were loaded with Fluo-4 (34) and added to PPACK/fragmin- or citrate-anticoagulated blood from the same genotype (10% labeled platelets). During blood flow over collagen, 16-bit digital fluorescence images were recorded at high speed ( $5 \text{ Hz}$ ) using an EM-CCD camera (31). Regions of interest rep-

resenting single adhered platelets were analyzed off-line for changes in fluorescence ( $F$ ) (34). Pseudorange  $F/F_0$  values were converted into nanomolar concentrations of  $[\text{Ca}^{2+}]_i$  using predefined calibration parameters (35). For quantitative purposes, traces from individual cells were superimposed so that  $[\text{Ca}^{2+}]_i$  rises started at the same frame number.

**Prothrombinase Activity**—Prothrombinase-stimulating activity was determined at linear assay conditions, as previously assessed for human platelets (36). Washed mouse platelets were diluted in HEPES buffer, pH 7.45, containing  $3 \text{ mM CaCl}_2$  to a count of  $4 \times 10^5/\text{ml}$  and incubated with  $0.5 \mu\text{M}$  prothrombin,  $2 \text{ nM}$  factor Va, and  $1 \text{ nM}$  factor Xa ( $37^\circ\text{C}$ ). Samples were taken after exactly 3 min and transferred to vials containing  $0.5 \text{ mM}$  thrombin substrate S2238 for chromogenic measurement of the thrombin formed.

**Thrombin Generation**—Thrombin generation was measured in citrate-anticoagulated PRP (28). The PRP, pooled from three animals with the same (chimeric) genotype, was diluted with autologous platelet-poor plasma to a count of  $1.5 \times 10^8$  platelets/ml. Samples were activated with convulxin ( $100 \text{ ng/ml}$ ), ionomycin ( $20 \mu\text{M}$ ), or vehicle for 15 min. Aliquots (4 volumes) were then transferred to a polystyrene 96-wells plate (Immulon 2HB, Dynex Technologies), already containing 1 volume of buffer A ( $20 \text{ mM}$  HEPES,  $140 \text{ mM NaCl}$ ,  $0.5\%$  BSA, and  $6 \text{ pM}$  tissue factor). Coagulation was

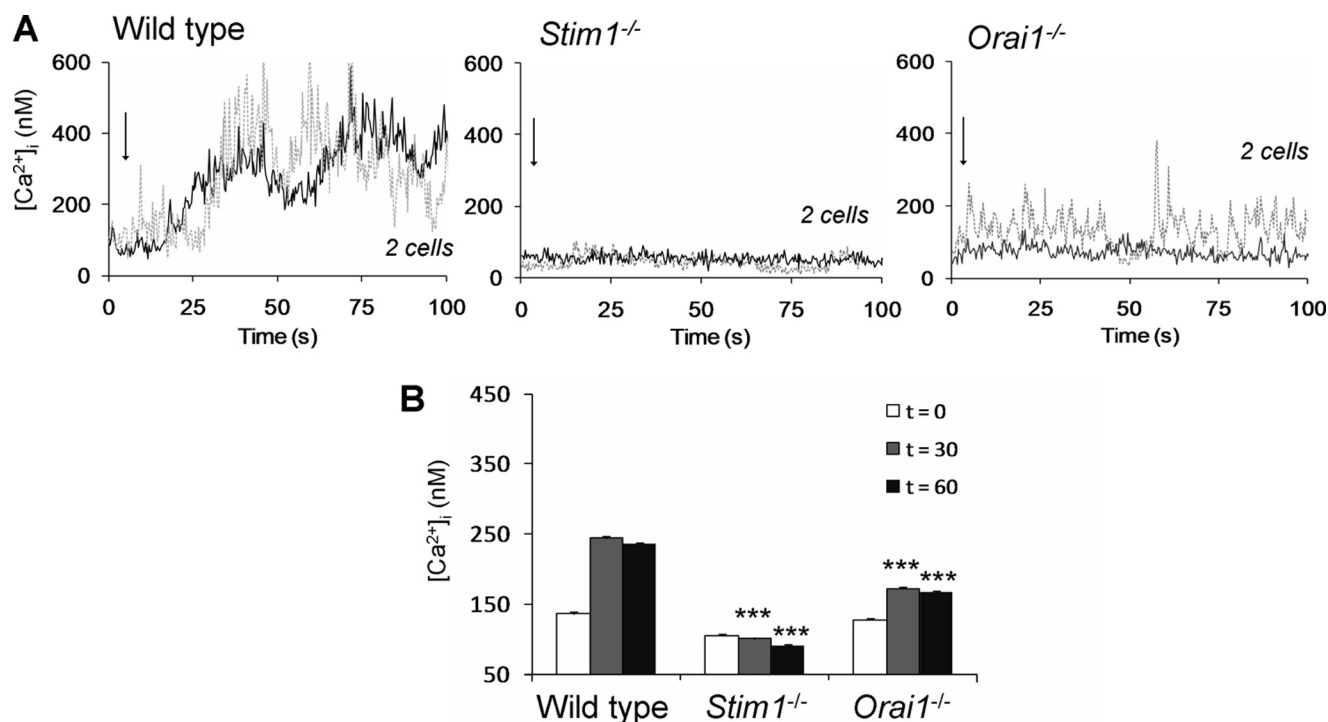
started by adding 1 volume of buffer B ( $2.5 \text{ mM Z-GGR-AMC}$ ,  $20 \text{ mM HEPES}$ ,  $140 \text{ mM NaCl}$ ,  $100 \text{ mM CaCl}_2$ , and  $6\%$  BSA). First-derivative curves were converted into nanomolar thrombin concentrations using a calibrator for human  $\alpha$ -thrombin (28). All analyses were in duplicate.

**Statistics**—Differences between groups were tested for significance with the nonparametric Mann-Whitney  $U$  test. Paired data were compared with Student's  $t$  test. The statistical package for social sciences (SPSS 15.0) was used.

