Received: 14 January 2023 | Accepted: 7 November 2023

DOI: 10.1111/bjh.19210

ORIGINAL PAPER

Platelets, Thrombosis and Haemostasis

SARAF overexpression impairs thrombin-induced Ca^{2+} homeostasis in neonatal platelets

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Funding information
Instituto de Salud Carlos III and FEDER, Grant/Award Number: PI14/01956 and PI18/0316; Junta-Extremadura FEDER, Grant/Award Number: GR21008 and IB18020; Ministerio de Ciencia e Innovación, Grant/Award Number: PID2019-104084GB-C21; ERDF

Summary

Neonatal platelets present a reduced response to the platelet agonist, thrombin (Thr), thus resulting in a deficient Thr-induced aggregation. These alterations are more pronounced in premature newborns. Here, our aim was to uncover the causes underneath the impaired Ca^{2+} homeostasis described in neonatal platelets. Both Ca^{2+} mobilization and Ca^{2+} influx in response to Thr are decreased in neonatal platelets compared to maternal and control woman platelets. In neonatal platelets, we observed impaired Ca^{2+} mobilization in response to the PAR-1 agonist (SFLLRN) or by blocking SERCA3 function with tert-butylhydroquinone. Regarding SOCE, the STIM1 regulatory protein, SARAF, was found overexpressed in neonatal platelets, promoting an increase in STIM1/SARAF interaction even under resting conditions. Additionally, higher interaction between SARAF and PDCD61/ALG2 was also observed, reducing SARAF ubiquitination and prolonging its half-life. These results were reproduced by overexpressing SARAF in MEG01 and DAMI cells. Finally, we also observed that pannexin 1 permeability is enhanced in response to Thr in control woman and maternal platelets, but not in neonatal platelets, hence, leading to the deregulation of the Ca^{2+} entry found in neonatal platelets. Summarizing, we show that in neonatal platelets both Ca^{2+} accumulation in the intracellular stores and Thr-evoked Ca^{2+} entry through either capacitative channels or non-selective channels are altered in neonatal platelets, contributing to deregulated Ca^{2+} homeostasis in neonatal platelets and leading to the altered aggregation observed in these subjects.

KEYWORDS
Ca^{2+} homeostasis, neonatal platelets, pannexin1, PDCD61/ALG2, SARAF, TBHQ

INTRODUCTION

Neonatal platelets exhibit alterations in intracellular pathways, which contribute to the widely described alteration of the platelet aggregation.[1–3] These alterations are more recurrent in premature babies.[1–3] An abnormal concentration of pro-coagulant factors has also been confirmed in the peripheral blood of preterm and full-term neonates, but these alterations do not last long and, after 6 months, most newborns present haematological values similar to those found in adults.[4] Furthermore, a reduced response to certain agonist has been described in neonatal platelets, which is due to alterations in the activity of some surface agonist receptors or their respective downstream signalling pathways. Among others, neonatal platelets present a reduced response to ADP, epinephrine, collagen, thrombin (Thr) and thromboxane analogues (TXA_2).[5–7] A very recent study has reported differences between neonatal and maternal

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platelets regarding the content of alpha and dense granules and their secretion rates. These differences were observed in response to collagen or Thr-receptor agonists like CRP, SFLLRN and AYPGKF, but not in response to ADP.[6] In line with this, we have previously described an impaired CLEC-2 and GPVI activation in neonatal platelets.[8] Interestingly, dense granule content was described to be reduced in neonatal platelets, when observed by electron microscopy, but this was not corroborated by other research group using different experimental techniques.[9] Alternatively, a decreased release of serotonin and other platelet agonists (such as Ca^{2+}, ATP and ADP) stored in the dense granules has been demonstrated.[10,11]

Despite the alterations described above, full-term neonates do not usually present clinical haemorrhagic episodes.[5] In fact, a recent publication has even suggested that to reduce neonatal platelet activity may protect newborns against thrombotic episodes.[5] Accordingly, neonatal platelets are often exposed to a pro-thrombotic surrounding environment due to the presence of an elevated amount of ultra-long von Willebrand factor and elevated haematocrit in the umbilical cord blood.[12–14] On the other hand, to understand these discrepancies in the physiology of neonatal platelets in response to Thr, we should consider the plethora of intracellular signalling pathways activated downstream of Thr receptors. The most relevant pathways activated by Thr are described below: (1) Thr activates protease-activated receptors (PARs; PAR-1 and PAR-4),[15,16] which among others, induces the activation of Ca^{2+}-dependent mechanisms downstream of phospholipase C (PLC); furthermore, PARs activate kinases like p115RhoGEF/RhoA, and the SKF (Lyn) pathways like PI3K, Akt, NOS/PKG and MAPK.[17] Additionally, PAR activation promotes the externalization of the integrin αIIbβ3 to the plasma membrane through a well-known inside-out mechanism.[18] (2) Leucine-rich glycoprotein receptor GPIb-IX-V may be activated by the Von Willebrand factor and other agonists.[19,20] Furthermore, Thr activates tyrosine kinases through GPIb that are important for the platelet function.[21–23] In line with this, using specific agonists and inhibitors of these receptors, we have demonstrated that the dense tubular system (DTS) and the acidic granules (AGs) may act as different intracellular Ca^{2+} stores in human platelets.[24] These Ca^{2+} stores can be differentially depleted according to the Thr concentrations used and/or in response to ADP.[24] Thus, activation of platelets with ADP, mainly mobilizes Ca^{2+} from DTS; meanwhile, low concentration of Thr (0.001–0.01 U/mL) activates GPIb-IX-V that mobilized Ca^{2+} mainly from the AGs. Finally, activation of PAR-1 and PAR-4 by Thr evokes Ca^{2+} from both stores, DTS and AGs, presenting PAR-1 much more affinity for Thr than PAR-4.[15,24]

These mechanisms have barely been investigated in neonatal platelets. In fact, regarding the Ca^{2+} homeostasis in neonatal platelets, previous studies have shown altered Ca^{2+} release in response to Thr and collagen.[25,26] Recently, our group have published a comparative transcriptome analysis between neonatal and adult platelets, revealing alterations of many genes involved in Ca^{2+} homeostasis and other proteins indirectly linked to Ca^{2+} signalling pathways, but these have not yet been deeply investigated.[27] As result, the mechanism of thapsigargin (TG)-evoked store-operated Ca^{2+} entry (SOCE) can be deregulated in neonatal platelets. Among other possible explanations, we have recently described that these subjects may present a slower Ca^{2+}-dependent inactivation (SCID),[28] but its physiological relevance in neonatal platelets remains unexplored. Finally, TG has been widely used to evaluate the SOCE mechanism in many cell types, but the intracellular mechanisms activated by this drug are not considered physiological. Therefore, transposition of the observations and conclusions obtained from those results to paediatric medicine would be very limited. Here, we aim to elucidate possible alterations of key proteins that regulate Ca^{2+} homeostasis in response to physiological agonist such as Thr, collagen or ADP in neonatal platelets.

MATERIALS AND METHODS

Materials and methods, together with the description of the subjects recruited for this study have been included in the Supporting Information (Data S1).

RESULTS

Neonatal platelets present an altered aggregation and Ca^{2+} homeostasis in response to Thr

Human platelets from control women (C), mothers (M) and neonates (N) were isolated and resuspended in HBS (Hepes buffered saline) medium containing 1 mM of $CaCl_2$ and apyrase (40 U/mL). As depicted in Figure 1A, aggregation experiments revealed the existence of an alteration in the activation of the neonatal platelets in response to Thr at low (0.1 U/mL; Figure 1A.1) or high (1 U/mL; Figure 1A.2) concentrations. Percentages of aggregation were reduced by 40% ± 15% and 20% ± 6% in maternal platelets, or by 70% ± 15% and 40% ± 8% in neonatal platelets, when platelets were stimulated with 0.1 and 1 U/mL of Thr respectively (Figure 1A; $p < 0.05$, $n = 4$). Conversely, depletion of Ca^{2+} stores using TG at low concentration (200 nM) was unable to evoke aggregation in adult platelets, which required higher TG concentration (5 μM) to obtain almost 50% of the platelet aggregation values found in adult platelets stimulated with Thr (Figure S1A; $n = 3$–5). The contribution of Thr-induced extra-cellular Ca^{2+} entry to platelet aggregation in neonatal and maternal platelets is presented in Figure S1B, where it is shown that in the presence of the non-permeable calcium chelator in the extra-cellular medium (100 μM of EGTA), Ca^{2+} entry is avoided and, thus, platelets from both types of subjects exhibited an impaired aggregation ($n = 3$, dotted traces).

