

Streptococcus pyogenes Activates Human Platelets via Streptolysin S-Mediated Calcium Ion Influx

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Keywords

Streptococcus pyogenes · Streptolysin S · Platelets · Calcium

Abstract

Introduction: *Streptococcus pyogenes* (group A streptococcus, GAS) is an exclusively human pathogen. It causes a wide spectrum of diseases, ranging from mild infections such as pharyngitis to severe life-threatening conditions such as streptococcal toxic shock syndrome (STSS). Thrombocytopenia is a common feature of STSS and is associated with severe outcome. GAS produce a plethora of virulence factors, including streptolysin S (SLS), which has lytic as well as immunomodulatory properties. However, its role in platelet activation remains unclear. **Methods:** Washed human platelets were infected with GAS wild-type and SLS-deficient mutant ($\Delta sagA$) strains. Platelet activation was assessed by measuring degranulation (CD62P expression). The role of calcium influx and the involvement of purinergic type 2 receptors (P2R) in platelet activation by GAS were assessed using chemical antagonists and calcium chelators. **Results:** GAS activate human platelets via SLS-mediated calcium influx, marked by increased surface expression of CD62P. IVIG treatment improved platelet viability in wild-type infections but failed to prevent SLS-mediated activation. Blocking of P2 receptors via suramin or NF449 as well as the

use of calcium chelators reduced SLS-mediated platelet activation. **Conclusion:** This study identified SLS as an M-protein and consequently a serotype-independent activator of human platelets. While IVIG partially improved platelet viability in GAS infections, its inability to prevent excessive platelet activation underscores the need for additional treatment options in severe GAS infections.

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Published by S. Karger AG, Basel

Introduction

Infections caused by an exclusively human pathogen *S. pyogenes* (group A streptococci, GAS) can lead to a broad spectrum of diseases, ranging from mild cases such as pharyngitis and impetigo to serious invasive infections [1]. GAS are a leading cause of monomicrobial necrotizing skin and soft tissue infections (NSTIs), which are often complicated by streptococcal toxic shock syndrome (STSS) [2]. STSS is particularly associated with excessive systemic hyper-inflammation, leading to multi-organ failure [3]. Such a severe presentation of GAS infections is attributed to both host as well as bacterial factors [4]. The secreted virulence factors, including superantigens, host protein-degrading proteases, and

cytolysins are believed to play a crucial role in the severity of GAS infections [5].

GAS secrete two cytolysins, cholesterol-dependent streptolysin O (SLO) and streptolysin S (SLS). In contrast to SLO, SLS is a small (2.7 kDa) non-immunogenic peptide [6, 7]. The SLS-associated gene (*sag*) locus comprises nine genes (*sagABCDEFGHI*), of which the *sagA* gene encodes the premature form of SLS. After processing and release, the mature SLS acts as a receptor-dependent cytolysin [8]. The peptide is responsible for the β -hemolytic phenotype of GAS [8], through targeting the anion channel Band 3 on red blood cells and thereby inducing osmotic imbalance that leads to cell lysis [9]. In addition to its lytic function, SLS also exhibits modulatory actions. It directly activates nociceptors on neurons, leading to the release of calcitonin gene-related peptide into infected tissues [10]. Such actions induce severe pain, which is a hallmark of NSTIs [11]. In addition, SLS was shown to impact signaling in keratinocytes by triggering programmed cell death and inflammatory cascades [12].

Platelets are the second most abundant blood cell type in circulation. Despite their role in hemostasis, several immunomodulatory functions are ascribed [13]. In GAS infections, platelet activation through M-protein-fibrinogen and IgG interaction triggers thrombus formation [14], leading to increased consumption of platelets and coagulation factors, which in turn results in coagulopathy [15, 16]. It was shown that M1, M3, and M5 GAS serotypes utilize such mechanism, with M1 being the most efficient [14]. Furthermore, it is reported that STSS caused by GAS [17, 18] as well as by closely related *Streptococcus dysgalactiae* subspecies *equisimilis* [19] is often associated with reduced platelet counts. Low platelet counts are linked to the poor outcome of invasive streptococcal infections [20].

Platelet activation is achieved via the interaction of a diverse range of ligands with different classes of surface receptors. Thrombin is a potent trigger of platelet activation and is recognized by the protease-activated receptors (PAR) 1 and 4 [21, 22]. Upon binding of a ligand, PARs further contribute to platelet function by promoting degranulation and inducing platelet procoagulant activity [23]. The released granule content, which includes thrombin, ADP, and thromboxane among others, leads to further amplification of platelet activation [24]. Another class of platelet receptors are purinergic type 2 receptors (P2Rs) [25]. The P2Rs are classified into two subclasses, P2X and P2Y receptors. While P2X receptors act as ion channels and are activated by extracellular ATP, P2Y receptors are mostly reactive to ADP [26]. To date, three different P2Rs are well described in human platelets:

P₂X₁, P₂Y₁, and P₂Y₁₂. All of them are directly or indirectly involved in Ca²⁺ signaling [27–29]. It was shown that P₂X₁R activation amplifies platelet aggregation mediated by thrombin and ADP-activated P₂Y₁R [30, 31]. Furthermore, P₂X₁ and P₂Y₁₂ appear to promote platelet-mediated bactericidal effects in staphylococcal infections [32], thus contributing to immune responses against pathogens.