## RESULTS

**Deficiency in Platelet *STIM1* or *Orai1* Impedes GPVI-mediated PS Exposure and Thrombus Formation**—For the experiments, chimeric mice were generated by transplantation of

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**FIGURE 2. Deficiency in STIM1 or Orai1 impedes GPVI-dependent Ca<sup>2+</sup> responses of collagen-adhered platelets under flow.** PPACK/heparin-anticoagulated blood of wild type or chimeric *Stim1*<sup>-/-</sup> or *Orai1*<sup>-/-</sup> mice was supplemented with 10% Fluo-4-loaded platelets of the same genotype. Blood samples were flowed over collagen at 1000 s<sup>-1</sup>, and fluorescence images from the collagen surface were recorded at 5 Hz. *A*, single-cell rises in Ca<sup>2+</sup> of two representative platelets per genotype. Arrows indicate time point of adhesion. *B*, quantitative analysis of Ca<sup>2+</sup> responses at 30 and 60 s after initial Ca<sup>2+</sup> rises. Means ± S.E. are shown. *n* = 35–45 cells; \*\*\*, *p* < 0.001 compared with wild type.

irradiated wild type animals with *Stim1*<sup>-/-</sup>, *Orai1*<sup>-/-</sup>, or wild type bone marrow cells. Platelets from these mice, isolated at least 4 weeks after transplantation, were checked for the absence of STIM1 protein or Orai1 transcript (supplemental Fig. 1, *A* and *B*). The platelets from chimeric *Stim1*<sup>-/-</sup> and *Orai1*<sup>-/-</sup> mice were greatly impaired in Ca<sup>2+</sup> rises evoked by the GPVI agonist, CRP (a triple-helical collagen peptide), or by thrombin, most markedly when extracellular CaCl<sub>2</sub> was present (supplemental Fig. 1*C*), which is in full agreement with earlier data (24, 25). In addition, *Stim1*<sup>-/-</sup> platelets showed reduced Ca<sup>2+</sup> rises in the absence of CaCl<sub>2</sub> and Ca<sup>2+</sup> entry, supporting the concept that STIM1 controls the filling state of Ca<sup>2+</sup> stores (24).

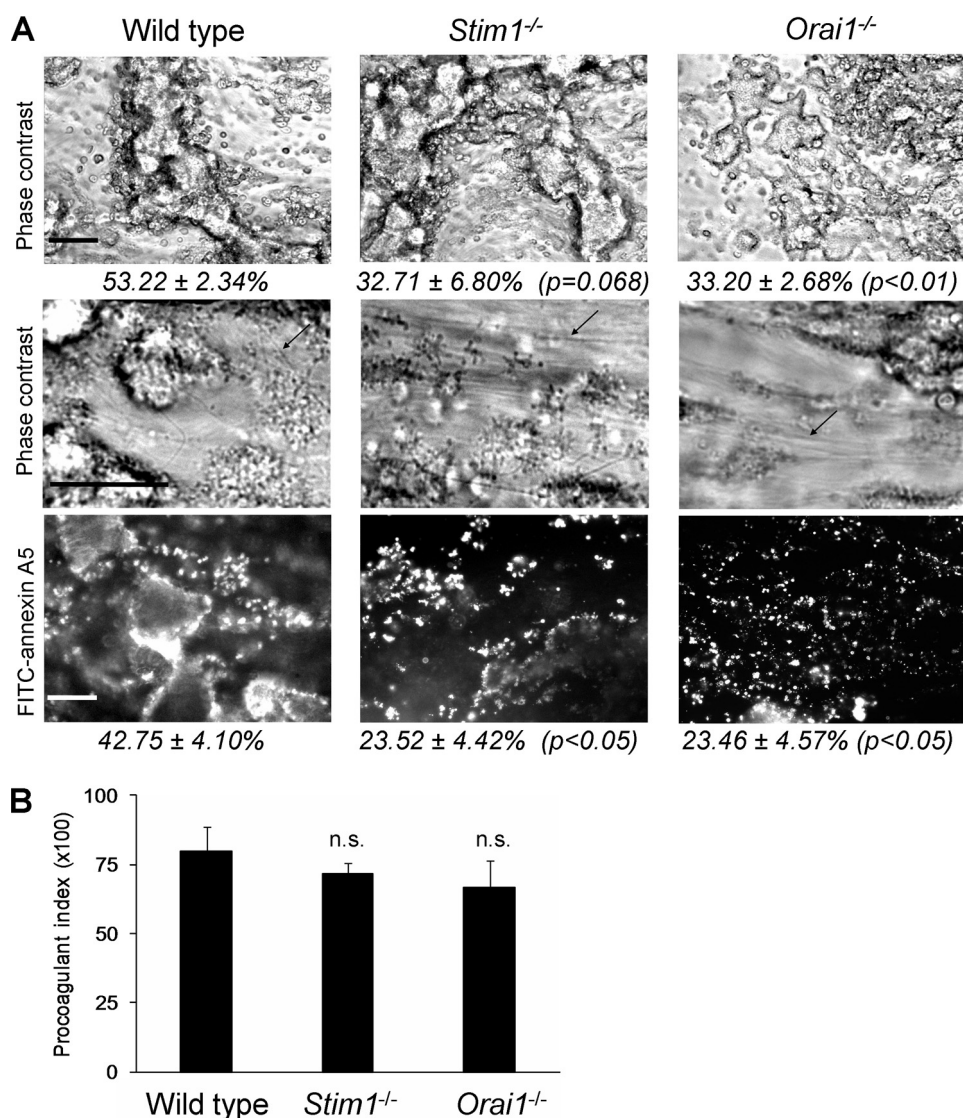
Blood from these chimeric mice was anticoagulated with PPACK/heparin and perfused at high shear flow rate over a collagen surface. By using these thrombin inhibitors, blood coagulation was ablated, whereas high, physiological concentrations of free Ca<sup>2+</sup> and Mg<sup>2+</sup> were maintained. In this test of collagen-induced thrombus formation, platelets in contact with the collagen fibers become activated via GPVI and respond by Ca<sup>2+</sup> elevation, PS exposure, and secretion of autocrine mediators, as a result platelet aggregate formation (5, 33, 37). Strikingly, with blood from chimeric *Stim1*<sup>-/-</sup> and *Orai1*<sup>-/-</sup> mice, no or only small aggregates were formed, whereas PS exposure was almost completely absent, as apparent from the lack of staining with FITC-labeled annexin A5 (Fig. 1*A*). This contrasted to the large aggregates and many PS-exposing platelets observed with blood from corresponding wild type mice. Analysis of microscopic images pointed to a markedly reduced deposition of *Stim1*<sup>-/-</sup> (–70%) and *Orai1*<sup>-/-</sup> (–46%) platelets

compared with wild type. The procoagulant index, *i.e.* the relative formation of PS-exposing platelets compared with all adhered platelets (33), was also greatly reduced in either knock-out (Fig. 1*B*).