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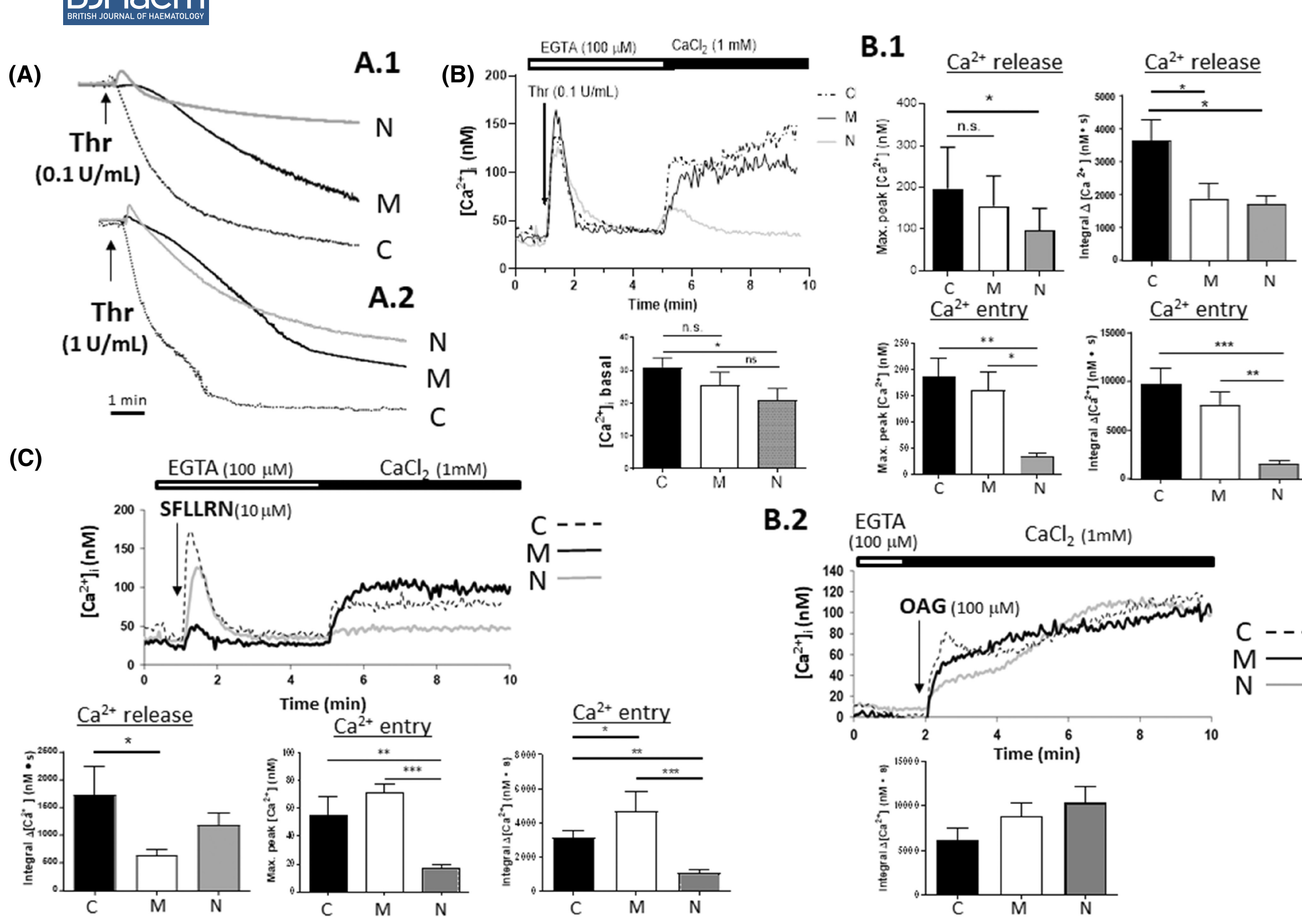

FIGURE 1 Platelet aggregation and Ca^{2+} homeostasis in response to agonists is altered in neonatal platelets. (A) Platelets drawn from control women (C), mothers (M) and neonates (N) were previously suspended in HBS buffer containing apyrase and 1 mM of $CaCl_2$. 400 µL of platelet samples suspended in HBS medium (supplemented with 1 mM of $CaCl_2$) were stimulated with either 0.1 U/mL (A.1) or 1 U/mL (A.2) of Thr and, subsequently, aggregation curves were recorded during the following 10 min using an aggregometer. (B) Fura-2-loaded platelets isolated from all experimental groups were stimulated in a Ca^{2+}-free medium (100 µM of EGTA was added) with Thr [0.1 U/mL; (B.1)] and, alternatively, with OAG in presence 1 mM $CaCl_2$ [100 µM; (B.2)] that were used to assess the basal cytosolic Ca^{2+} concentration and the activation of SOCE or non-SOCE Ca^{2+} entry respectively. Finally, the platelets were stimulated with the PAR-1 agonist [SFLLRN, 10 µM; (C)], and changes in Ca^{2+} release and Ca^{2+} entry were monitored as described in the "Materials and methods" section. Ca^{2+} basal, Ca^{2+} peaks and the integral under the curves were determined during 4 min and were represented in the beside graph bars as mean ± SEM of 5–10 experiments performed using independent subjects. (One-way ANOVA with Dunnett's post hoc test were used.) *, ** and *** represent $p < 0.05$ to $p < 0.001$ with respect to Ca^{2+} values found in control woman platelets.

Altered Ca^{2+} release from different intracellular Ca^{2+} pools in neonatal platelets

Platelet aggregation is promoted by the variations in intracellular calcium concentration ($[Ca^{2+}]_i$), which among other things, control protein kinase activation, granule secretion and cytoskeleton reorganization.[29–33] Therefore, human platelets loaded with the Ca^{2+} fluorescent dye, fura-2, were stimulated according to the protocols described in the "Materials and methods" section. As depicted in Figure 1B.1, basal cytosolic Ca^{2+} concentration was reduced in neonatal platelets compared with platelets from control women; meanwhile, both maternal and neonatal platelets exhibited a reduced Thr-evoked Ca^{2+} release (0.1 U/mL was used) with respect to the control women. Conversely, only neonatal platelets presented a significant reduction in the Thr-induced Ca^{2+}-entry according to the analysis of initial maximum peaks and integrals under the curves (see Figure 1B.1 and histograms below; $n = 6$, $p < 0.001$).

As described in the "Introduction", section Thr evokes PAR activation leading to the generation of IP_3 and diacyl glycerol (DAG).[34,35] Subsequently, DAG activates non-capacitative Ca^{2+} entry (non-SOCE) at the plasma membrane in human platelets through the activation of the Ca^{2+} channels such as TRPC3 and TRPC6.[35,36] Therefore, we used the synthetic analogue 1-oleoyl-2-acetyl-glycerol as a surrogate for DAG[35,36] to find out whether non-SOCE could be altered in neonatal platelets. As shown in Figure 1B.2, non-significant differences were observed among the three experimental groups analysed ($p > 0.05$; $n = 4$). On the other hand, it has been described that Thr activates several receptors at the plasma membrane of human platelets, like PAR-1, PAR-4 and GPIb-IX-V; hence, we used SFLLRN to assess the existence of possible alterations in Ca^{2+} homeostasis downstream of PAR-1,

which is the high-affinity Thr receptor.[24,37,38] Thus, platelet stimulation with SFLLRN in a Ca^{2+}-free medium (100 μM of EGTA was added) resulted in reduced Ca^{2+} release compared with control woman platelets (Figure 1C; $p < 0.05$; $n = 5–7$), but non-statistical differences could be observed in neonatal platelets. Interestingly, neonatal platelets exhibited a reduction in Ca^{2+} entry driven by PAR-1 activation. A 40% ± 8% and 55% ± 5% reduction in SFLLRN-induced Ca^{2+} entry in neonatal platelets was observed compared with the values found in platelets from the control and maternal groups ($p < 0.01$ and $p < 0.001$, respectively, $n = 4–6$). Finally, in agreement with previous results reported in the literature where PAR-4 hyporeactivity has been described in neonatal platelets,[39] PAR-4 agonist (AYFPKF 750 μM) was unable to mobilize Ca^{2+} from the intracellular stores in neonatal platelets (Figure S2A). These findings were also corroborated by preincubation of neonatal platelets for 30 min with 5 μM of SHC79797 (PAR-1 antagonist) that impaired Ca^{2+} release evoked by Thr (0.1 U/mL) (Figure S2B).

In line with these results, previous studies have described that human adult platelets have distinct intracellular Ca^{2+} stores that can activate different types of Ca^{2+} entry into platelets.[32,40] In fact, our research group has previously described that these stores can selectively be depleted using different concentrations of Thr (ranging from 0.005 to 0.1 U/mL).[42] Therefore, to elucidate whether neonatal platelets may exhibit altered intracellular Ca^{2+} pools with respect to adult platelets,[41] we activated platelets with a low concentration of Thr (0.1 U/mL) combined with two sarcoendoplasmic Ca^{2+}-ATPases (SERCAs) blockers, TG and tert-butylhydroquinone (TBHQ). As reported in the literature, TG at very low concentration (10 nM) depletes DTS from adult platelets because SERCA2b is found in DTS membranes; meanwhile, 20 μM of TBHQ blocks SERCA3 function and, subsequently, depletes Ca^{2+} stored in the AGs, where the SERCA3 isoform has been localized.[24,45,46] As shown in Figure 2A, TBHQ (20 μM) was unable to mobilize Ca^{2+} from AGs in neonatal platelets compared with platelets from maternal or control woman ($p < 0.05$, $n = 4$); similarly, Ca^{2+} entry due to emptying of AGs was also significantly reduced by 80% ± 10% compared with adult platelets ($p < 0.001$; $n = 2–4$). In addition, preincubation of neonatal platelets for 5 min with TBHQ (20 μM) in a Ca^{2+}-free HBS medium (100 μM of EGTA was added) and, subsequent, stimulation with Thr (0.1 U/mL) presented a significant reduction of 20% ± 8% in the Ca^{2+} mobilization compared with adult platelets [$p < 0.05$; compare black-bars in the graph bars of the Figure 2B(B.1–B.3)]. Furthermore, to corroborate the existence of an alteration in the ability of SERCA3 to refill AGs, platelets from all experimental groups were incubated with TG (10 nM) to deplete DTS and, subsequently, Thr was added.[32,43] This pharmacological treatment revealed that neonatal platelets contained a greater amount of Ca^{2+} stored in DTS, while AGs in neonatal platelets would store a smaller amount of Ca^{2+}. Reduction in AGs Ca^{2+} content was further demonstrated by analysing the decays of the Ca^{2+} curves after preincubation of platelets with TG (10 nM), being these results used for comparison between neonatal platelets and maternal or control woman platelets (Figure S2D). Interestingly, no significant changes were found between groups in the maximum Ca^{2+} peak evoked by Thr, independently of the SERCA antagonist used (Figure S2E). Finally, we explored the possibility that increased SOCE compensates the reduced Thr-induced Ca^{2+} entry found in neonatal platelets. Therefore, platelets from all experimental groups were stimulated with a combination of TG (200 nM) + Thr (0.1 U/mL) to induce depletion of intracellular stores and 5 min later, 1 mM of $CaCl_2$ was added to determinate SOCE. As depicted in Figure S2C, we validated this hypothesis by confirming the existence of a compensatory mechanism, as neonatal platelets exhibited a similar Ca^{2+} entry than control woman platelets (grey vs. dotted traces in Figure S2C and beside histogram). However, maternal platelets showed reduced Ca^{2+} entry compared with neonatal and control woman platelets (Black-solid trace and white boxes in the beside histogram; 20% ± 8% and 25% ± 14% compared with neonatal and control woman platelets respectively; $p < 0.05$, $n = 4$). As expected, reduced Ca^{2+} mobilization from intracellular stores in response to both agents (TG and TBHQ) was observed in neonatal platelets, but the values were not statistically different between the different experimental groups (see grey trace and grey boxes respectively, in Figure S2C).