It is already known that GAS are potent platelet activators via the M1-protein and fibrinogen-IgG axis [16, 33]. However, investigations on other specific and particularly serotype-independent mechanisms of GAS-platelet interaction are scarce. Here, we show that SLS activates human platelets by mediating calcium ion influx.

Methods

Platelet Preparation

Washed human platelets were prepared as described previously [34]. In brief, platelet-rich plasma (PRP) was prepared from ACD-A anticoagulated whole blood from a defined set of healthy volunteers, who were not taking any antiplatelet or non-steroidal anti-inflammatory drugs. The PRP was washed two times in Tyrode's buffer containing 2.5 U/mL apyrase, 1 U/mL hirudin, 0.35% BSA, and 0.1% glucose (pH 6.3). Lastly, the platelet pellet was suspended in a bicarbonate-based suspension buffer supplemented with 2.12 mM MgCl₂, 1.96 mM CaCl₂, 0.35% BSA, and 0.1% glucose (pH 7.2). The platelet count was adjusted to 300,000 platelets/ μ L.

Bacterial Strains

The following GAS strains were used: 5448 [35], 5448 Δ *slo* [36], and 5448 Δ *sagA* [37]. All strains were cultivated in Todd-Hewitt broth (Carl Roth) supplemented with 1.5% (w/v) yeast extract (Carl Roth) at 37°C.

Platelet Infections, Erythrocytes, and PBMC Stimulations

All experiments were carried out in Tyrode's buffer containing Ca²⁺ and Mg²⁺. The platelets (7.5×10^6) were infected with 5448, 5448 Δ *slo*, and 5448 Δ *sagA* at a multiplicity of infections (MOIs) of 0.01/0.1/1.

To assess SLS and SLO expression in Tyrode's buffer, the same amount of bacteria was incubated in Tyrode's buffer for 30 min, supernatants were collected and erythrocytes or PBMCs (1.0×10^5) were incubated with the supernatants at 1:4 dilution for 30 min or 24 h, respectively. PBMCs were isolated from buffy coats by

Lymphoprep (Axis-Shields) gradient centrifugation as previously described [38, 39].

SLO and SLS hemolytic activity was measured by use of the conventional erythrocyte lysis activity assay as previously published [40] and the activities were related to a Triton-X100 lysis control. Tyrode's buffer was used as a negative control.

Cytolytic activities were assessed via flow cytometry [39]. Briefly, dead cells were labeled using the Zombie Aqua Fixable Viability Kit (BioLegend). Unspecific binding of immunoglobulins was blocked by using Human TruStain FcX (BioLegend) according to the manufacturer's instructions. Incubations of cells with titrated amounts of CD45 (BV605, HI30) monoclonal antibody were carried out for 30 min at 4°C in the dark. Cells were washed between each staining step and fixed using the Cyto-Fast Fix/Perm Buffer Set (BioLegend).

Furthermore, bacterial supernatants were separated by 12% SDS-PAGE and transferred to a PVDF membrane. As molecular mass markers, pre-stained protein standards (Bio-Rad) were used. The membranes were blocked with 5% skim milk and incubated with primary antibodies (1 mg/mL IVIG), washed and then incubated with secondary antibodies (anti-human IgG horseradish peroxidase linked Fab fragment; GE Healthcare). Positive binding was detected by Super Signal West Femto maximum sensitivity substrate (Thermo-Scientific) [41].

Platelet Activation Assay

The washed human platelets were infected as described above. In a subset of experiments, platelets were subsequently stimulated with 20 μ M thrombin receptor activator peptide (TRAP)-6 5 min prior to the end of infections. The platelets were stained with FITC-labeled mouse anti-human CD42a and PE-Cy5-labeled mouse anti-human CD62P (both BD Biosciences) for 10 min at RT. Platelets were fixed with 2% paraformaldehyde for 30 min. The activation of platelets was assessed by measuring CD62P expression of CD42a-positive cells using a FACSCalibur or FACSARIA III flow cytometer (Becton Dickinson) and CellQuestPro 6.0 or FACS Diva software, respectively. Data were analyzed using FCS Express 7 software (De Novo Software). The gating strategy for selected infectious conditions is shown in online supplementary Figure 1 (for all online suppl. material, see <https://doi.org/10.1159/000544951>).

