Indian Journal of Hematology and Blood Transfusion
https://doi.org/10.1007/s12288-024-01875-y

ORIGINAL ARTICLE

Ablation of CD38 Alleviates the Secondary Aggregation of Platelets

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Received: 10 March 2024 / Accepted: 12 September 2024

Abstract
CD38 is an ectoenzyme that plays an essential role in mobilizing intracellular Ca^{2+}. Here, we intend to demonstrate the role of CD38 in platelet primary and secondary aggregation and suggesting the need to inhibit primary aggregation for cardiovascular patients. Mouse platelets were used in this study. Platelet aggregation, in vitro thrombus formation, release reactions, and calcium signalling experiments were performed in response to thrombin, a normal agonist of platelets. For aggregation, pathway-specific inhibitors were used to differentiate between primary and secondary aggregation. In an in vitro setting, the formation of a thrombus revealed a distinctive pattern on the collagen-coated surface when comparing two types of platelets. Platelets positive for CD38 exhibited smaller yet more aggregated platelets than CD38-negative platelets. Moreover, in vitro, the aggregation process exhibited distinct patterns for the two types of platelets. By employing various inhibitors, we were able to distinguish between the primary and secondary aggregation pathways, both upstream and downstream. The release reaction and calcium signalling were valuable for identifying primary and secondary aggregation events based on their respective time frames. Distinct variations in thrombus formation and aggregation patterns suggest the involvement of CD38. The significant difference in the second minute of calcium signalling and release reaction evidently established a distinct time interphase between primary and secondary aggregation.

Keywords Primary and secondary aggregation · CD38 · Platelet · Calcium · Aggregation

Introduction

CD38, a multifunctional enzyme, is well known to synthesize two potent calcium mobilizing messengers, cADPR and NAADP, which target intracellular calcium stores, the ER, and acidic organelles, respectively. An increase in the cytosolic concentration ($[Ca^{2+}]i$) is hence an important event that maintains physiological responses within the narrow limit of Ca^{2+}. The $[Ca^{2+}]i$ signal represents a balance between the adequate activation of the responses and the avoidance of $[Ca^{2+}]i$ levels that may be toxic to the cell.

The ultimate fate of the platelet, irrespective of the stimulus, is to aggregate at the injury site. The surface of platelets is rich in various kinds of receptors that communicate between the extracellular and intracellular space. Once the signal is relayed to the surface, a series of cascade pathways are initiated, leading to the activation of platelets, followed by shape changes, adhesion, primary aggregation (PA), release reactions, and secondary aggregation (SA) [1]. These series of physiological events are closely related phenomena that depend upon each other and cause what is known as platelet aggregation. Aggregated platelets at the site of injury are thus the physical barrier that forms a hemostatic plug and thus prevents blood loss.

The formation of a stable hemostatic plug, known as a thrombus, occurs as an integrated process of platelet aggregation relating PA and SA. PA results from the direct interaction of a stimulus with its receptor [2, 3] and ends with calcium ion release from the ER. while SA involves the release of dense granule contents, such as serotonin and ADP [4], and the activation of a pathway converting arachidonic acid into prostaglandins, which are further processed into thromboxane (TXA2) [5–8] and end with the formation of thrombotic plug by intercalating with clotting factors. The literature has documented these progressive changes in

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Published online: 19 September 2024

platelets; however, the precise time points at which these changes occur in PAs and SAs have not been determined. PA can be initiated by various agonists, which then cause a release reaction; thus, the release of ADP initiates SA. However, the calcium signal that is initiated in PAs also continues to stimulate SA via CD38, and the release of ADP potentiates and maintains this calcium signal, causing complete biphasic aggregation.

Although the presence of CD38 on the platelet membrane is well established, efforts have been made to elucidate its role and physiological response in platelets. Previous reports have described the involvement of CD38 in procoagulant activity [9] and the role of PKC in CD38 internalization [10]. Likewise, various cell lines have been shown to express CD38 and modulate physiological responses via [Ca2+]*i*. In the present study, we aimed to illustrate the significance of CD38 as a positive modulator of PA and SA in terms of calcium signalling and dense granule secretion.

Materials and Methods

Reagents: Thrombin, R-136, Xestospongin C, and ATP assay kits were purchased from Sigma.