Mouse platelets were loaded with Fluo-4 and back-added to blood of the same genotype to measure Ca<sup>2+</sup> rises in the cells during blood flow over collagen. Wild type platelets showed high Ca<sup>2+</sup> responses shortly after adhesion (Fig. 2*A*). However, adhered *Stim1*<sup>-/-</sup> platelets gave only minute Ca<sup>2+</sup> spikes, in a manner resembling the low responses of *Fcer1g*<sup>-/-</sup> platelets, known to lack GPVI signaling activity (34). Many of the adhered *Orai1*<sup>-/-</sup> platelets gave similar, minute Ca<sup>2+</sup> spikes (~50%), but the remaining *Orai1*<sup>-/-</sup> cells showed a short series of medium amplitude spikes (Fig. 2*A*). Quantitative analysis demonstrated a nearly complete ablation of the average Ca<sup>2+</sup> signal in *Stim1*<sup>-/-</sup> platelets and a greatly reduced signal in *Orai1*<sup>-/-</sup> platelets (Fig. 2*B*). Together, these results point to important roles of both STIM1 and Orai1 in collagen-dependent Ca<sup>2+</sup> signaling, PS exposure, and thrombus formation under noncoagulant conditions.

**Deficiency in STIM1 or Orai1 Does Not Abolish PS Exposure and Thrombus Formation under Coagulant Conditions—**GPVI-induced platelet activation has been shown to control collagen-dependent thrombus formation also under conditions favoring coagulation (29). To determine the role of STIM1 and Orai1 in this setting, citrate-anticoagulated blood from chimeric animals was co-infused with CaCl<sub>2</sub>/MgCl<sub>2</sub> to achieve again millimolar free Ca<sup>2+</sup> and Mg<sup>2+</sup> concentrations. Perfusion of the recalcified blood over collagen leads to onset of coagulation via collagen-dependent activation of factor XII





**FIGURE 3. Deficiency in *STIM1* or *Orai1* permits GPVI-dependent thrombus formation and PS exposure in the presence of coagulation.** Citrate-anticoagulated blood of the indicated mice was recalcified with  $\text{CaCl}_2/\text{MgCl}_2$  and flowed over collagen for 4 min. Thrombi with platelets and fibrin (arrows) were poststained with FITC-annexin A5. **A**, representative phase contrast and fluorescence images after 4 min (scale bars, 50  $\mu\text{m}$ ). Percentages in *italic* indicate area covered with (fluorescent) platelets. **B**, procoagulant index of relative number of PS-exposing platelets. Means  $\pm$  S.E. (error bars) are shown. *n* = 5–7; *n.s.*, difference between groups not significant.

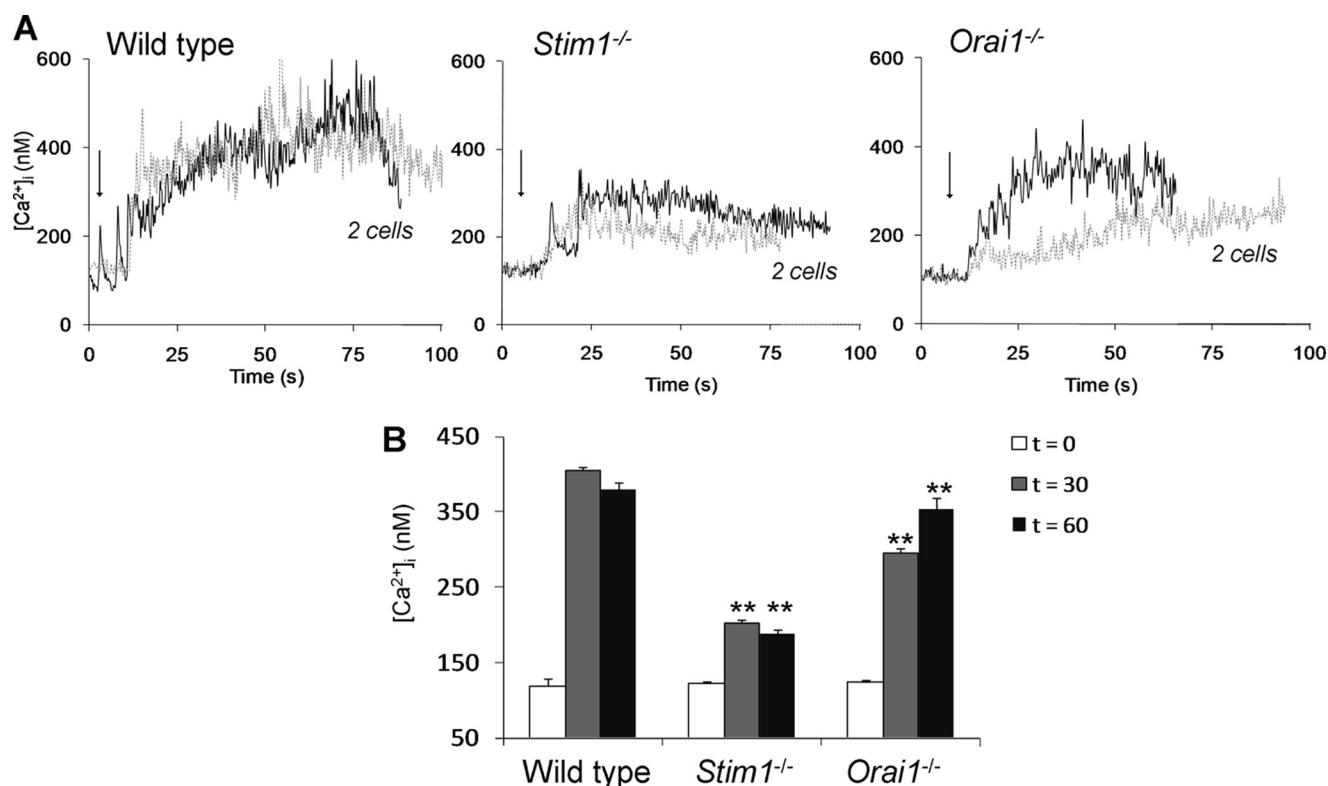
(31). With wild type blood, massive and dense thrombi (clots) were formed within 4 min of perfusion, which were covered with PS-exposing platelets and connected by fibrin fibers (Fig. 3A). Surprisingly, dense fibrin-containing thrombi were also formed with *Stim1*<sup>-/-</sup> or *Orai1*<sup>-/-</sup> blood. These thrombi were also surrounded by PS-exposing platelets, but to a lesser degree compared with wild type blood. However, the procoagulant index was similarly high for images recorded from wild type, *Stim1*<sup>-/-</sup>, and *Orai1*<sup>-/-</sup> thrombi (Fig. 3B). These results thus indicate that neither *STIM1* nor *Orai1* is essential for collagen-dependent thrombus formation and PS exposure under coagulant conditions.