All experiments performed with IVIG (98% IgG; Privigen, CSL Behring) included prior platelet incubation

with monoclonal antibody IV.3 (1:15, 45 min, 37°C) to avoid Fc γ RIIa-dependent activation. The bacteria were subjected to 1 mg $\times$ mL⁻¹ IVIG treatment for 30 min before infection.

Initially, platelet viability was assessed using the RealTime-Glo Cell Viability Assay kit (Promega) for over 120 min. Reagents were prepared according to the manufacturer's instructions. Human washed platelets were infected with GAS, as described above. The luminescence was measured 1 min after the plate was mixed and shaken (300 rpm for 3 s); measurements were taken every 180 s thereafter. PBS and TritonX-100 (Sigma-Aldrich) were used as the negative and positive controls, respectively. All experiments were conducted in duplicates.

Inhibition of Platelet Activation

All infections in inhibition studies were performed in the presence of IVIG. Prior to infections, platelets were pre-incubated with different agents for 30 min. The following chemicals were used: ethylenediaminetetraacetic acid (EDTA) or ethylene glycol bis(β -aminoethylether) tetraacetic acid (EGTA) to chelate extracellular Ca²⁺; general P2R antagonist suramin (Sigma-Aldrich); specific P₂X₁R antagonist NF449 (Cayman Chemicals); TPRC6 receptor antagonist BI749327 (Focus Biomolecules).

Calcium Mobilization Assay

Ca²⁺ mobilization was measured using Fluo-4 AM as previously described with minor modifications [42]. In brief, platelets were washed and suspended in Ca²⁺ and Mg²⁺ free PBS (pH 7.4) containing 5 μ M Fluo-4 AM (Molecular Probes). The suspensions were incubated for 10 min at 37°C. Next, CaCl₂ was added to a final concentration of 2 mM and platelets were transferred to a 96-well F-bottom plate to measure baseline fluorescence at 520 nm for 1 min using a FLUOstar Omega fluorometer (BMC LabTech). Subsequently, bacteria (MOI 1) and the respective controls (PBS and TRAP-6) were added to platelets. The fluorescence was recorded every 30 s for 15 min. The amount of free intracellular Ca²⁺ induced by GAS infections was normalized to PBS control at the respective measurement.

Statistics

If not otherwise indicated, statistically significant differences were determined using the Kruskal-Wallis test with Dunn's posttest. Statistics were performed using GraphPad Prism version 7 (GraphPad software). A *p* value <0.05 was considered significant.

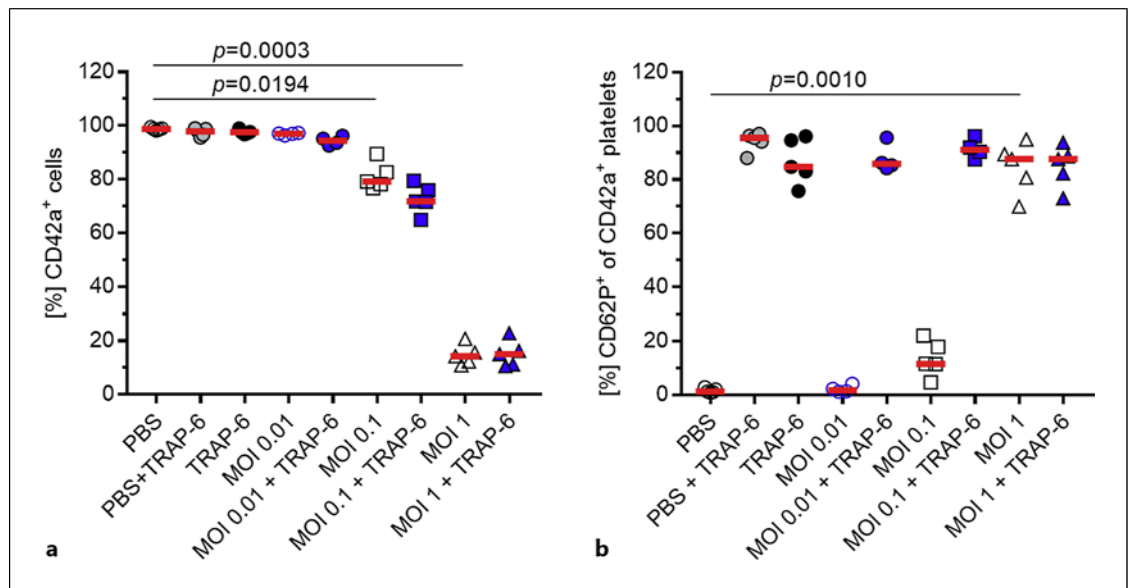


Fig. 1. Infection-dose-dependent killing of platelets by *S. pyogenes*. Washed human platelets were infected with GAS 5448 wild-type strain at MOI 0.01, 0.1, or 1.0 and platelet viability (a) and activation (b) were assessed. Platelet viability (a) and activation (b) were measured via flow cytometry using FITC-labeled CD42a and PE-Cy5-labeled CD62P antibodies, respectively. TRAP-6 (20 μ M) and PBS were used as

positive and negative controls, respectively. Alternatively, 5 min prior to the end of infection, TRAP-6 (20 μ M) was added. Each dot in a, b represents one independent experiment with cells from one donor ($n = 5$). Horizontal lines depict median values. The level of significance was determined using the Kruskal-Wallis test with Dunn's posttest. RLU, relative luminescence units.