Platelet preparation: The mice were bred and maintained at the university's animal facility. Blood was drawn by cardiac puncture into acid citrate dextrose (ACD) (20 mM citric acid, 110 mM sodium citrate, and 5 mM glucose) at a 1:10 ratio v/v. Platelets were prepared as previously described [9, 11]. In brief, ACD blood was centrifuged at 225 RCF for 6 min at room temperature. Plate-rich plasma (PRP) was removed from the top in a separate tube containing 200 µl of modified Tyrode's buffer (TB). PRP was collected in one tube. With quick centrifugation at 1400 RCF for 40 s, the RBC and WBC that may be present were removed with a couple of quick spins. The PRP was then transferred to another tube and centrifuged at 2000 RCF for 6 min. Pelleted platelets were resuspended to the required density in TB, and the platelet count was adjusted to 0.5×10^9 per ml via an optical method as previously described [12, 13]. Platelets in TB were then rested for 30 min at 37 °C before they were used for the experiment.

Platelet aggregation: Aggregation assays, as previously described [14–16], were performed with a Thermomax microplate reader (Molecular Devices Spectra Max Plus) connected to a computer with SOFTMAX Plus 4.0. The equipment was turned on for at least 30 min before being preheated to 37 °C. Briefly, in a clear flat-bottomed 96-well plate, 100 ul of TB-suspended platelets or incubated with various inhibitors was added, and the cells were stimulated with the agonist or the same volume of vehicle solution (control) by using a multipipette. The plate was stirred for 5 s in a microplate reader, where the turbidity change at 650 nm was measured in units of absorbance every 15 s with agitation for 3 s between readings for 10 min. The absorbance value thus obtained was converted to % of light transmission (LT%).

Measurement of Intracellular Ca^{2+}: Measurement of $[Ca^{2+}]i$. TB-washed platelets were incubated with 1% BSA and 5 µM Fluo3 AM for 40 min. After the incubation, the Fluo3-loaded platelets were stimulated with agonist or vehicle solution. An increase in the Fluo3 AM fluorescence was measured at 485 nm excitation and 538 nm emission using a fluorescence plate reader Fluorescence was recorded for another 10 min with intervals of 15 s and agitation for 3 s between the readings. For the calculation of $[Ca^{2+}]i$, the method described earlier [17] was used.

ATP assay: The platelets were suspended in TB or pre-incubated with BAPTA (an intracellular calcium chelator) and stimulated with thrombin for the desired time at 37 °C in EP tubes. After the desired period, the tubes were spun at 15,000 RCF for 3 min at 4 °C. Supernatant: 800 µl was transferred to other tubes containing ice-cold PCA, vortexed well and left on ice. After centrifugation at 15,000 RCF, the supernatant was transferred to another tube, and the PCA was removed with K_2HCO_3 at a ratio of 3:1. After centrifugation, ATP in the supernatant was measured with a luciferase luciferin mixture with Lumat LB 9507. The values obtained were plotted against the standard value of ATP.

In vitro *thrombus formation*: In vitro, thrombus formation on the surface of collagen was assessed. Hundred µl of 50 µg/ml collagen was added to the 4-well cell culture dish and incubated for 3 h. TB-suspended platelets (100 µl) were added to each well and stimulated with an agonist or the same volume of vehicle (control). The dish was left on a slow-moving shaker for 10 min. After 10 min, the extra platelets were removed. Phase contrast images from 6 random microscopic fields were acquired by using a Nikon Eclipse TE2000-S. Thrombi observed in the images were visually counted.

Statistics: Statistical analysis was carried out on the raw data using SigmaPlot 9 by using an unpaired *Student's t test*, and $P < 0.05$ was considered to indicate statistical significance. The values are expressed as the means, and *n* indicates the number of times the experiments were repeated with other sets of freshly prepared platelets.

Result

CD38 is Required for In Vitro Thrombus Formation

In vitro, thrombus formation was assessed using washed platelets from $CD38^{+/+}$ and $CD38^{-/-}$ mice. Compared with that in $CD38^{+/+}$ mice, the formation of thrombi in $CD38^{-/-}$ mice treated with thrombin was profoundly

reduced (Fig. 1A). $CD38^{+/+}$ platelets exhibited densely packed platelet thrombi, suggesting the completion of the biphasic aggregation process. Usually, such a thrombus does not disaggregate. However, platelets lacking CD38 form a "large" thrombus in which platelets are loosely attached, confirming that the platelets are arrested at the PA. Increasing the dose of thrombin did not significantly change the outcome of this experiment. These findings indicate that the lack of CD38 abrogates SA. The lack of interaction in $CD38^{-/-}$ cells prompted us to assess the degranulation efficacy of the platelets to determine the approximate duration/interphase of PA and SA.