Fluo-4-loaded platelets were then used to determine rises in  $\text{Ca}^{2+}$  under the same conditions of flow and coagulation. Wild type platelets, adhered to collagen, showed prolonged, high  $\text{Ca}^{2+}$  responses (Fig. 4A), which were higher in level than those

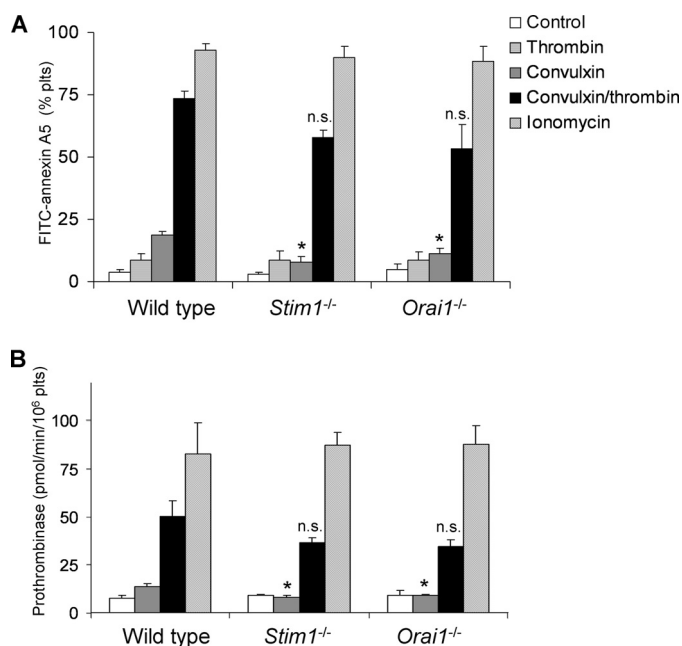
measured in anticoagulated blood (Fig. 2B). With the thrombin inhibitor hirudin present, the mean  $\text{Ca}^{2+}$  rises reduced from about 400 to 250 nM, which confirmed the contribution of thrombin to the  $\text{Ca}^{2+}$  signal. Interestingly, in recalcified blood, collagen-adhered *Stim1*<sup>-/-</sup> and *Orai1*<sup>-/-</sup> platelets also showed prolonged  $\text{Ca}^{2+}$  responses, but these remained lower in magnitude than those of wild type platelets (Fig. 4B). The effect of *STIM1* knock-out was again more pronounced than that of *Orai1* deficiency. Hence, under these conditions of *in situ* formation of thrombin, platelet  $\text{Ca}^{2+}$  responses seem to be sufficiently high for PS exposure even in the absence of the *STIM1*-*Orai1*-SOCE pathway.

*Deficiency in *STIM1* or *Orai1* Does Not Affect GPVI-Induced PS Exposure and Prothrombinase Activity with Thrombin Present*—To investigate this further, washed platelets were stimulated with collagen (GPVI)- and/or thrombin-receptor agonist, and staining of the cells was observed with the PS probe, FITC-annexin A5. Extracellular  $\text{CaCl}_2$  was present to allow  $\text{Ca}^{2+}$  entry. The GPVI agonist convulxin was used for these studies (12), which is similarly effective as CRP in wild type and *Orai1*-deficient platelets (25). Flow cytometric analysis showed that convulxin stimulation of wild type platelets resulted in about 20% PS-exposing platelets (Fig. 5A). This fraction increased to 73% with thrombin as co-agonist, whereas thrombin alone had no more than little effect. Stimulation of platelets from chimeric *Stim1*<sup>-/-</sup> and *Orai1*<sup>-/-</sup> mice with convulxin resulted in impaired PS exposure, comparable with stimulation with thrombin alone. However, combined stimulation with convulxin and thrombin resulted in 53–58% PS-positive platelets, which was similar to that of wild type platelets. Control experiments indicated that all platelets responded nearly completely when stimulated with the  $\text{Ca}^{2+}$  ionophore, ionomycin. As an alternative approach, we studied the capacity of platelets to support prothrombinase activity, *i.e.* the PS-dependent cleavage of prothrombin by factors Xa and Va (13). In wild type platelets, but not *Stim1*<sup>-/-</sup> or *Orai1*<sup>-/-</sup> platelets, convulxin caused a moderate increase in prothrombinase activity (Fig. 5B). The combination of convulxin and thrombin induced a 5-fold increased prothrombinase activity with wild type platelets and a slightly lower increase with knock-out platelets, but

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**FIGURE 4. Deficiency in *STIM1* or *Orai1* partly reduces Ca<sup>2+</sup> signaling in the presence of coagulation.** Citrate-anticoagulated blood of the indicated mice was supplemented with 10% Fluo-4-loaded platelets of the same genotype and recalcified. During blood flow over collagen, fluorescence images from the collagen surface were recorded at 5 Hz. *A*, single-cell rises in [Ca<sup>2+</sup>]<sub>i</sub> of two representative platelets per genotype. Arrows indicate time point of adhesion. *B*, quantitative analysis of Ca<sup>2+</sup> responses at 30 and 60 s after initial [Ca<sup>2+</sup>]<sub>i</sub> rises. Means ± S.E. (error bars) are shown. *n* = 15–29 cells; \*\*, *p* < 0.01 compared with wild type.