Results

GAS Activate Platelets via SLS

To assess the viability and activation of human platelets in response to GAS infections, cell suspensions were infected with the wild-type strain 5448 at different MOIs. Initial viability analyses included a luminescence-based approach. However, the bacteria incorporated the substrate (online suppl. Fig. 2), and therefore, the viability was assessed based on the increasing/decreasing frequencies of CD42a⁺ cells within the total GAS-platelet population instead (Fig. 1a). These analyses revealed that GAS kill and also activate dose-dependent human platelets (Fig. 1a, b). While MOI 0.01 had no effect on both processes, high-dose infections (MOI 1) almost completely killed the platelets (Fig. 1a). The remaining negligible viable population was fully activated in high-dose infections (Fig. 1b). To determine whether additional activation could be achieved, TRAP-6 was added to infections 5 min prior to the termination of the experiment. The frequencies of CD62P⁺ cells increased when low MOIs (0.01 and 0.1) were used. The platelets remained unresponsive to additional TRAP-6 stimulation at MOI 1 (Fig. 1b).

To potentially improve platelet viability, isogenic mutants lacking single streptolysins SLO (5448 Δ slo) or SLS (5448 Δ sagA) were used in the next set of experiments. First approach included MOI 0.1 infections for up to 120 min. The analyses revealed that (i) the majority of platelets remained viable (online suppl. Fig. 3a), (ii) only wild-type infections activated up to 40% of the platelet population (online suppl. Fig. 3b), and (iii) no bactericidal effects were observed (online suppl. Fig. 3c). In the second approach, the infectious dose was increased to MOI 1 and infection time was reduced to 30 min. Again, the viability dropped in all infectious conditions (Fig. 2a). In contrast, while a high proportion of the remaining platelets were activated in 5448 and 5448 Δ slo infections, the 5448 Δ sagA mutant failed to activate the cells (Fig. 2b). In congruence with the results above, all bacterial strains remained viable (Fig. 2c).

To confirm that both streptolysins are released by the bacteria under chosen conditions (MOI 1), the bacteria was incubated in Tyrode's buffer for 30 min and the supernatants were either directly subjected to Western blot analyses or hemolytic and cytolytic assays (online suppl. Fig. 4). While protein analysis showed that the bacteria release certain factors, they remained