Interestingly, we observed a possible alteration in the storage capability and Ca^{2+} mobilization of AGs in neonatal platelets. As depicted in the Figure S3A, Ca^{2+} mobilization in response to the lysosomal disruptor, dipeptide glycyl-l-phenylalanine 2-naphthylamide (GPN, 50 μM) followed by the addition of TBHQ, was reduced in neonatal platelets compared with maternal platelets. Given that calcium is pumped in the AGs by the activity of SERCA3 and the acidic nature of these granules, we further analysed the possible alteration in the acidic nature of these granules using LysoSensor-green, a pH-sensitive dye that is directed to the acidic compartments and, thus, is widely used to stain lysosomes.[45–47] Our results demonstrated an alteration in neonatal platelet AGs as the amount of LysoSensor-green accumulated in these AGs was reduced in neonatal platelets compared with platelets from maternal and control women (see confocal images in Figure S3C; Blue: DTS stained with ER-Tracker, Green: AGs stained with LysoSensor-green). As a result, the subsequent addition of GPN to platelets caused a rapid reduction in LysoSensor-green fluorescence in maternal platelets; meanwhile, fluorescence in neonatal platelets unchanged (Figure S3B; Video S1). This could also be explained by a reduction in cathepsin expression and/or activity in neonatal platelets AGs, but this would deserve further investigation.

Impaired interaction of the key SOCE components in neonatal platelets

We analysed possible alterations in the molecular mechanisms underlying Thr-induced Ca^{2+} entry in neonatal

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platelets. In fact, different Ca^{2+} stores may regulate different Ca^{2+} entry pathways, since both types of platelet stores (DTS and AGs) contain STIM1 in their membranes[43–46] which is the main regulator of SOCE.[43,44] Hence, we first analysed the content of Orai1 channel (main SOCE channel in platelets); thus, no statistical differences in Orai1 expression were found between neonatal and maternal platelets (Figure 3A; $p > 0.05$; $N = 6$). Conversely, differences

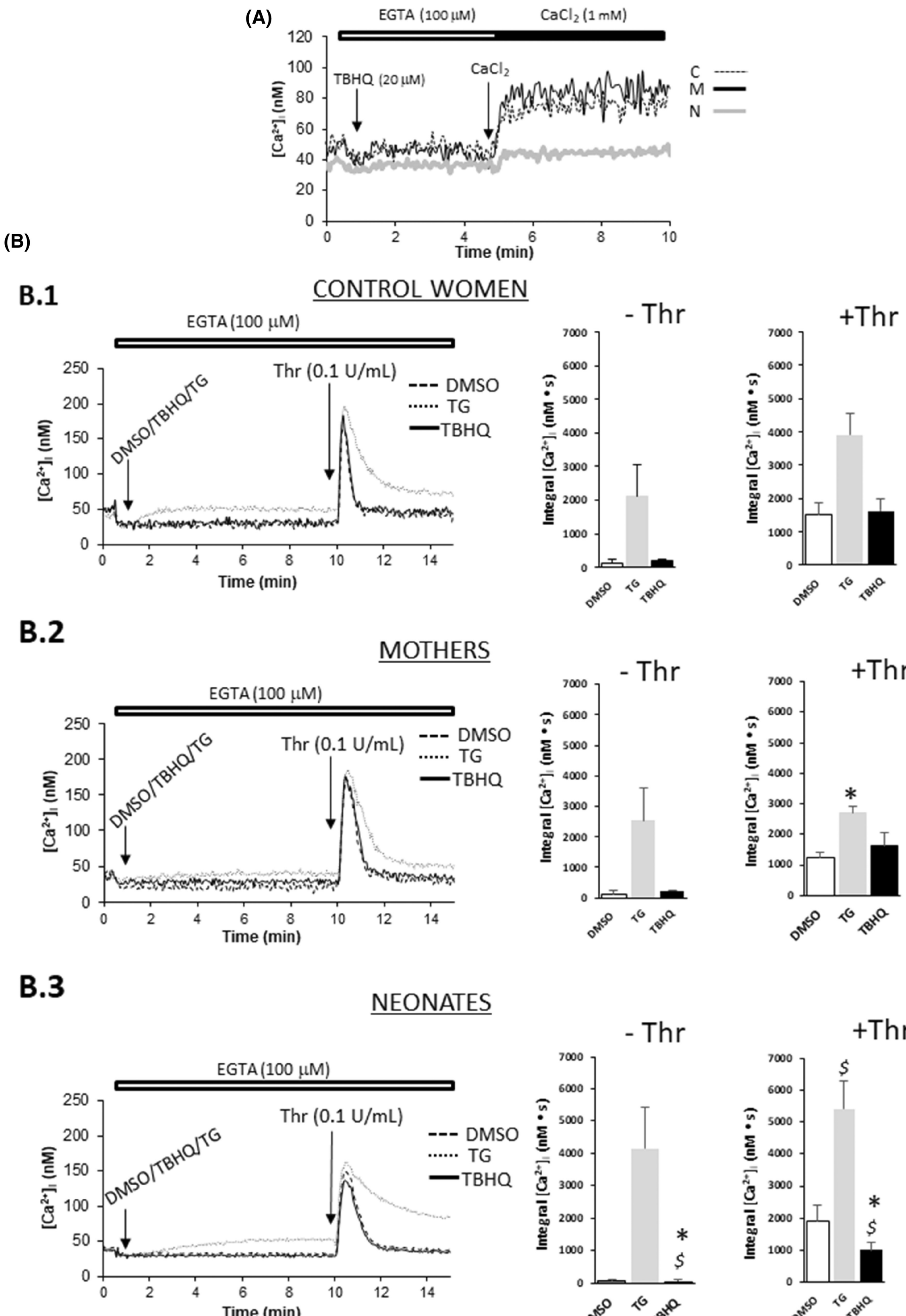

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FIGURE 2 Ca^{2+} mobilization from the acidic granules (AGs) is altered in neonatal platelets. (A) Ca^{2+} content in the AGs of the platelets from control women (C), maternal women (M) and newborns (N) was determined by incubating the fura-2-loaded platelets with tert-butylhydroquinone (TBHQ, 20 μM). Graph is representative of 3–4 independent experiments. (B) Extra-cellular Ca^{2+} was removed by adding 100 μM of EGTA and, subsequently, intracellular Ca^{2+} stores (AGs and DTS) of control woman (B.1), maternal (B.2) and neonatal platelets (B.3) were selectively depleted incubating for 10 min with either low-concentration of TG (10 nM; doted-line), TBHQ (20 μM; black-solid line), or remained unaltered in the presence of DMSO. Finally, Ca^{2+} content that remained within the stores was mobilized by incubating platelets with Thr (0.1 U/mL) for 5 min. Integral of the areas under the curves was calculated for 4 min and was used for comparison between the different experimental groups, as shown beside the graph bars that represent the mean ± SEM of three to four independent experiments. (Kruskall–Wallis and Dunn's tests were used.) "*" Represents $p < 0.05$ with respect to control woman platelets; "$" represents $p < 0.05$ as compared to maternal platelets.

in STIM1 expression, as well as the presence of an alternative STIM1 band were previously reported by our group in neonatal platelets.[45] Additionally, we analysed SARAF, a STIM1 regulator during SOCE. SARAF has been described as the main factor responsible for the mechanism of SCDI.[28,46] In line with the results presented above, a nearly twofold increase in SARAF/STIM1 association was found in neonatal platelets under resting conditions compared with adult platelets. Subsequently, the interaction between these proteins did not change in the presence of Thr with respect to the values found in resting neonatal platelets (compare Lanes 1 and 3 with 7 and 9 in the WB (western blotting) of SARAF and the beside graph bar; Figure 3B; $p < 0.05$, $n = 5–10$). Similarly, alteration of SARAF/STIM1 coupling was also found in maternal platelets.