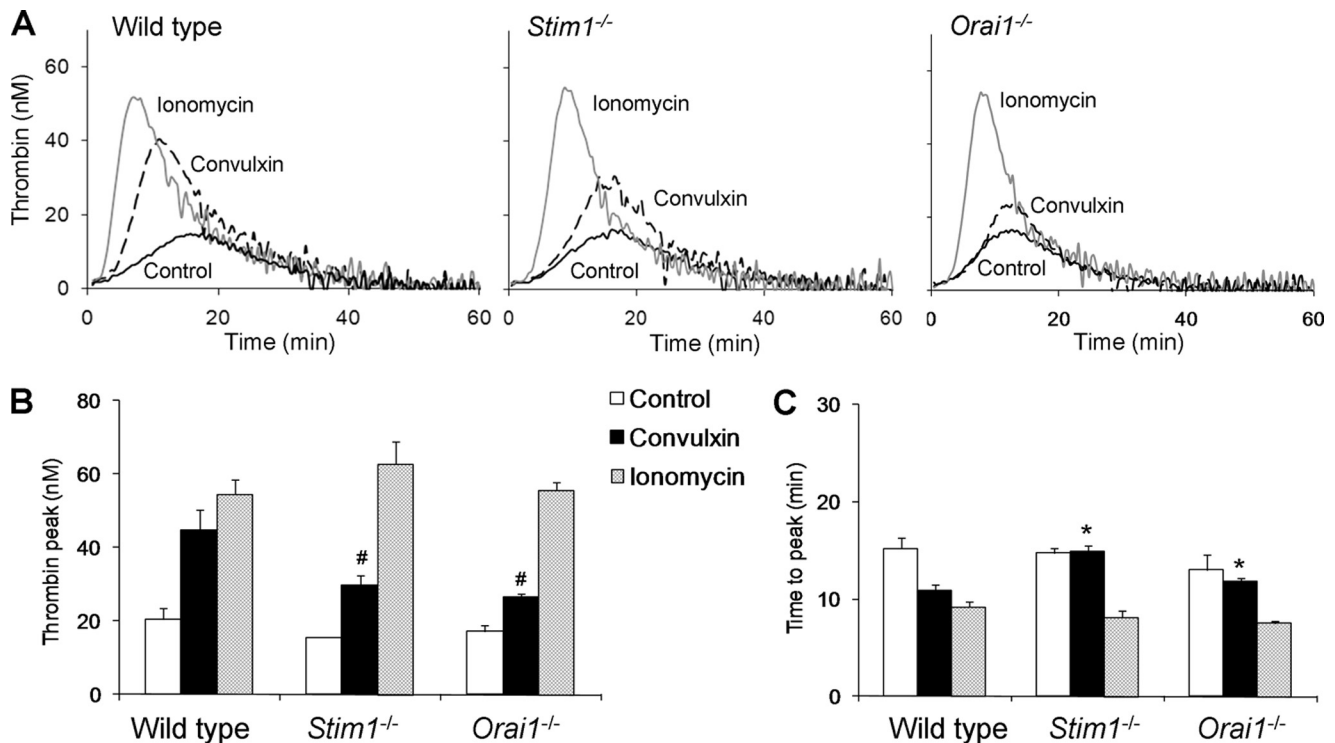


**FIGURE 5. Deficiency in *STIM1* or *Orai1* permits GPVI-dependent procoagulant activity in the presence of thrombin.** Platelets in buffer containing 2 mM CaCl<sub>2</sub> (1 × 10<sup>8</sup>/ml) were stimulated for 10 min with convulxin (100 ng/ml), thrombin (4 nM), or ionomycin (20 μM), as indicated. *A*, flow cytometric analysis of binding of FITC-annexin A5 to platelets (*n* = 5). *B*, determination of prothrombinase activity of platelet suspensions. Activity is expressed as pmol of thrombin/min per 10<sup>6</sup> platelets (*n* = 7–8). Means ± S.E. (error bars) are shown. \*, *p* < 0.05; *n.s.*, not significant compared with wild type.

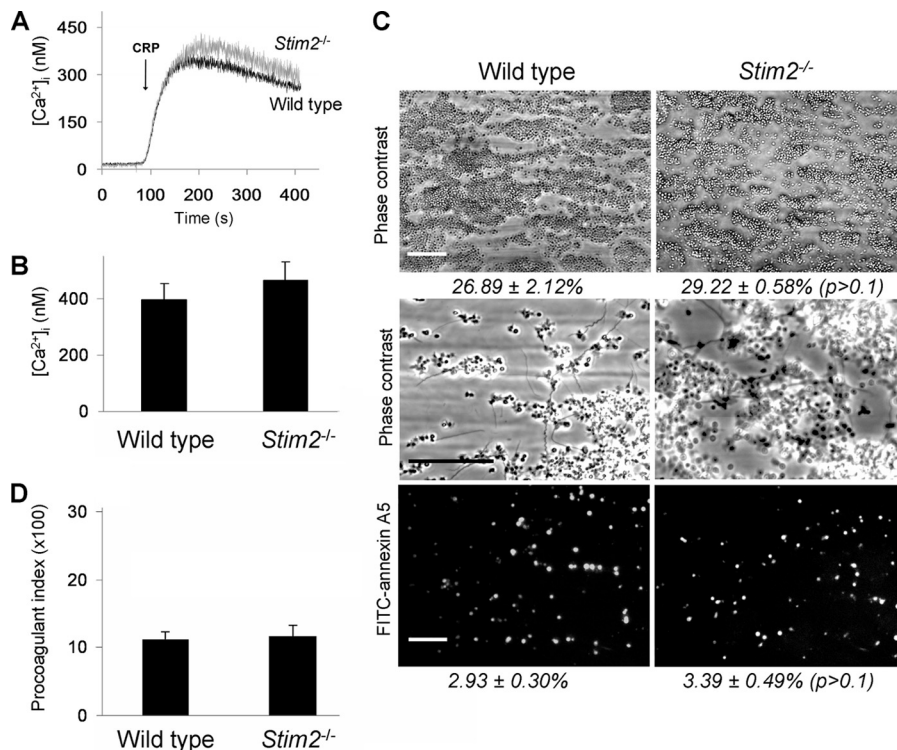
this was not significantly different from wild type. Stimulation with ionomycin/CaCl<sub>2</sub> caused high, maximal prothrombinase activity in all groups.