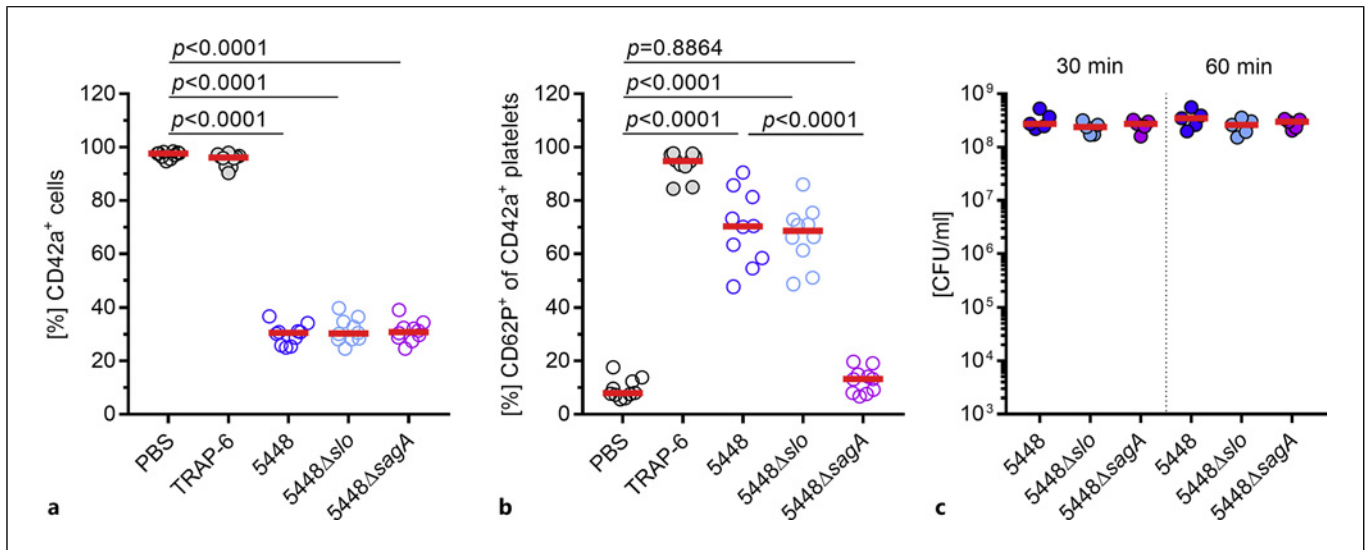


Fig. 2. SLS activates platelets. Washed human platelets were infected with GAS strains at MOI 1 for 30 min and platelet viability (**a**) and activation (**b**) were assessed via flow cytometry using FITC-labeled CD42a and PE-Cy5-labeled CD62P antibodies, respectively. TRAP-6 (20 μ M) and PBS were used as positive and negative controls, respectively. Each dot in **a** and **b** represents one independent experiment with

cells from one donor ($n = 10$). **c** CFU of the indicated GAS strains after 30 and 60 min of platelet infection at MOI 1. Each dot represents one independent experiment with washed platelets from one donor ($n = 5$). Horizontal lines depict median values. The level of significance was determined using the Kruskal-Wallis test with Dunn's posttest. CFU, colony-forming unit.

inconclusive in terms of SLO (online suppl. Fig. 4a), potentially due to the release of a limited amount, hemolysis as well as the cytotoxicity assays indirectly confirmed that both factors, SLS and SLO, are released (online suppl. Fig. 4b, c). Based on these observations, all subsequent infection experiments were performed with MOI 1 for 30 min.

IVIG Neutralizes the Cytotoxic Effect of GAS but Does Not Prevent SLS-Mediated Activation

Since the use of single streptolysin mutants did not improve platelet viability, IVIG, a commonly used adjunctive therapeutic agent in streptococcal NSTIs and STSS [43], which neutralizes a plethora of proteinous streptococcal virulence factors including toxins, was used in all subsequent experiments. Initially, two approaches were tested: (i) platelets were infected with GAS and IVIG was added 5 min post infections (Fig. 3a) or (ii) GAS were incubated with IVIG prior to platelet infections (Fig. 4a). Both approaches improved platelet viability, characterized by increased frequencies of CD42⁺ cells (Fig. 3, 4b). However, the second approach was slightly more effective (Fig. 4). In contrast, IVIG treatment did not neutralize 5448 wild-type as well as 5448Δslo-mediated

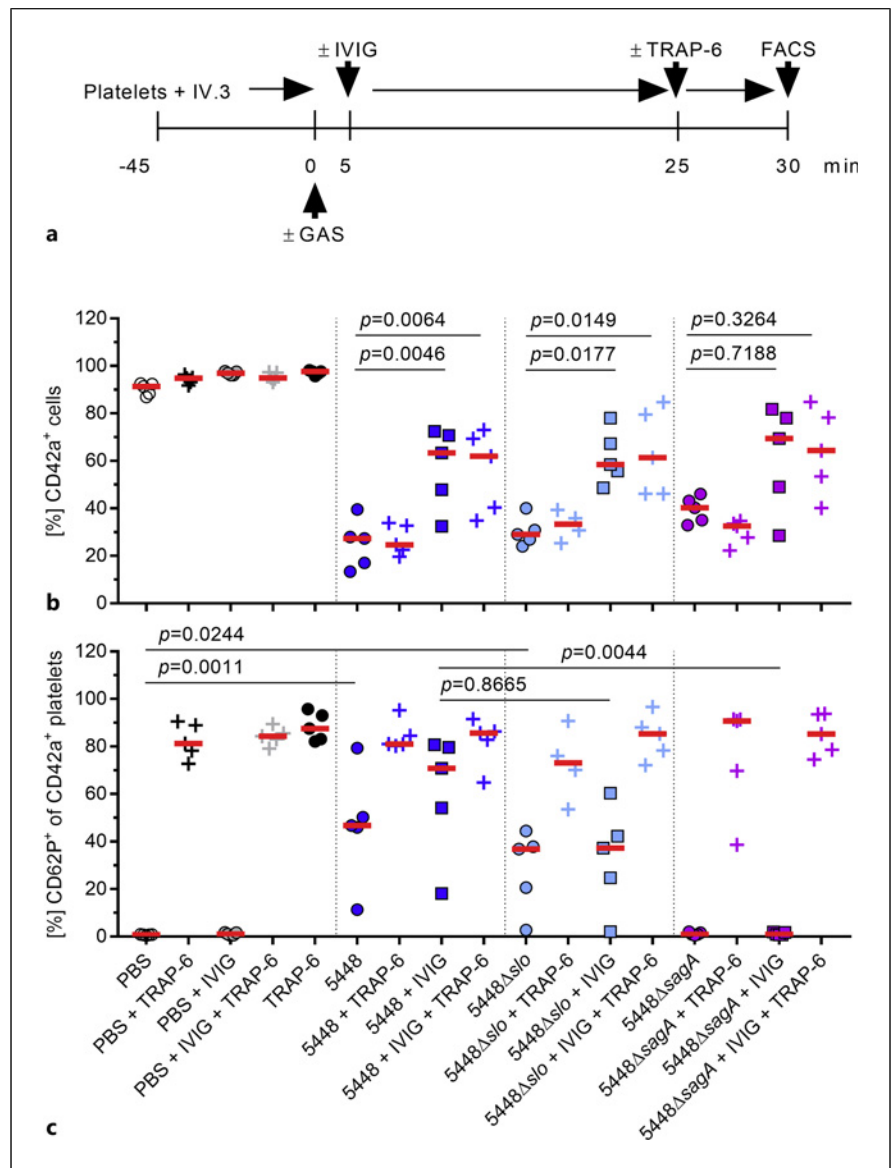
platelet activation (Fig. 3, 4c). High frequencies of CD62P⁺ cells were observed. In congruence with the results above, 5448ΔsagA mutant strain completely failed to activate human platelets (Fig. 3, 4c). Nonetheless, the addition of TRAP-6 to 5448ΔsagA infections activated platelets confirming their viability and responsiveness. Based on these results, all subsequent experiments were performed with 5448 wild-type and 5448ΔsagA mutant strains in the presence of IVIG.