Dense Granule Secretion of ATP

ATP secretion from dense granules was temporally controlled in platelets following treatment with thrombin. ATP release peaked within 2 min (Fig. 2). However, upon preincubation of the platelets with BAPTA, an intracellular Ca^{2+} scavenger, nucleotide release was significantly abolished. These data support the idea that the second minute of aggregation is an interphase of PA and SA. Dense granule secretion is known to be Ca^{2+} dependent, so our next experiment was based on Ca^{2+} release.

Calcium Measurement

The time duration was assessed using washed platelets from $CD38^{+/+}$ and $CD38^{-/-}$ mice, and $Ca2^{+}$ release in the platelets was measured. Calcium release between the two types of platelets significantly decreased after 2 min (Fig. 3). As CD38 synthesizes two potent calcium mobilizing messengers, cADPR and NAADP, using the inhibitors 8-Br-cADPR and bafilomycin A1, these nucleotides abolish the calcium signal (Data published earlier [9]).

Platelet Aggregation

Our previous finding of dense granules and calcium secretion indicated that the second minute of aggregation is somehow an interphase of PA and SA. However, thrombin stimulation of G protein-coupled receptors simultaneously activates two diverse groups of enzymes, PLC and phosphatidylinositol 3-kinase (PI3K). Gα interacts with PLC, and Gβγ mediates the activation of PI3K. Both of these pathways suggest substantial redundancy in platelet signalling pathways as far as the aggregation of platelets is concerned. However, a distinct difference lies in their ability to mobilize intracellular calcium. Similarly, PLC