The procoagulant response of platelets was also studied in natural plasma environment, by activating PRP with tissue factor/CaCl<sub>2</sub> and measuring generation of thrombin (5). Similar thrombin generation curves were obtained with PRP from wild type, *Stim1*<sup>-/-</sup>, and *Orai1*<sup>-/-</sup> mice (Fig. 6*A*). In all groups, prestimulation with ionomycin caused a 3–4-fold increase in thrombin generation, indicating that the knock-out platelets were normally capable to support thrombin generation (Fig. 6*B*). Prestimulation with convulxin resulted in a significant increase in thrombin peak height in all groups, but in this case *STIM1*- or *Orai1*-deficient PRP was less effective than wild type PRP compared with prestimulation with ionomycin. The time-to-peak was less shortened in convulxin-stimulated knock-out PRP compared with wild type (Fig. 6*C*). Taken together, these results show that, in the presence of thrombin (either externally added or formed *in situ*), GPVI-dependent PS exposure and procoagulant activity are only partly affected by deficiency in platelet *STIM1* or *Orai1*.

**Roles of Ca<sup>2+</sup> Entry Channels Other than *Orai1***—The modest roles of *STIM1* and *Orai1* in GPVI-dependent platelet procoagulant activity in the presence of thrombin suggest the presence of a compensating Ca<sup>2+</sup> entry mechanism, that, for instance, could be triggered in a receptor-operated fashion. In other cell types, it has been shown also that *STIM2* regulates Ca<sup>2+</sup> entry by controlling cytoplasmic Ca<sup>2+</sup> levels (38). Because



**FIGURE 6. Deficiency in *STIM1* or *Orai1* reduces GPVI-dependent thrombin generation.** Citrate-anticoagulated PRP ( $1 \times 10^8$  platelets/ml) of the indicated mice was preincubated with vehicle solvent (control), convulxin (100 ng/ml), or ionomycin (20  $\mu$ M). Thrombin generation was triggered with tissue factor/ $\text{CaCl}_2$ . *A*, representative thrombin generation curves per genotype. *B*, quantification of thrombin peak height. *C*, quantification of time to peak. Means  $\pm$  S.E. (error bars) are shown.  $n = 4-6$ . #,  $p < 0.05$  compared with wild type (ionomycin); \*,  $p < 0.05$  compared with control.



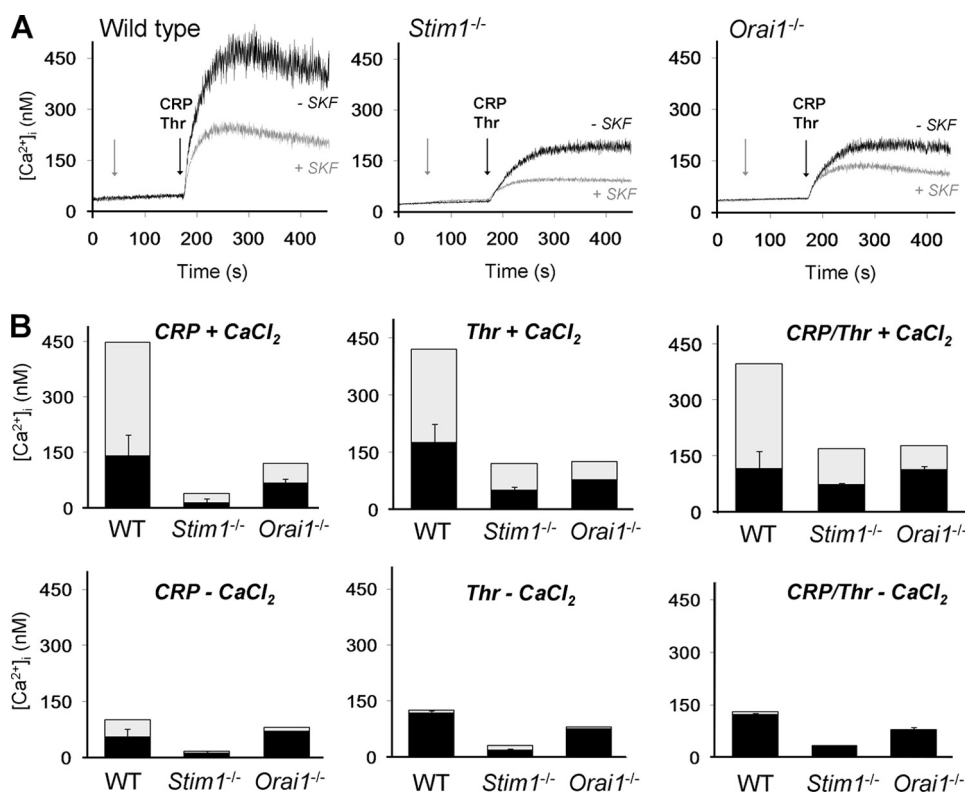
**FIGURE 7. Unchanged GPVI-dependent  $\text{Ca}^{2+}$  responses and thrombus formation of *STIM2*-deficient platelets.** *A*, representative  $\text{Ca}^{2+}$  rises of Fura-2-loaded platelets from wild type and *Stim2*<sup>-/-</sup> mice, induced by CRP (10  $\mu$ g/ml) plus  $\text{CaCl}_2$  (1 mM). *B*, quantification of maximal  $\text{Ca}^{2+}$  rises. *C* and *D*, thrombus formation after flow of PPACK/heparin-anticoagulated blood over collagen at high shear rate (4 min). *C*, representative contrast images captured at low (top panels) or high (middle panels) magnification. Bottom panels, fluorescence images after staining with FITC-annexin A5 (scale bars, 50  $\mu$ m). *D*, procoagulant index of relative number of PS-exposing platelets. Means  $\pm$  S.E. (error bars) are shown.  $n = 4-6$ . The difference between groups was not significant.