SLS Induces Continuous Ca²⁺ Influx in Human Platelets

As Ca²⁺ signaling plays a key role in platelet activation [44], the effect of SLS on Ca²⁺ mobilization in platelet infections was assessed. Intracellular Ca²⁺ (iCa²⁺) dynamics were monitored using a Fluo-4 AM assay. Infections with 5448 resulted in a significant continuous increase of iCa²⁺ as compared to the PBS control (Fig. 5a). In contrast, while TRAP-6 induced high levels of iCa²⁺ within a short time, only minor iCa²⁺ accumulation was noted in 5448ΔsagA infections (Fig. 5a).

Next, Ca²⁺ influx was inhibited by chelating the extracellular source. Two reagents were used, EDTA and EGTA (Fig. 5b–d). High concentrations of EDTA

Fig. 3. Platelet viability is improved by the addition of IVIG 5 min post infection. **a** Schematic overview of the experimental setup. Platelet viability (**b**) and activation (**c**) were measured via flow cytometry using FITC-labeled CD42a and PE-Cy5-labeled CD62P antibodies, respectively. TRAP-6 (20 μ M) and PBS were used as positive and negative controls, respectively. Alternatively, 5 min prior to the end of infection, TRAP-6 (20 μ M) was added. Each dot in **b, c** represents one independent experiment with cells from one donor ($n = 5$). Horizontal lines depict median values. The level of significance was determined using the Kruskal-Wallis test with Dunn's posttest.