SARAF overexpression reduces SOCE in the platelet precursor cell lines

SARAF overexpression in neonatal platelets has been previously described by our group,[27] which may lead to an increased SCDI.[28,46] Therefore, we analysed whether SARAF could also be overexpressed in platelet cell-linage, and in pro-megacaryoblastic MEG-01 and DAMI cells. No significant differences in SARAF content were observed in mononuclear cells isolated from peripheral blood samples of mothers and their newborns (Figure 3C, Lanes 2–5, and right-hand side bar graph; $n = 3$). Interestingly, during the analysis we could notice that SARAF was expressed more in MEG01 and DAMI cells (which are considered more differentiated cells than MEG01 cells) than in neonatal platelets (Compare Lane 1 vs. 6 and 7 and see graph bar in the right-hand side of Figure 3C, $N = 3$). In addition, we quantified SARAF expression in peripheral circulating $CD34^+/CD41^+$ pro-megakaryocytes and mature megakaryocytes by flow-cytometry (using an Amnis ImageStream XMk II Imaging Cell Sorter(R)) and the results were also corroborated by confocal microscopy (using a Carl Zeiss® confocal microscope and the Zen 3.4 Blue software). Thus, CD34/CD41-positive cells isolated from the maternal peripheral blood presented an increase in the SARAF staining compared with cells isolated from control women, although these values were not statistically significant (Figure 3D,E, $N = 4$).

Finally, we overexpressed SARAF in DAMI cells resulting in a non-significant 20% ± 15% reduction in the Thr-evoked Ca^{2+} entry (Thr: 0.1 U/mL; Figure 4A; $p > 0.05$; $n = 6$). Conversely, a highly significant 50% ± 10% reduction in the Thr-evoked Ca^{2+} entry was found using high concentration of Thr (0.5 U/mL) in cells overexpressing SARAF (Figure 4A; $p < 0.001$; $n = 6$). As demonstrated in Figure S4A, by using GEM-CEPIA that is a Ca^{2+} sensor dye directed towards the endoplasmic reticulum,[47] we evidenced that although Thr (0.1 U/mL) evoked small changes in the fura-2 fluorescence, it was able to mobilize Ca^{2+} from intracellular stores in MEG01 cells. Interestingly, ER-Ca^{2+} mobilized by Thr (0.1 U/mL) is similar to that observed in DAMI cells using higher Thr concentration (0.5 U/mL). Furthermore, in Figure S4B, GEM-CEPIA was clearly retained within the ER of MEG01 cells and revealed the existence of ER-invagination in the cell nuclei. This later has also been described in other cell types like HEK293 cells, but its physiological relevance remains unknown. On the other hand, SARAF overexpression in MEG01 cells reduced Thr-evoked Ca^{2+} entry, independently of the Thr concentration used (see Ca^{2+} traces and the bar-graphs presented in Figure 4A,B).

Post-translational modification of SARAF in response to Thr

Although the important role of SARAF as a regulatory factor during SOCE has been widely demonstrated, the post-translational modifications that affect function remain poorly investigated. Hence, we analysed the phosphorylation and degradation of SARAF by kinases and proteases respectively. First, as Thr activates several tyrosine kinases (like members of the Src family), we analysed SARAF phosphorylation at tyrosine resides in DAMI cells overexpressing SARAF. As depicted in Figure 5A, changes in tyrosine phosphorylation were observed in response to Thr (0.1 U/mL) in mock-transferred and in SARAF-overexpressing DAMI cells; nonetheless, these results lack statistical significance. Hence, we were unable to draw any relevant conclusions regarding the functional relevance of this post-translational modification of SARAF during Thr-evoked SOCE (Figure 5A, $p > 0.05$, $n = 4–6$). On the other hand, a very recent study has demonstrated that SARAF may be down-regulated by ubiquitination, which has been described to be prevented by the coupling of SARAF to PDCD61/ALG2.[48] We first confirmed the expression of PDCD61/ALG2 in platelets, without finding statistical differences between the three experimental groups, neither at mRNA nor at protein levels (Figure 5B.1, $p < 0.05$; $n = 8–12$). Furthermore, we found no alteration in the expression of PDCD61/ALG2 (see right-hand side dot-plot graphs in Figure 5B.1). Hence, we next assessed the

association between SARAF and PDCD61/ALG2 in platelets, and found an elevated coupling between the two proteins under resting conditions in maternal and neonatal platelets compared to the values found in platelets from control women. As depicted in Figure 5B.2, Thr increased the interaction between PDCD61/ALG2 and SARAF in neonatal

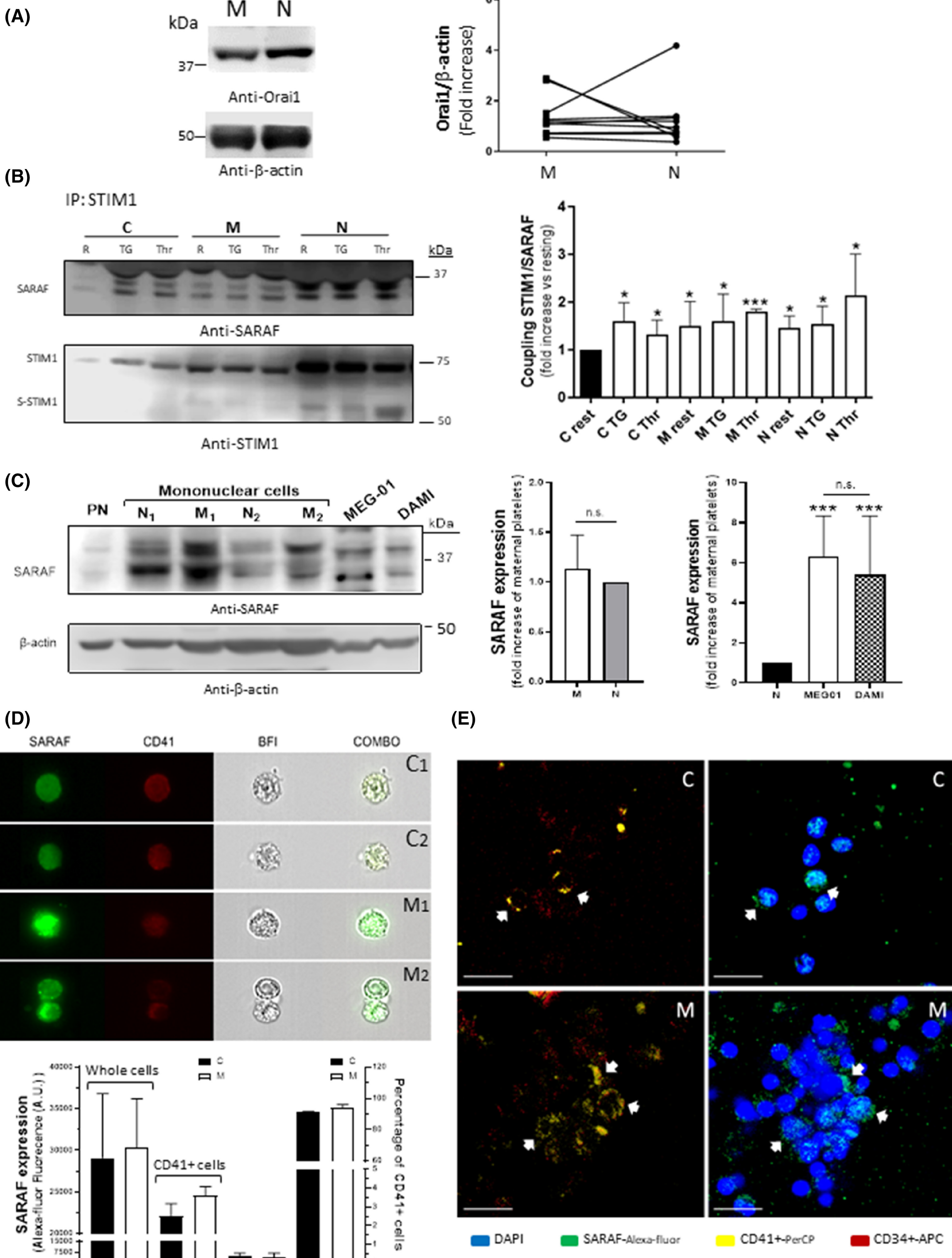

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FIGURE 3 Neonatal platelets present an altered SOCE due to the enhanced STIM1 inhibition by SARAF. (A) Changes in Orai1 expression were analysed in maternal and neonatal platelets under resting conditions by WB, as has been described in the "Materials and methods" section. Images are representative of four independent WBs using platelets from 5 to 10 subjects. Beside dot-plot represents Orai1 content in neonatal platelets compared with the values found in their own mothers upon being normalized with respect to the β-actin values. (B) Platelets from control women (C), mothers (M) and neonates (N) remained under resting conditions in a Ca^{2+}-free medium (R or rest) or were stimulated for 5 min with Thr (0.1 U/mL) or TG (200 nM), as indicated and, finally, they were lysed using ice-cold NP-40 lysis buffer. Following, platelet samples were immunoprecipitated using an anti-STIM1 antibody (2 μg/mL) and agarose beads. Next day, samples were solved by WB using an anti-SARAF antibody according to the conditions described in the "Materials and methods" section. Membranes were reprobed with an anti-STIM1 antibody that was considered as protein loading control. Images are representative of 4–6 independent WBs. Beside bar graphs represent the fold increase compared with platelets from control women under resting conditions. *, *** $p < 0.05$ and $p < 0.001$ as compared with the values found in control woman platelets under resting conditions. (Kruskall–Wallis and Dunn's tests were used). (C) The portion containing the mononuclear cell was isolated from maternal and neonatal blood samples using histopaque-1077 and, following, they were fixed in LB. WB using an anti-SARAF antibody was performed in parallel with samples from neonatal platelets (PN) that were used as control of SARAF expression and, further, samples from platelet progenitor cells (MEG01 and DAMI cells) were simultaneously analysed. Following, anti-β-actin antibody was used as protein loading control to normalize the values of SARAF expression in each sample. Images are representative of 3–4 experiments using blood samples from different subjects. Right-hand side graph represents mean ± SEM of the SARAF fold increase compared to the content found in neonatal platelets. ***$p < 0.001$. (Kruskall–Wallis and Dunn's tests were used). (D) Flow cytometry combined with cell images was used to analyse the expression of SARAF in $CD34^+$/$CD41^+$ cells, which were isolated and stained following the protocol described in the "Materials and methods" section. Images are representative of around 1000 cells detected in four different blood samples from control women and mothers. Left *Y*-axis in the bottom graph bar represents the values of SARAF expression by considering the arbitrary units of fluorescence emitted by the secondary anti-rabbit Ig G antibody conjugated with Alexa Fluor, which was evaluated using the entire cell population (whole cells) or after selecting the $CD41^+$ cells; meanwhile, right *Y*-axis represents the percentage of $CD41^+$ cells before and after performing the cells selection using the fluorescence of the CD41-PercP 5.5 antibody. (E) Cells isolated from control women and mothers were staining using the same protocol than in (D) and were observed under confocal fluorescence microscope at middle cell plane. Images are representative of four different experimental preparations obtained from processing cells of three independent subjects. White arrows point to $CD41^+$ cells and bar represents 50 nm.

platelets as compared to the values found under resting conditions that were lower than in adult platelets (1.8 ± 0.5-fold increase was observed in Thr-stimulated neonatal platelets with respect to resting platelets; $p < 0.05$); meanwhile, changes in the coupling of SARAF to PDCD61/ALG2 were not observed neither in control women nor in maternal platelets.