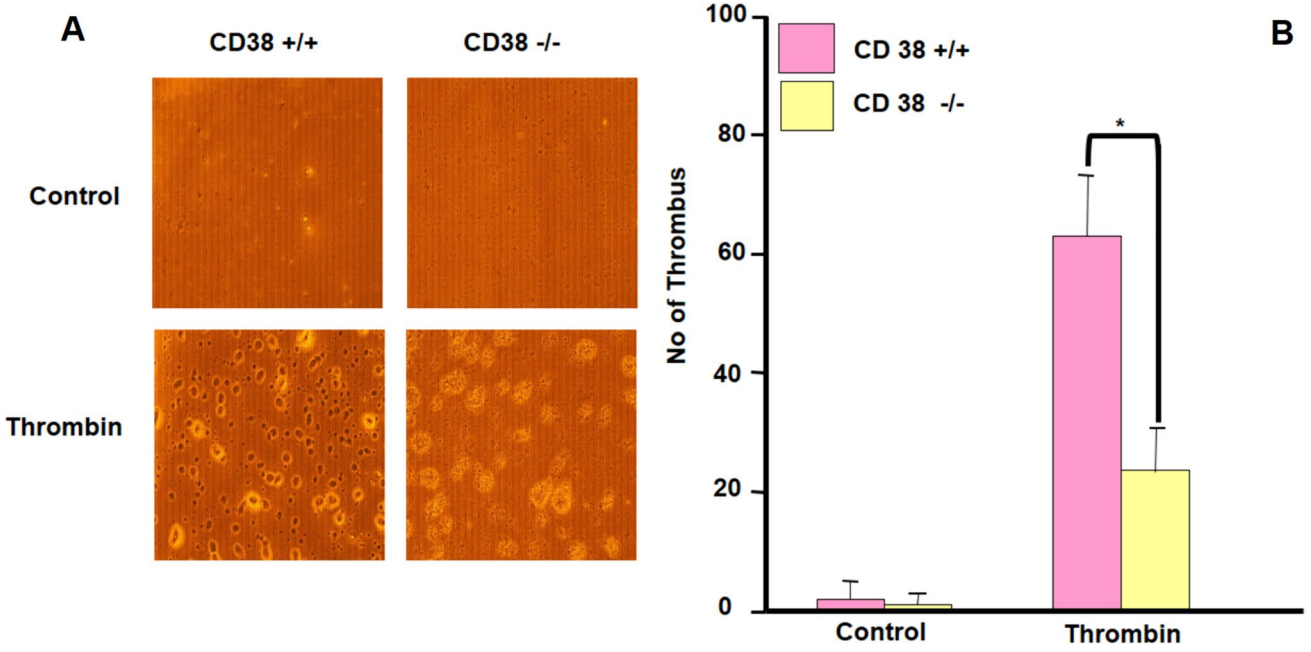

Fig. 1 In vitro thrombus formation: Sedimented and washed platelets from $CD38^{+/+}$ and $CD38^{-/-}$ mice were simultaneously processed, suspended in TB and added to 4-well flat-bottomed plates. At an appropriate concentration, 0.5 units/ml thrombin or the same volume of buffer from the control was added to the wells. The plate was left at room temperature on a slow-moving shaker. After 10 min, the reaction was stopped by the addition of 3% ice-cold formalin, and the mixture was removed after 15 min along with the addition of nonattached platelets. Phase contrast images from 5 random microscopic fields were acquired using a Nikon Eclipse TS100 at a total magnification of 100× , and images were acquired. Aggregated clusters of platelets were visually counted (**A**) and are plotted in a histogram (**B**). Quantitative data (mean ± SD) from four different experiments are shown in the bar graph. A significant reduction in thrombus formation was noted between the $CD38^{+/+}$ and $CD38^{-/-}$ platelets. $^{*}P < 0.03$

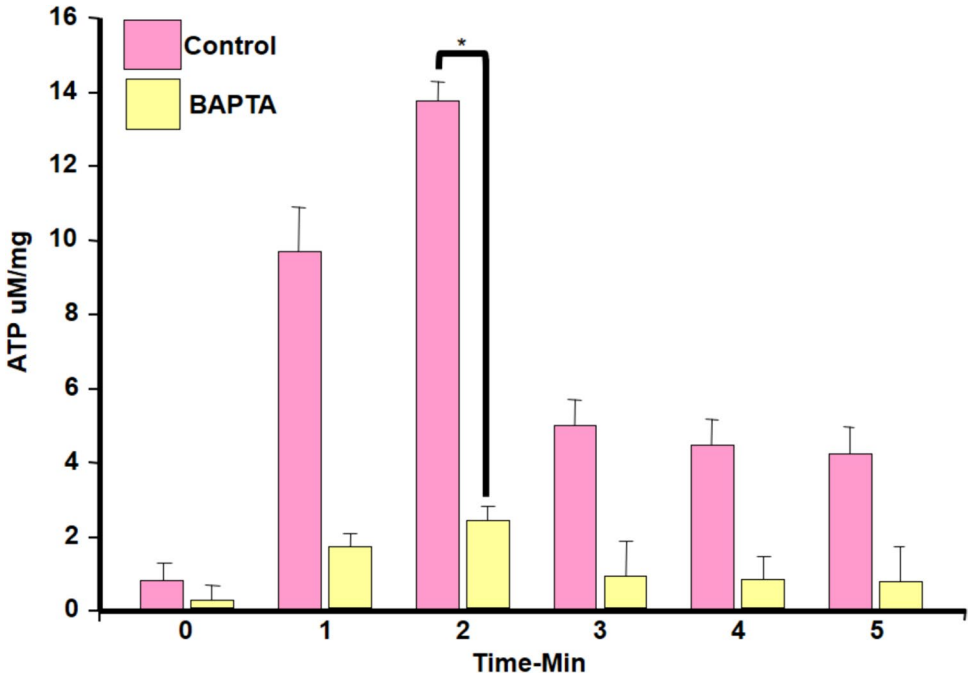

Fig. 2 Dense granule secretion: Platelets of CD38$^{+/+}$ mice were preincubated with or without BAPTA and stimulated with 0.5 units/ml thrombin for the indicated time, after which the amount of secreted ATP in the supernatant was assessed by luminometry. Quantitative data (mean ± SD) from five experiments are shown in the bar graph. The maximum secretion of ATP occurs at 2 min. *$P < 0.02$