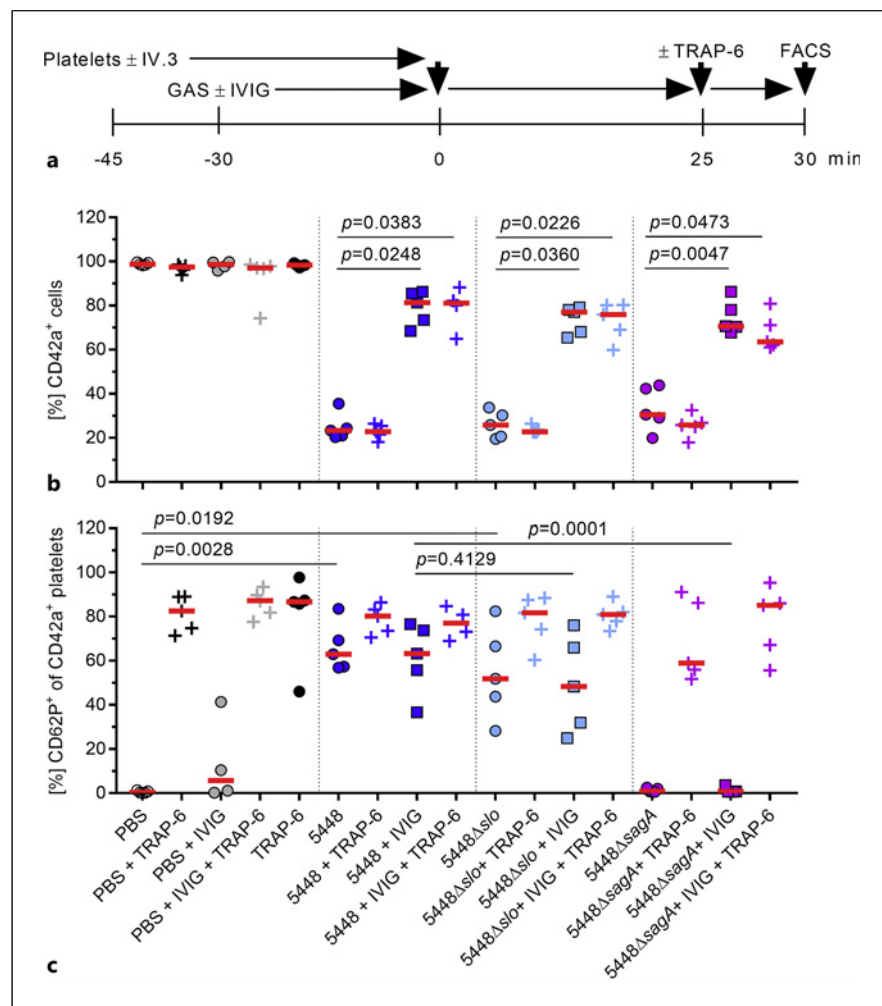


(10 mM) completely abolished wild-type mediated platelet activation (Fig. 5c). Next, EGTA, which has a higher affinity for Ca^{2+} , was used. A dose-dependent reduction of platelet activation was observed in 5448 infections (Fig. 5d). In both scenarios, (i) no activation in 5448Δsaga infections was observed, (ii) TRAP-6 responsiveness by platelets remained unaffected by the chelating agents, and (iii) both agents had a minor effect on additional TRAP-6 responsiveness of infected platelets (Fig. 5c, d). These analyses revealed that SLS targets platelets via direct activation of Ca^{2+} influx, which is a thrombin receptor-independent pathway.

SLS Activates Platelets via P2 Receptors

$\text{P}_2\text{X}_1\text{R}$ and TRPC6 are receptor-operated calcium entry channels in platelets [44, 45] and potential targets of SLS. To explore whether these receptors are targeted by SLS, chemical antagonists were used in a series of experiments (Fig. 6, 7). The specific antagonist for TRPC6, BI749327, did not reduce platelet activation in 5448 infections (Fig. 6). In contrast, a general P2R antagonist, suramin, as well as NF449, a suramin analog that is highly selective for $\text{P}_2\text{X}_1\text{R}$ [46, 47], dose-dependently reduced platelet activation by the wild-type strain 5448 (Fig. 7). In control experiments, (i) the highest dosages of the inhibitors had no activating effects, (ii) the platelets

Fig. 4. Platelet viability is further improved by the addition of IVIG prior to infections. **a** Schematic overview of the experimental setup. Platelet viability (**b**) and activation (**c**) were measured via flow cytometry using FITC-labeled CD42a and PE-Cy5-labeled CD62P antibodies, respectively. TRAP-6 (20 μ M) and PBS were used as positive and negative controls, respectively. Alternatively, 5 min prior to the end of infection, TRAP-6 (20 μ M) was added. Each dot in **b, c** represents one independent experiment with cells from one donor ($n = 5$). Horizontal lines depict median values. The level of significance was determined using the Kruskal-Wallis test with Dunn's posttest.



remained responsive to additional TRAP-6 stimulations, and (iii) 5448 Δ sagA did not activate human platelets (Fig. 6, 7).

Discussion

Severe GAS infections are often associated with coagulopathy and low platelet counts [17–19]. While GAS are known to activate platelets in a serotype-dependent fashion, the general activating mechanisms remain elusive. Here, we show that GAS induce platelet activation through SLS-mediated P2R activation and subsequent calcium influx.

Our results are in congruence with other studies that confirm the ability of GAS to quickly activate human platelets [14, 16, 33, 48]. To study rather general and M-protein-independent mechanisms, we used washed platelets instead of PRP [16]. Indeed, this approach revealed

that GAS activate human platelets without utilizing the previously described M-protein-fibrinogen-IgG axis [16]. However, since both streptolysins were active in wild-type infections, a tremendous lytic effect was observed. Single knock-outs of each streptolysin did not improve platelet viability. However, the SLS mutant strain completely failed to activate the remaining viable platelet population. To neutralize SLO and other streptococcal virulence factors, we used IVIG in all infectious conditions. IVIG is commonly used as an adjunct therapy in severe GAS infections [43, 49, 50] to neutralize bacterial toxins, modulate the immune response, and improve pathogen clearance [51]. In experimental GAS infections, IVIG has been shown to completely neutralize SLO and other streptococcal virulence factors except for SLS [52]. Consequently, the use of IVIG neutralized SLO-mediated killing of platelets but did not prevent SLS-triggered activation in our experimental setup. However, IVIG did not completely neutralize the