Finally, in mock-transfected DAMI cells stimulated for 1 min with Thr (0.1 U/mL), a dissociation of the PDCD61/ALG2/SARAF complex was detected (finding almost 54% ± 7% less interaction after Thr stimulation; See Figure 5B.3, $p < 0.01$; $n = 5$–7) and, subsequently, almost a twofold increase in the SARAF ubiquitination could be observed under these experimental conditions ($p < 0.05$; $n = 5$–7). Conversely, in DAMI cells overexpressing SARAF, we could not observe significant changes in the coupling SARAF to PDCD61/ALG2 after being stimulated with Thr (0.1 U/mL; Figure 5B.3; $p < 0.05$; $n = 5$–7). Although changes in the ubiquitination of SARAF were observed in response to Thr in these cells, those changes lack statistical significance ($p > 0.05$; $n = 5$–7).

Pannexin1 channel permeability is altered in neonatal platelets

Although SARAF alteration may explain the reduced Ca^{2+} entry through modification of the SOCE components, an alternative explanation for the deficient Ca^{2+} entry in neonatal platelets could be alteration of the ATP-permeable channel pannexin1 (PANX1). Indeed, the role of PANX1 in platelet function in response to Thr has been underestimated during the last decade. Recently, enhanced Ca^{2+} entry due to activation of PANX1 in response to Thr or collagen has been reported in adult platelets[49,50]; however, direct PANX1 permeation to Ca^{2+} remains elusive.[49–51] With this in mind, we analysed the expression of this channel in platelets by qRT-PCR, and observed a reduced expression of *PANX1* mRNA in neonatal platelets compared to adult (Figure 6A.1; $p < 0.001$; $n = 10$); conversely, analysis of PANX1 at protein level by WB reported non-statistical differences between the three experimental groups considered (Figure 6A.2; $p > 0.05$, $n = 5$). PANX1 expression in neonatal platelets was also corroborated by MALDITOF/TOF analysis, which positively identified two different peptides of PANX1 (Figure 6B).

Changes in PANX1 permeability in response to Thr was evaluated using a calcein-based protocol.[49] Platelets drawn from the three experimental groups were incubated with 2 μM of calcein-AM for 30 min. Then, calcein-loaded platelets were stimulated with Thr (0.1 U/mL) in the absence or presence of 100 μM of probenecid, the PANX1 antagonist and platelets were finally fixed with paraformaldehyde every 2 min while we reproduced the experimental protocol used for the Ca^{2+} experiments.[49] As shown in Figure 6C, Thr induced a time-dependent depletion of the intracellular calcein content in control woman and maternal platelets; thus, more than 50% of the calcein content was lost 10 min after addition of Thr to the extra-cellular medium and in presence of extra-cellular $CaCl_2$ (1 mM; $p < 0.001$; $n = 4$). Conversely, calcein fluorescence remained almost unchanged throughout the experiment in neonatal platelets. Interestingly, release of calcein from platelets was promoted by the addition of Ca^{2+} to the extra-cellular medium (5 min after platelet stimulation with Thr), revealing a direct link between Ca^{2+} entry and PANX1 permeability, in agreement with previous data found in the literature.[49] Furthermore, preincubation for 30 min of probenecid (100 μM) significantly reduced the calcein release from both maternal and control platelets and, as expected, this drug did not affect the calcein content of neonatal platelets. These results confirmed that in adult

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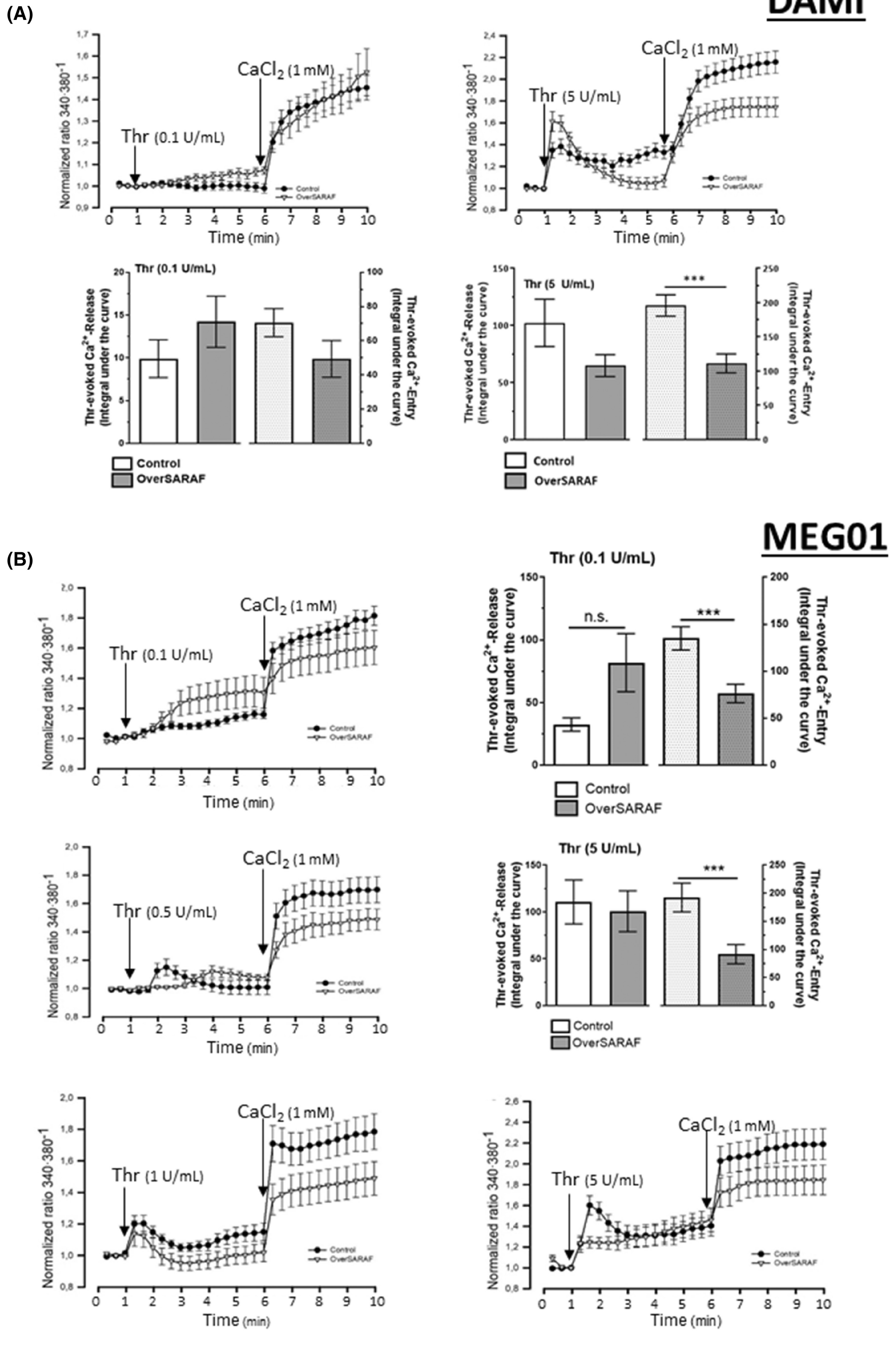

FIGURE 4 SARAF overexpression alters Ca^{2+} homeostasis in DAMI and MEG01 cells. DAMI (A) and MEG01 (B) cells were transfected for 24 h with the overexpression plasmids of SARAF (grey boxes) or the empty vector (Control; white boxes). Cells were then loaded with fura-2/AM and changes in the $[Ca^{2+}]_i$ evoked by cell stimulation with Thr were determined as explained in the "Materials and methods" section. Ca^{2+} curves in the graph are representative of 5–6 independent experiments. The beside bar-graphs represent the mean ± SEM. of the integral under the curves of the Thr-induced Ca^{2+} release (left axes non-dotted boxes) and Ca^{2+} entry as indicated (right axes, dotted boxes). (One-way ANOVA with Dunnett's post hoc test were used.) ***$p < 0.01$ compared to control cells non-transfected with the plasmid of SARAF.

platelets, calcein permeation occurs through PANX1 channels as previously proposed by others[49] and, thus, neonatal platelets present an alteration in this mechanism downstream of Thr receptor.