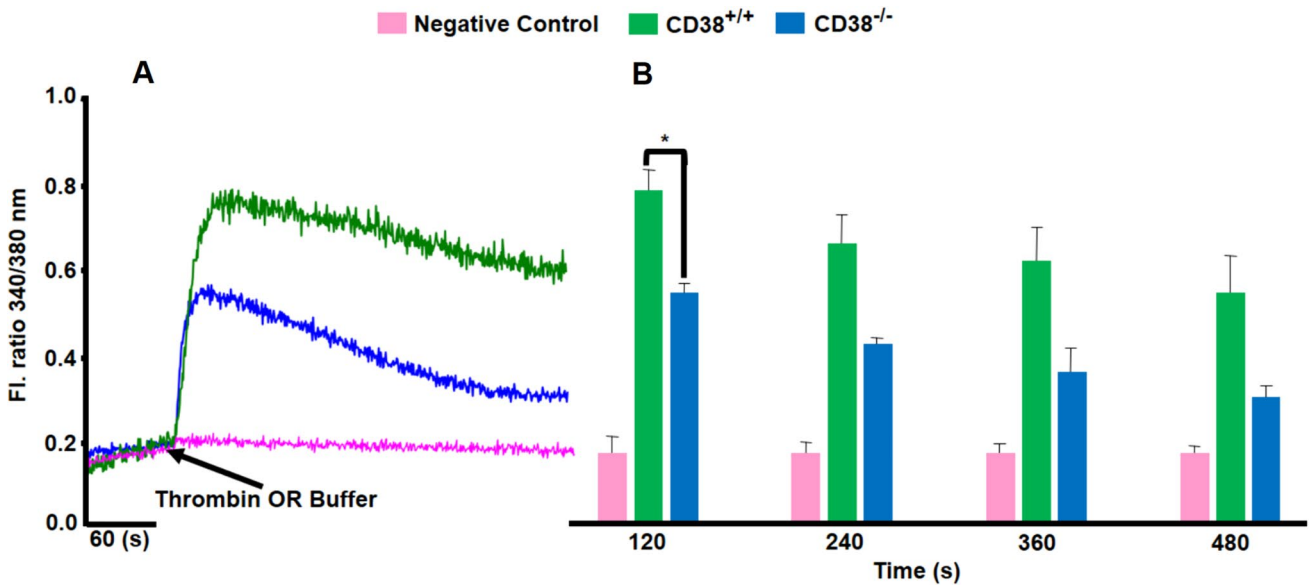

Fig. 3 Calcium measurement: Washed platelets from CD38$^{+/+}$ and CD38$^{-/-}$ mice were simultaneously processed and stimulated with 0.5 U of thrombin for $[Ca^{2+}]i$ measurement (**A**). A significant difference was observed at 2 min between the two platelet groups. The quantitative data (mean ± SD) from three independent experiments are shown in the bar graph (**B**), which represents the mean value (SD) of the fluorescence (FL) ratio of $[Ca_2]i$. *$p < 0.04$

activation also bifurcates to IP3 and DAG in terms of calcium mobilization and hence mechanistically affects platelet aggregation. Washed platelets were preincubated with the PLC inhibitor U-73122, an inositol trisphosphate inhibitor; Xestospongin C, a PKC inhibitor; or R-136 and coincubated with U-73122 and R-136 to assess the patterns of aggregation. Aggregation abrogates with XC, and coincubation with U and R. The PKC inhibitor allows some aggregation in the first couple of minutes, which continues to occur in the latter time period at the same amplitude. On the other hand, PLC inhibitors completely abrogate the first half, and some aggregation can be observed in the latter half of the reaction. This interesting pattern suggests that PLC and PKC play distinct roles in

aggregation, with PLC playing a role in the first couple of minutes and PKC playing a role in downstream pathways. Compared with the positive control, the second minute seemed to be an important interphase of upstream and downstream pathways. However, the results of $CD38^{+/+}$ and $CD38^{-/-}$ platelet aggregation were also the same, as expected. After the first spike of 60 s, it diminished in the 2nd minute and continued to remain the same throughout the observed period (Fig. 4C). Figure 4D shows a histogram of the same data.

Discussion

CD38 is a type II transmembrane protein known to synthesize cADPR and NAADP. Both of these nucleotides have established firm evidence of their ability to mobilize intracellular calcium from the internal calcium store. Ca^{2+} influx and $[Ca^{2+}]i$ mobilization [18, 19] have long been considered prerequisites for platelet aggregation; however, intercellular calcium communication enables platelet aggregation and thrombus growth [20]. Similarly, cytosolic calcium changes during platelet adhesion and cohesion under flow conditions [21]. The most potent and physiological stimuli about to do