mouse platelets highly express this isoform (supplemental Fig. 1B), we studied the possibility that *STIM2*-mediated  $\text{Ca}^{2+}$  regulation controls the extent of PS exposure. Mice homozygously deficient in *STIM2* are viable and have normal platelet counts (27). Stimulation of Fura-2-loaded *Stim2*<sup>-/-</sup> platelets showed unaltered  $\text{Ca}^{2+}$  responses upon stimulation of GPVI (Fig. 7, *A* and *B*) or thrombin-receptors (not shown). Flow perfusion experiments over collagen, performed with blood from the *Stim2*<sup>-/-</sup> mice, indicated the formation of large thrombi and the presence of many PS-exposing platelets (Fig. 7C). Furthermore, detailed image analysis pointed to an unchanged procoagulant index of *Stim2*<sup>-/-</sup> thrombi compared with *Stim2*<sup>+/+</sup> thrombi (Fig. 7D).

The putative role of other  $\text{Ca}^{2+}$  entry channels was investigated by using the established  $\text{Ca}^{2+}$  entry blocker SKF96365 (39). To compare with earlier established  $\text{Ca}^{2+}$  traces (supplemental Fig. 1), Fura-2-loaded platelets were activated with GPVI agonist CRP, thrombin, or a



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**FIGURE 8. Residual receptor-induced Ca<sup>2+</sup> entry in *STIM1*- and *Orai1*-deficient platelets.** Calcium responses were measured of Fura-2-loaded platelets from wild type (WT), chimeric *Stim1*<sup>-/-</sup>, or *Orai1*<sup>-/-</sup> mice, induced by CRP (10  $\mu$ g/ml) and/or thrombin (Thr, 0.9 nM) and CaCl<sub>2</sub> (1 mM), as indicated. *A*, representative traces of SKF96365 (SKF, 100  $\mu$ M) effect on Ca<sup>2+</sup> rises. Arrows indicate addition of SKF96365 and agonist(s), respectively. *B*, quantitative effect of SKF96365 on Ca<sup>2+</sup> rises. Total bars give rises without SKF96365; black bars indicate rises with SKF96365; and gray bars represent inhibition by SKF96365 (means  $\pm$  S.E.,  $n = 3-4$ ).

combination of both. In wild type platelets, pretreatment with SKF96365 inhibited the Ca<sup>2+</sup> responses to all agonists with 60–70%, only if CaCl<sub>2</sub> was present in the incubation medium (Fig. 8A). On the other hand, when no CaCl<sub>2</sub> was added and Ca<sup>2+</sup> entry was prevented, SKF96365 did not have a significant effect (Fig. 8B). Pretreatment with SKF96365 had a similar, 70% inhibitory effect on the Ca<sup>2+</sup> response evoked by convulxin, again only in the presence of CaCl<sub>2</sub>. Strikingly, in *Stim1*<sup>-/-</sup> and *Orai1*<sup>-/-</sup> platelets, SKF96365 still suppressed both the CRP- and thrombin-induced Ca<sup>2+</sup> responses by 40–60%, again if extracellular CaCl<sub>2</sub> was present (Fig. 8B). For all genotypes, the inhibition by SKF96365 was significant in the presence of CaCl<sub>2</sub> ( $p < 0.001$ ), but not without CaCl<sub>2</sub> ( $p = 0.05-0.38$ ). The *Stim1*<sup>-/-</sup> and *Orai1*<sup>-/-</sup> platelets responded similarly, but the former had a lower residual Ca<sup>2+</sup> signal in the presence of SKF96365, which is explained by the reduced Ca<sup>2+</sup> store content in these cells. Comparison of time traces of knock-out platelets shows that the SKF96365-inhibitable Ca<sup>2+</sup> signal is a relatively slow component, operating after ~10–60 s (Fig. 8A).

Further experiments demonstrated that the SKF96365-inhibitable Ca<sup>2+</sup> entry pathway was important for procoagulant activity because it suppressed the GPVI/thrombin-mediated PS exposure in both wild type ( $-73 \pm 8\%$ ) and *Orai1*<sup>-/-</sup> platelets ( $-34 \pm 6\%$ ,  $p < 0.05$ ). Together, these data point to the involvement of a second Ca<sup>2+</sup> entry path-

way in platelet PS exposure that is different from the *Orai1* channels.

## DISCUSSION

This study examines the contribution of recently discovered Ca<sup>2+</sup> entry pathways to the regulation of platelet procoagulant activity and thrombus formation. A first key finding is that deficiency in the Ca<sup>2+</sup> sensor *STIM1* or the Ca<sup>2+</sup>-selective channel *Orai1* greatly reduces GPVI-dependent PS exposure and thrombus formation under conditions of flow and absence of coagulation. This corresponds to a major reduction in the Ca<sup>2+</sup> responses of collagen-adhered *Stim1*<sup>-/-</sup> and *Orai1*<sup>-/-</sup> platelets. Thus, it can be concluded that the reduced GPVI-induced Ca<sup>2+</sup> signaling leads to impaired PS exposure and platelet aggregation. Interestingly, platelets from *Stim2*<sup>-/-</sup> mice are normally active in thrombus formation and PS exposure under the same flow conditions, indicating that the *STIM2* homolog is not implicated in these platelet responses.