Additionally, we performed immunoprecipitation assays to determine whether STIM1 could regulate PANX1, and as shown in Figure 6D (upper panel), STIM1/PANX1 coupling was neither found in maternal (M) nor in neonatal (N) platelets. Accordingly, MALDITOF/TOF analysis of neonatal platelet samples immunoprecipitated with the anti-STIM1 antibody confirmed the lack of PANX1 (data not shown). These results led us to conclude that STIM1 does not regulate PANX1 activation in human platelets. On the other hand, p60Src phosphorylation of PANX1 at Y198 has been reported.[52] Therefore, neonatal and maternal platelet samples were maintained in resting conditions or stimulated with Thr (0.1 U/mL), and after IP protocols with the anti-PANX1 antibody, protein phosphorylation profiles were analysed by WB using anti-phosphor-tyrosine (4G10)-antibody. Results presented in Figure 6D (middle panel) revealed that PANX1 is phosphorylated at the tyrosine residues during Thr stimulation in maternal platelets, but the data were not robust among subjects (three to fourfold increase was observed; $p < 0.05$, $n = 3–6$). Conversely, in response to Thr, the activation of PANX1 through this mechanism was not observed in neonatal platelets. On the other hand, the physiological relevance of PANX1 in the platelet aggregation is shown in Figure 6E, where it is shown that preincubation of platelets from control women with 100 μM of probenecid for 30 min significantly reduced by 30% ± 10% the percentage of aggregation evoked by Thr stimulation ($n = 4$).

Finally, we investigated whether other platelet agonists, such as collagen or ADP, could also activate PANX1 permeability and, subsequently, whether PANX1 could be involved in the platelet aggregation in response to these agonists. PANX1-dependent calcein release in response to collagen 2 μg/mL was observed in adult platelets (Figure S5A.1,A.2). Furthermore, treatment of platelets with probenecid (400 μM for 30 min at 37°C) partially reversed the calcein leakage through PANX1, as well as aggregation in response to collagen in platelets from control women (Right-hand side bar-graphs in Figure S5A.2,B; $n = 4–6$). The incapacity of collagen (2 μg/mL) to mobilize calcium from intracellular stores, confirmed that PANX1 permeability is independent of the Ca^{2+} content in the stores, which agree with the fact that STIM1 did not associate with PANX1 during this process, as previously shown (Figure 6D; Figure S5B). Conversely, neither collagen nor ADP was able to induce a reduction in the calcein content in neonatal platelets (Figure S5A.2,A.3). Furthermore, a reduction in the Ca^{2+} mobilization evoked by ADP (10 μM) was observed in neonatal platelets compared with platelets from control women, which is in agreement with previous studies reported in the literature (Figure S5C).[50,53,54] Accordingly, although Ca^{2+} mobilization in response to ADP (10 μM) was significantly reduced in the presence of probenecid (400 μM) in platelets from control women and newborns (with lower concentration: 100 μM), probenecid was unable to impair ADP-induced platelet aggregation (Figure S5C). We also observed a reduced neonatal platelet aggregation in response to ADP stimulus.

DISCUSSION

Neonatal platelets show alterations downstream of several platelet receptors (CLEC-2, GPVI and PAR-4[6,8]), leading to reduced platelet activation. In fact, aggregation in neonatal platelets is altered in response to collagen, ATP and Thr, as shown in this and previous studies performed by others. In fact, physicians and researchers have proposed that these alterations may have a protective role in neonatal platelets, as they contribute to balance the surrounding pro-thrombotic environment.[55] Here, we analysed Thr-stimulated platelet aggregation, finding alterations in the aggregation rates of neonatal platelets with respect to adults using low concentration of Thr (0.1 U/mL), which was the agonist concentration used throughout the present study. Additionally, we enlighten that Ca^{2+} entry is required to evoke full platelet aggregation in both maternal and neonatal platelets that is consistent with previous publication using adult platelets.[15,16] We then explored possible changes in Ca^{2+} homeostasis in neonatal platelets downstream of Thr receptors; thus, our results indicate that platelets from maternal and control women exhibited a similar Thr-evoked Ca^{2+} entry, but neonatal platelets presented an alteration in both Thr-evoked Ca^{2+} release and Ca^{2+} entry.

Human platelets contain different types of intracellular Ca^{2+} pools (such as AGs and/or dense granules) that have different properties respect to the well-known DTS. In fact, the presence of SERCA3 in their membranes, instead of SERCA2b, has been stablished; moreover, both stores contain STIM1 and STIM2 in human platelets.[43] Therefore, SOCE activation may differ in response to the Ca^{2+} store selectively depleted using the different SERCA blockers at the appropriate concentration, as we and others have reported.[32,42,56] Furthermore, given the different affinity of Thr receptors exposed to different concentrations of Thr, distinct Ca^{2+} stores could be selectively depleted, triggering activation of different pathways to operate SOCE.[32,40] Therefore, using pharmacological agents such as TG or TBHQ at the appropriate concentration, we have demonstrated the reduction in Ca^{2+} content in the AGs in neonatal platelets and, subsequently, TBHQ was unable to evoke Ca^{2+} mobilization from these stores. In addition, SERCA3 activity may be also altered in neonatal platelets, as suggested by the reduced areas under the curves observed in the Ca^{2+} experiments and the decays of the Ca^{2+}-curves in response to Thr after being pretreated with a low concentration of TG. This reduction in the curves after Thr stimulation was not observed in adult platelets. The reduction in Ca^{2+} content in the AGs could explain these discrepancies reported in the literature regarding the ultrastructure of neonatal platelets mentioned in the introduction, as well as the lack of response

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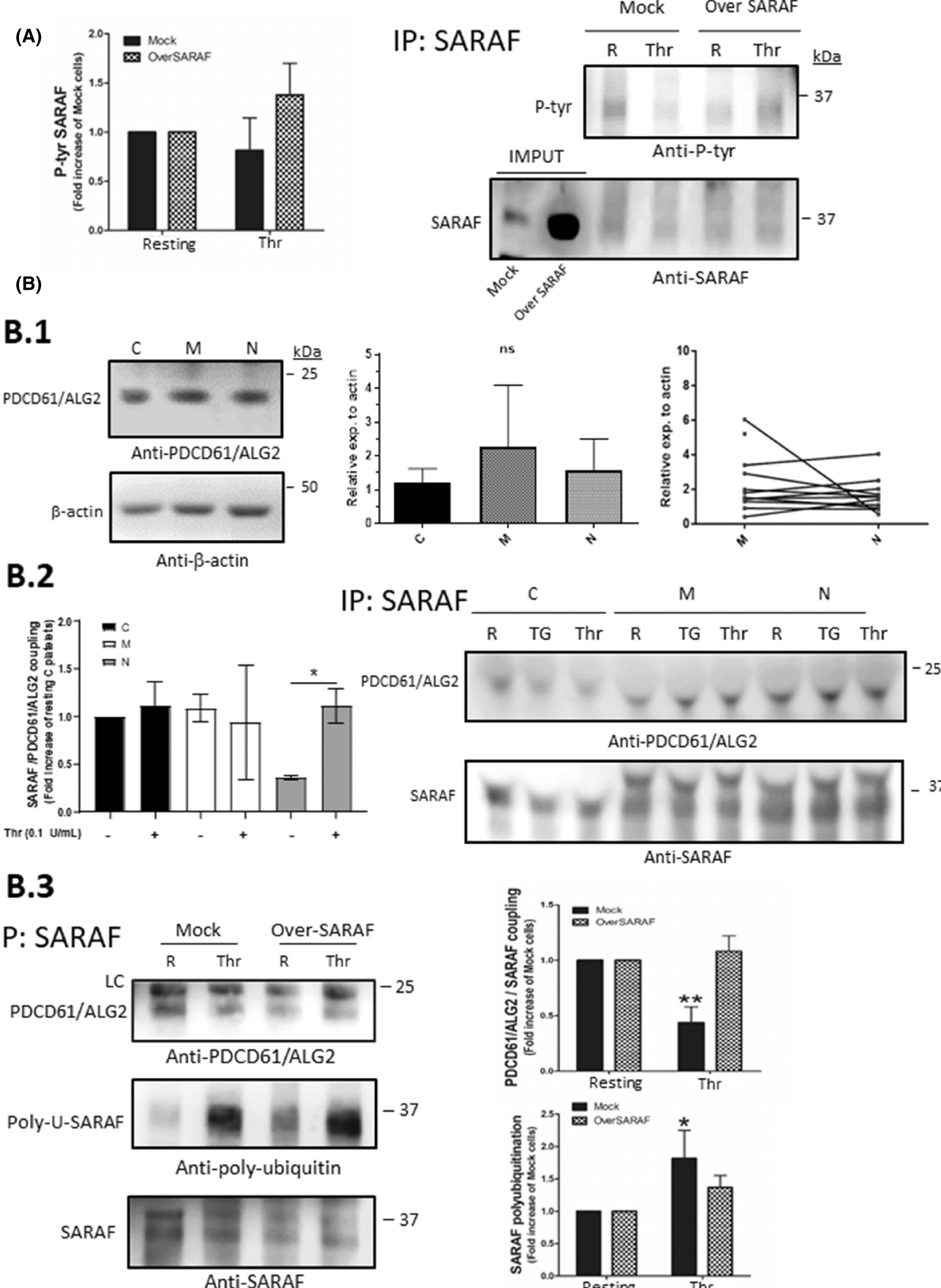

of neonatal platelets to ADP and ATP,[13,57] as these two platelet agonists are stored in these platelet granules. Differences in the amount of Ca^{2+} stored in AGs may also explain the differences in the accumulation of LysoSensor-green in maternal and neonatal platelets observed using either confocal microscopy or spectrofluorophotometry. Indeed, changes in