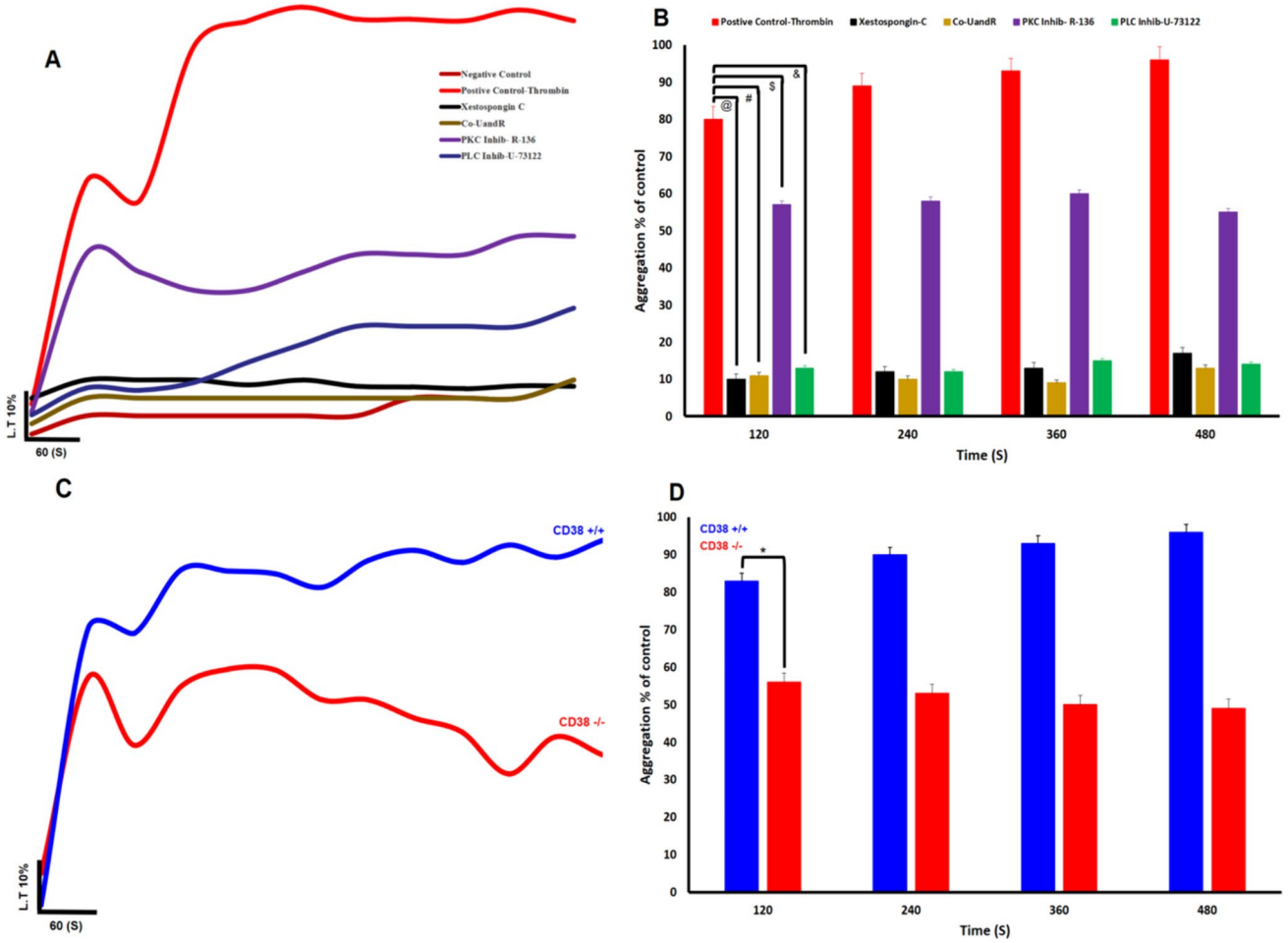

Fig. 4 Platelet aggregation: Sedimented and washed $CD38^{+/+}$ platelets were suspended in TB and added to 96-well flat-bottomed plates after preincubation with the relevant inhibitors. An appropriate concentration and volume of thrombin were added to each well, and the plates were subjected to analysis with a prewarmed ThermoMax microplate reader (SOFT-Max PRO version 4, Life Sciences Edition). Light transmission was measured every 15 min at 650 nm for 10 min. The plate was stirred for 5 s after every 15 s (**A**). For statistical purposes, the same reading histogram (**B**) was plotted for a specific time gap. The quantitative data in the bar histogram are presented as the means ± SDs of three independent experiments. Compared with that of the positive control, significantly reduced aggregation was observed after preincubation with the inhibitor. @ $P < 0.01$, # $P < 0.01$, \$ $P < 0.03$, and & $P < 0.01$. Similarly, the aggregation pattern of $CD38^{+/+}$ and $CD38^{-/-}$ washed platelets mimicked that of the PKC inhibitor (**C**), and after 2 min, the aggregation pattern pronouncedly decreased (**D**)

so are thrombin and ADP. Therefore, in this study, we used thrombin as the most potent agonist of platelets.