Detailed single-cell analysis yet points to discernible differences between the genotypes, in that a subpopulation of the collagen-adhered *Orai1*<sup>-/-</sup> platelets, but not of the *Stim1*<sup>-/-</sup> platelets, displays residual spiking in Ca<sup>2+</sup>. This residual Ca<sup>2+</sup> signal will be a consequence of the appreciable GPVI-induced Ca<sup>2+</sup> mobilization from internal stores of *Orai1*-deficient platelet (25), in contrast to the markedly reduced Ca<sup>2+</sup> store mobilization of *STIM1*-deficient platelets (24). This difference between the genotypes was confirmed in CRP- and thrombin-induced activation studies with Fura-2-loaded platelets. The present findings agree with the recent evidence that a dysfunctional R93W mutation of the *Orai1* channel leads to partial impairment of GPVI-induced PS exposure (26). Collectively, our data underline the importance of both SOCE-regulating proteins in shear-dependent platelet activation by collagen under conditions where the contribution of thrombin is absent or limited.

The second key finding is that, under conditions of flow and coagulation, deficiency in platelet *STIM1* or *Orai1* does not abolish the capability to PS exposure and formation of a fibrin-containing thrombus. Under these conditions, collagen-adhered *Stim1*<sup>-/-</sup> and *Orai1*<sup>-/-</sup> platelets still show prolonged rises in Ca<sup>2+</sup>, although these are lower in magnitude compared with wild type platelets. Although the number of PS-exposing *Stim1*<sup>-/-</sup> and *Orai1*<sup>-/-</sup> platelets is still reduced, the procoagulant index is similar for all genotypes. This suggests that the GPVI-induced SOCE pathway mediated by *STIM1* and *Orai1* becomes redundant in the regulation of thrombus formation in



cases where thrombin acts as a co-agonist. This conclusion is supported by the data showing that stimulation of isolated *Stim1*<sup>-/-</sup> or *Orai1*<sup>-/-</sup> platelets with a combination of GPVI and thrombin receptor agonists results in near unchanged PS exposure and prothrombinase activity. Furthermore, these platelets are partly inhibited in tissue factor-triggered thrombin generation when stimulated with GPVI ligand. It has been shown that polyphosphates released from platelets significantly contribute to thrombin generation in PRP by activating factor XII (40). The above findings suggest that the polyphosphate contribution is still effective in the absence of *STIM1* or *Orai1*.

The standard protocol for assessment of SOCE is measurement of entry of extracellular Ca<sup>2+</sup> after Ca<sup>2+</sup> store depletion, e.g. by the reticular Ca<sup>2+</sup>-ATPase inhibitor thapsigargin. This Ca<sup>2+</sup> entry is almost totally blocked in *Stim1*<sup>-/-</sup> and *Orai1*<sup>-/-</sup> platelets, implying that the *STIM1*-*Orai1* interaction forms the principal SOCE mechanism (24, 25). A similar conclusion has been drawn for other secretory cells, e.g. T cells and mast cells (41). However, it now appears that both *Stim1*<sup>-/-</sup> and *Orai1*<sup>-/-</sup> platelets show significant Ca<sup>2+</sup> responses and PS exposure, when co-stimulated via GPVI and thrombin receptors. This is suggestive for the presence of another, compensatory mechanism of Ca<sup>2+</sup> entry, acting in a (thrombin) receptor-operated fashion.

This hypothesis is supported by our finding that the imidazole antagonist SKF96365, which is a well studied inhibitor of Ca<sup>2+</sup> entry in platelets (39), is still capable of suppressing the Ca<sup>2+</sup> and procoagulant responses of platelets deficient in *STIM1* or *Orai1*. Unfortunately, this compound cannot be used in measurements of thrombus formation because it rapidly inactivates in the presence of blood plasma (data not shown). In Fura-2-loaded *Stim1*<sup>-/-</sup> and *Orai1*<sup>-/-</sup> platelets, SKF96365 still inhibits 40–60% of the Ca<sup>2+</sup> signal independently of the agonist (GPVI ligand and/or thrombin). The slow, non-*Orai1* Ca<sup>2+</sup> entry most likely does not involve the TRPC1 channel (16) because *Trpc1*<sup>-/-</sup> platelets have a fully intact Ca<sup>2+</sup> signaling machinery (42). Other candidate target proteins of SKF96365 are the non-SOCE channels, TRPC6 and *Orai3*, both of which act in a receptor-operated way and are expressed in platelets (17, 19). Further studies are required to identify this channel.

A pending question is whether the SOCE mechanism *per se* is implicated in the transmembrane scrambling of phospholipids, which is the underlying event of PS exposure. Such a role of SOCE has been proposed by others (43). However, the current findings do not support this because *Stim1*<sup>-/-</sup> and *Orai1*<sup>-/-</sup> platelets (both devoid of SOCE) almost normally expose PS upon combined collagen- and thrombin-receptor stimulation. This is in agreement with the finding that platelets from Scott syndrome patients, which have a defect in PS exposure, show unchanged Ca<sup>2+</sup> signals in response to collagen/thrombin (44). Together, this strongly argues against a role of SOCE or other Ca<sup>2+</sup> channels as phospholipid scramblase proteins.

The present findings support the idea that the *Orai1* channel is an attractive target for pharmacological treatment of thrombosis. Previous results have demonstrated that the deficiency of *Orai1* in mouse platelets protects from arterial thrombus formation, particularly in thrombosis models that are collagen-dependent (24, 25). Because collagen-independent thrombosis

models mostly rely on tissue factor exposure and thrombin generation (6), it is plausible that specific targeting of *Orai1* (or *STIM1*) restricts platelet procoagulant activity and thrombus formation only under conditions, where the role of tissue factor is limited. Hence, targeting of *Orai1* may be more effective to prevent thrombosis in arteries, where (tissue factor-dependent) thrombin accumulation is confined by the high shear flow conditions. In contrast, blockage of *Orai1* may not impair bleeding from wounds where tissue factor is abundantly exposed. Indeed, mice deficient in *Orai1* have only a mild prolongation of the tail bleeding time (25). Whether inhibition of the non-*Orai1* (non-SOCE) channels targeted by SKF96365 also provides anti-thrombotic protection still needs to be investigated.

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