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FIGURE 5 Post-translational modification of SARAF is altered in neonatal platelets. (A) DAMI cells were transfected either with a mock empty vector (Mock) or with the SARAF overexpression plasmid. Upon confirmation of SARAF overexpression in these cells (IMPUT), they were maintained under resting conditions (R) or were stimulated for 5 min with Thr (0.1 U/mL) in a Ca^{2+}-free medium (100 µM of EGTA was added) and, subsequently, they were lysed by mixing with ice-cold NP-40 buffer. Immunoprecipitated complexes with the anti-SARAF antibody were solved by WB and incubating the membranes with an anti-phosphor-tyr (4G10) antibody. Reprobing of the membranes was done with the anti-SARAF antibody that was used as protein loading control. Image is representative of four independent IPs ($p > 0.05$: Non-significant). (B) Platelets from either control women (C), mothers (M), neonates (N) were lysed with LB in resting conditions and, following, WBs were done using an anti-PDCD61/ALG2 antibody. (B.1) Left-hand side bar graph represents PDCD61/ALG expression after normalization with β-actin; meanwhile, right-hand side bar graph represents comparison between platelets from the mothers and their own babies. (B.2) Platelets from the three experimental groups were kept under resting conditions or stimulated with Thr (0.1 U/mL) and, following, samples were lysed with ice-cold NP-40 buffer. The resulting samples were immunoprecipitated overnight using the anti-SARAF antibody. Next day, immunoprecipitated complexes were analysed by WB using an anti-PDCD61/ALG2 antibody as described in the "Materials and methods" section. Anti-SARAF antibody was used for reprobing the membranes and data were considered as protein loading control. Graph bar represents the fold increase of the coupling between both proteins in control women and under resting conditions ($p < 0.05$; $n = 3–4$). (B.3) DAMI cells were treated as in (A) and lysed with ice-cold NP-40 buffer; following, immunoprecipitation of the samples was completed overnight by using the anti-SARAF antibody. Following, WB was performed to analyse the immunoprecipitated protein complexes using an anti-PDCD61/ALG2 and an anti-poly-ubiquitin antibodies as described in the "Materials and methods" section. Images are representative of 6–8 independent experiments, while graph bars represent mean ± SEM. of fold increase in mock-transfected cells under resting conditions. (One-way ANOVA with Dunnett's post hoc test were used.) * and ** represent $p < 0.05$ and $p < 0.01$ compared to the mock-transfected cells under resting conditions.

the pH of AGs may facilitate the depletion of Ca^{2+} within these granules (evidenced by loss of the LysoSensor-green) which, together with the altered ability of SERCA3 to re-uptake Ca^{2+} into AGs, may lead to reduction in the Ca^{2+} content in AGs of neonatal platelets as demonstrated in the present study.

In line with this, we observed differences in the ability of different Thr receptors to mobilize Ca^{2+} from intracellular stores. Thus, PAR-1-induced Ca^{2+} mobilization in adult platelets was reduced in neonatal platelets, as revealed by SFLLRN. Additionally, incubation of platelets with the specific PAR-4 agonist, AYPGKF, failed to induce significant changes in cytosolic Ca^{2+} concentration, which is in agreement with previous studies.[6,8,39] Discrepancies in the PAR expression and function may explain the observed differences in Ca^{2+} mobilization in response to Thr in neonatal platelets, which has been previously reported.[26] Interestingly, reduced Thr-evoked Ca^{2+} mobilization was found in maternal platelets compared with platelets from control women, which may be explained by alteration in PAR-1-agonist, as evidence using SFLLRN; nonetheless, PAR-1-mediated activation of Ca^{2+} entry appears not to be altered in maternal platelets, in contrast to the alteration found in neonatal platelets.

Orai1-mediated SOCE mechanism is driven by STIM1-dependent activation, which is promoted by the depletion of intracellular Ca^{2+} stores.[44] According to the literature, SARAF is responsible of keeping STIM1 in resting conformation, as well as reversing STIM1 activation upon SOCE activation, the so called SCDI mechanism. Therefore, we have analysed possible alteration in SARAF/STIM1 coupling. As expected, we observed an enhanced SARAF/STIM1 coupling in neonatal and maternal platelets under resting conditions that remains unchanged upon stimulation. These results were further corroborated by overexpressing SARAF in MEG01 and DAMI cells, resulting in a reduced Thr-evoked Ca^{2+} entry. Additionally, in DAMI cells and neonatal platelets, we observed an enhanced interaction between PDCD61/ALG2 and SARAF, which would protect SARAF from ubiquitination.[51] Recently, our research group reported that STIM1 must dissociate from SARAF to reach a fully active conformation (known as *extended conformation*), a process that involves EFHB.[28] However, the mechanism by which both proteins are interchanged is still unknown.[28] Here, we propose that ubiquitination of SARAF may facilitate STIM1 activation, which would allow its dissociation of STIM1 and, subsequently, STIM1 could then associate with EFHB to adopt the full extend conformation.[58] In line with this idea, PDCD61/ALG2 would be associated with SARAF and, therefore, SARAF would not be ubiquitinated and would remain coupled to STIM1 in resting conditions. Hence, we have demonstrated an enhanced association between STIM1/SARAF in neonatal platelets at resting and upon Thr stimulation compared to the values found in adult platelets. Consequently, STIM1 would be unable to interact with Orai1, which would explain the reduction in SOCE induced by Thr in neonatal platelets. In line with this, we previously detected a reduction in SOCE in the platelet cell-linage, which was initially explained by the overexpression of TRPA1 that is thought to have a negative role in SOCE[59]; but it could be additionally explained due to elevated SARAF expression (Figure 3C). Similarly, using flow cytometry and confocal images, we detected a tendency in higher SARAF expression in control $CD34^+$/$CD41^+$ cells isolated from maternal peripheral blood samples than in those from control woman samples, although these data were not statistically significant (Figure 3D,E). Unfortunately, we were not able to obtain bone marrow samples from these subjects, which would have provided more concise results, as well as large number of platelet precursor cells. Therefore, we try to consolidate the above presented results by using DAMI and MEG01 cells where we overexpressed SARAF (Figures 4 and 5).

Finally, we investigated the presence of an additional Ca^{2+} entry mechanism that could be altered in neonatal platelets. The rationale was that the differences found between maternal and neonatal platelets cannot be explained solely by the alteration observed in SOCE activation. In fact, maternal platelets presented Ca^{2+} entry values in response to Thr similar to those of control women; despite they showed elevated SARAF expression. Hence, we explored the contribution of

(A)

A.1

PANX1

****

Fold Change (2^-ΔΔCt method)

Adults

Neonates

C M N

PANX1

—50

Anti-PANX1

β-actin

—50

Anti-β actin

A.2

ns

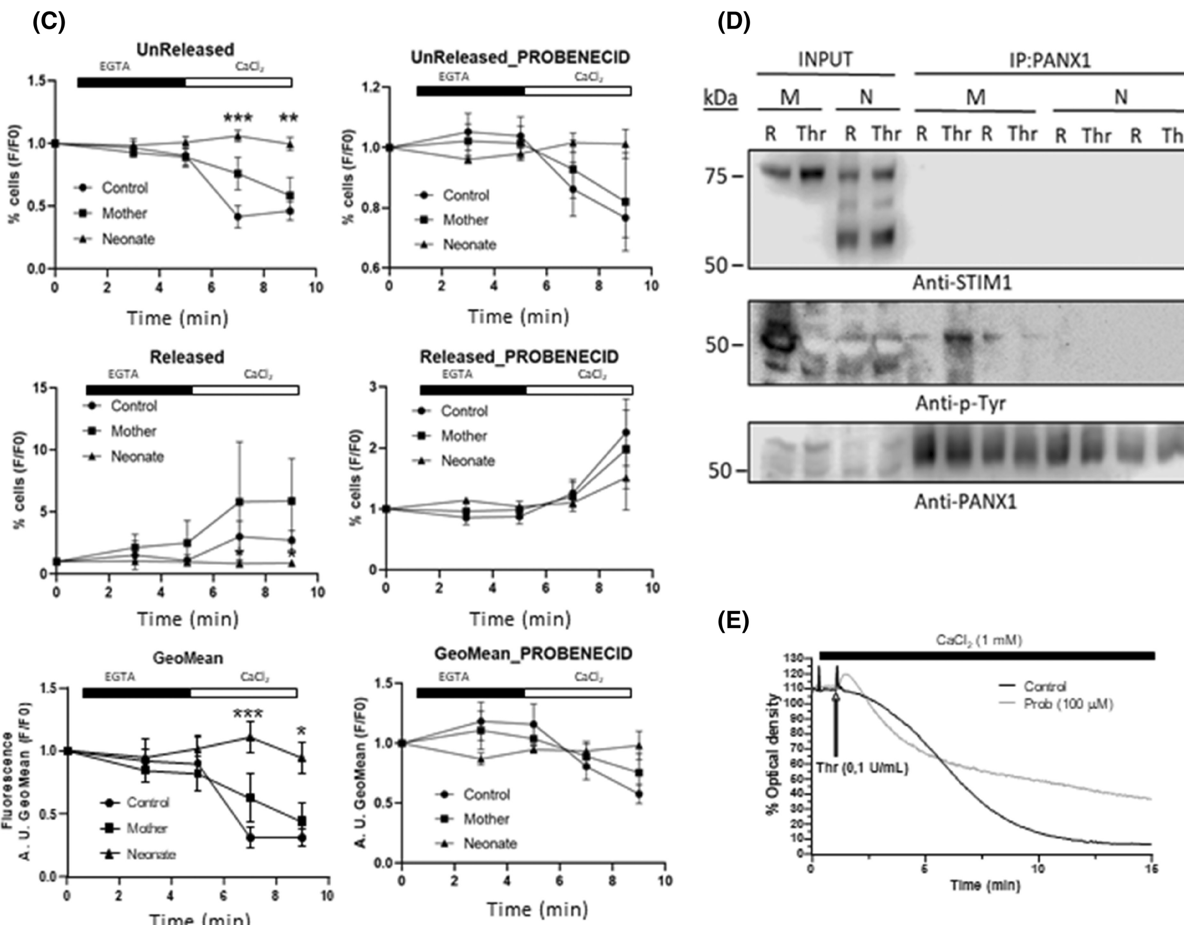
Relative exp. to actin

C M N

(B)