Primarily, platelet aggregation is classified as 1-PA, 2-Release of nucleotides, or SA waves, and these aggregation phases have been well documented in the literature [1]. Various diseases are also attributed to these phases, such as PA being impaired in Glanzmann's thrombasthenia [22], release of nucleotides being impaired in storage pool disorders, and defects in the COX pathway [3]. Calcium is the most important need in all of these phases. Hence, calcium-dependent and calcium-independent pathways mediate platelet shape changes [23].

In vitro, thrombus formation in 96-well plates and aggregation in our study were significantly impaired in the absence of CD38-deficient platelets, as these cells behaved unorthodoxly and could not form tightly packed thrombi as $CD38^{+/+}$. Ample redundancy of aggregation is present in platelets; however, knocking down calcium-dependent pathways results in delayed onset of platelet activation. Considering that $[Ca^{2+}]i$ is a prime factor for platelet activation upon thrombin stimulation, the same phenomena were demonstrated by Theresa et al. [24].

Oscillatory aggregation of $CD38^{-/-}$ cells enabled us to deduce that $[Ca^{2+}]i$ mobilization is required to form stable packed thrombi, which could be achieved only in the presence of $CD38^{+/+}$ cells to achieve biphasic aggregation of platelets. Therefore, we believe that PA augments SA and that calcium is the carrier of PA to SA.

The appropriate functioning of CD38 requires its translocation from the membrane to the cytosol, and this occurrence was impressively demonstrated earlier by different groups, including our lab members working on different cells [25–27]. Critical analysis revealed that this translocation is PKC-dependent and requires MHC IIA involvement [28].

Using different inhibitors of the upstream and downstream pathways of aggregation, we could completely abrogate aggregation with PLC and Xc inhibitors. However, PKC inhibitors could only ablate SA. Additionally, the use of blebbistatin, an inhibitor of MHC IIA, decreases the affinity of myosin for actin [29] and inhibits the translocation of CD38; hence, its performance is similar to that of an inhibitor of PKC [10]. However, our previous publication confirmed this pathway [9].

Most of the antiplatelets/antithrombotic drugs used by the cardiovascular patient are downstream inhibitors of specific pathways, leaving intact calcium signals which can still bypass and activate the platelet. Many equivocal results of redundancy in aggregation have been reported. This could only be confirmed when a PLC inhibitor or PI3K inhibitor was used; however, coincubation completely ablated the aggregation, and the calcium signal was completely knocked down in the presence of U73122 [10].

These results could easily be translated to human platelets, as a number of groups have reported similarities in the proteome, biological signalling cascade and ultrastructure of mouse and human platelets [30–32]. However, duplicating the same finding will undoubtedly strengthen the findings for clinical application. Various classes of anti-aggregating agents, such as clopidogrel and aspirin, are used by cardiovascular patients; the former inhibits the binding of ADP to its receptor [33], and the latter irreversibly inhibits the COX-1 enzyme, which is required for the formation of thromboxane [34, 35]. Both of these drugs inhibit SA, and therefore, the PA mechanism still prevails. Hence, an agent is needed to inhibit PA during calcium release. Such parallel experiments could further be validated using human platelets, and hence, the need for a more potent anti-aggregating agent could be debated in larger studies. Furthermore, in-depth analysis of aggregating pathways at the molecular level could be beneficial in understanding the interphase of PA and SA.

Conclusion

We conclude that PA and SA are involved in various sets of pathways. Calcium plays a pivotal role in the aggregation of platelets, and CD38, a potent calcium mobilizer, plays a crucial role and carries PA–SA. However, the discrete interphase lies around the 2nd minute, which is confirmed by the aggregation pattern, a significant difference in the calcium signal, and an increased amount of release of nucleotides, which would augment the aggregation in the SA.

Acknowledgements None.

Funding This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Data Availability Data are available on request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Consent for Publication The author approved and consented to publication.

Consent to Participate None.

Ethical Approval None.

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