SHOTGUN NEONATES

Protein Accession	Peptide	Unique	-10lgP	Mass	Length	ppm	m/z	z	RT	Area	Scan	#Spec	Start	End
P60709|ACTB	R.KYSVWIGGSILASLSTFQQMWISKQ EYDESGPSIVHR.K	N	101,43	4227,10	37	6,9	1057,792	4	73,03		53882	2	336	372
Q96RD7|PANX1	K.VYEILPTFDVLHFK.S	Y	**59,99**	1719,92	14	0,1	574,3152	3	56,86		39394	1	308	321
Q96RD7|PANX1	K.SSGQGIDPMLLLTNLGMIK.M	Y	**19,75**	1987,04	19	4,2	994,5355	2	70,06		51264	1	356	374



PANX1 permeability to the Ca^{2+} entry in platelets. PANX1 was first identified as a non-selective channel involved in ATP secretion and was considered as an alternative ATP-extrusion pathway different from granule secretion in human platelets.[49,52] PANX1 has already been associated with the Ca^{2+} entry activated downstream of Thr receptors in the platelet linage, but this mechanism has been widely ignored up today. Here, we show that PANX1 is constitutively expressed in neonatal platelets as confirmed by WB and MALDITOF/TOF. In addition, no alteration in PANX1

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FIGURE 6 Pannexin1 permeability is altered in neonatal platelets (A) (A.1) mRNAs of platelets from adults and newborns were isolated using a TRIzol-based protocol and, following, qRT-PCR was completed using Taqman-dyes and specific primers for pannexin1. Presented qRT-PCR data were normalized using β-actin as housekeeping control. ****$p<0.001$ compared to adult platelets. (A.2) Resting platelets isolated from the control women (C), mothers (M) and neonates (N) were lysed with LB (5% DTT) and, subsequently, WBs were completed using an anti-PANX1 antibody. Membranes were reprobed with anti-β-actin antibody as protein loading control. (n.s.: no significant, Kruskall–Wallis and Dunn's tests). (B) Whole neonatal platelet lysates were analysed by MALDITOF/TOF as described in the "Materials and methods" section. (C) Calcein loaded platelets from the three experimental groups were preincubated for 10 min with the vehicle or with 100 μM of probenecid and, following, the platelets were stimulated with Thr (0.1 U/mL) in a Ca^{2+}-free HBS medium (100 μM of EGTA was added) and 5 min later 1 mM of $CaCl_2$ was added to the extra-cellular medium. Platelet samples were taken every 2 min after the addition of Thr to platelets and were then fixed in ice-cold para-formaldehyde solution. Following, the samples of platelet were stained with an anti-CD41 fluorescent-antibody to ascertain the platelet calcein content by using flow-cytometry. Graphs represent the mean ± SEM of the percentage of calcein that was either retained or released by the platelets compared to the entire population, and, additionally, GeoMean (F/F_0) was also represented (bottom graphs). (One-way ANOVA with Dunnett's post hoc test were used.) *, ** and *** represent $p<0.05$, $p<0.01$ and $p<0.001$ compared to platelets under resting conditions. (D) Maternal (M) and neonatal platelets (N) remained under resting conditions (R) or were stimulated with Thr (0.1 U/mL) for 5 min and, following, they were fixed with ice-cold NP-40 buffer. Immunoprecipitation was performed using an anti-pannexin1 antibody (2 μg/mL). Next day, WBs were completed using an anti-STIM1 (top image) or anti-phosphor-Tyr antibodies (middle image) respectively. Finally, membranes were reprobed with an anti-PANX1 antibody that was used as protein loading control (bottom image). Images are representative of 2–4 independent WBs. (E) Control woman platelets were suspended in HBS buffer (supplemented with 1 mM of $CaCl_2$) and were kept under resting condition or were incubated for 30 min at 37°C with 100 μM of probenecid (Prob). After platelet incubation, they were stimulated for 15 min with Thr (0.1 U/mL) and platelet aggregation was monitored as described in the "Materials and methods" section. Traces are representative of four to six aggregation protocols performed using blood samples of three to four volunteers.

expression was found in the three experimental groups considered in the present study. However, PANX1 permeability was significantly reduced in neonatal platelets compared with platelets from control and maternal women, which could explain the difference in Ca^{2+} entry, despite Ca^{2+} does not necessarily enter cells through this channel. In fact, this issue is still under debate. Additionally, we evaluated the possible interaction between STIM1 and PANX1, but no association between the two proteins was found; therefore, the activation of Ca^{2+} entry by PANX1 activation in response to Thr stimulation would be independent of the intracellular pool Ca^{2+} content, as STIM1 would not participate in this signalling pathway. We also corroborate previous findings that PANX1 permeability is driven by collagen. In our hand, collagen was able to evoke PANX1 permeability in adult platelets but not in neonatal platelets. Finally, we do not observe tyrosine phosphorylation of PANX1 in neonatal platelets, which has been proposed as a mechanism of activation in response to Thr, and has been observed in some maternal platelet samples.[52] Therefore, based on the data presented above, although the direct Ca^{2+} permeability of PANX1 remains unresolved here, we can conclude that PANX1 permeability is directly linked to Ca^{2+} entry in human platelets; perhaps, due to the activation Src kinase family members that depend on Ca^{2+} changes to be fully activated. The contribution of PANX1 to the Thr- and collagen-evoked platelet aggregation was also evidenced here by incubating platelets with the PANX1 antagonist, probenecid (Figure S5). Conversely, ADP (10 μM) was not able to conduct PANX1 activation, since ADP-evoked aggregation was unaffected in presence of probenecid. In addition, activation of serine/threonine kinases have also been described involved in PANX1 phosphorylation, and they can also be activated by Ca^{2+}; however, the latter was not investigated here and will need further study in the future.

In summary, in neonatal platelets, we found an alteration in Ca^{2+} content and an impaired Thr-evoked Ca^{2+} mobilization from the intracellular stores. These alterations lead to the deregulation of Ca^{2+} entry in response to Thr. The overexpression of SARAF would result in an increased association with STIM1 in resting conditions, as observed in neonatal platelets. Therefore, we found an impaired signalling mechanism downstream of the different Thr receptors. In line with this, Thr was unable to evoke PDCD61/ALG2 dissociation from SARAF, which, in turn, prevents SARAF ubiquitination. Reduction of SARAF ubiquitination would lead to an increase in STIM1/SARAF coupling in the presence of the stimulus and, subsequently, it could explain the reduction in Thr-induced Ca^{2+} entry found in neonatal platelets. In addition, PANX1 permeability-dependent Ca^{2+} entry is also altered in neonatal platelets, helping to better explain differences observed in the Thr-induced Ca^{2+} entry between neonatal and maternal platelets, although both share an enhanced SARAF expression compared to platelets from control women.

AUTHOR CONTRIBUTIONS

Alejandro Berna-Erro has contributed to the intellectual conception, as well as performed and analysed most of the experiments and did data curation. Elena Delgado, Antonio J. Corbacho and Maria P. Granados were responsible for volunteer interview, providing the informative consents, performing the selection of the subjects and drawing the samples from umbilical cord blood and peripheral blood. Maria T. Vazquez-Godoy provided the additional clinical and/or demographic data from donor subjects and mother and neonatal information. Raul Teruel-Montoya and Francisca Ferrer-Marin performed the genetic array and most of the qRT-PCR determinations done in the platelet samples. Jose A. Tapia performed most of the cytometry analysis. Pedro C. Redondo wrote the manuscript, was responsible for the intellectual conception, data curation and their presentation.

ACKNOWLEDGEMENTS

We thank M. Gomez and S. Alvarado for their technical laboratory support and Dr R. Bobe (IMSERN) who revised

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the manuscript. We also thank the MALDITOF/TOF analysis and data curation done by Dr A. Paraleda from the Proteomics Core Facility of Biotechnology National Center-CSIC. Finally, we thank Dr A. Barrientos and the members of the STAB unit of the University of Extremadura for their contribution with some qRT-PCR analysis. This work has been supported by the Spanish government (MCIN) through a Grant PID2019-104084GB-C21 funded by MCIN/AEI/10.13039/501100011033 and ERDF A way of making Europe, and by the Junta-Extremadura-FEDER research grants (IB18020 and GR21008). R.T.-M. and F.F.-M. were supported by Instituto de Salud Carlos III and FEDER (PI14/01956 and PI18/0316).

CONFLICT OF INTEREST STATEMENT

Authors have no conflicts of interest to declare in the present manuscript.

DATA AVAILABILITY STATEMENT

Data as well as methodological details not included in the manuscript are available at pcr@unex.es.

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How to cite this article: Berna-Erro A, Granados MP, Teruel-Montoya R, Ferrer-Marin F, Delgado E, Corbacho AJ, et al. SARAF overexpression impairs thrombin-induced Ca^{2+} homeostasis in neonatal platelets. Br J Haematol. 2024;204(3):988–1004. https://doi.org/10.1111/bjh.19210

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