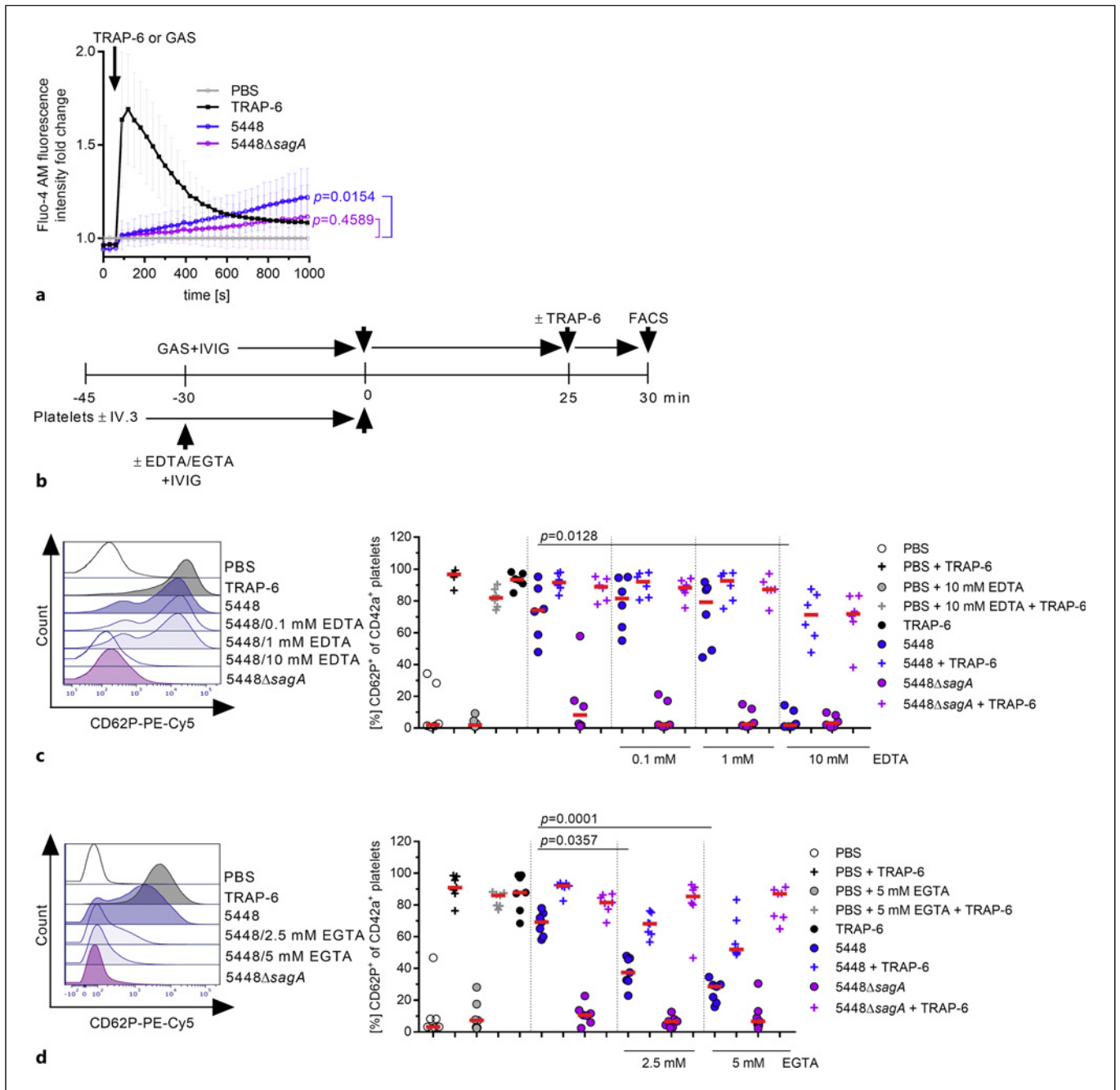


Fig. 5. SLS induces Ca^{2+} influx to activate platelets. **a** Kinetics of Ca^{2+} mobilization in platelets in response to GAS infections. After 60 s baseline measurement, controls and IVIG-treated bacteria (MOI 1) were added to the platelets ($\downarrow$). Curves represent the median values of 5 independent experiments with five donors ($n = 5$). The level of significance was determined by comparing endpoint measurements using the Kruskal-Wallis test with Dunn's posttest. **b** Schematic overview of the experimental setup. EDTA- (**c**) or EGTA-treated (**d**) washed platelets were infected with bacteria. Platelet activation was measured via

flow cytometry using PE-Cy5-labeled CD62P antibody. TRAP-6 (20 μM) and PBS were used as positive and negative controls, respectively. Alternatively, 5 min prior to the end of infection, TRAP-6 (20 μM) was added. Left panels and right panels in **c** and **d** show representative histograms and a summary of the data, respectively. Each dot in **c** and **d** represents one independent experiment with cells from one donor (**b**, $n = 6$; **c**, $n = 7$). Horizontal lines depict median values. The level of significance was determined using the Kruskal-Wallis test with Dunn's posttest.

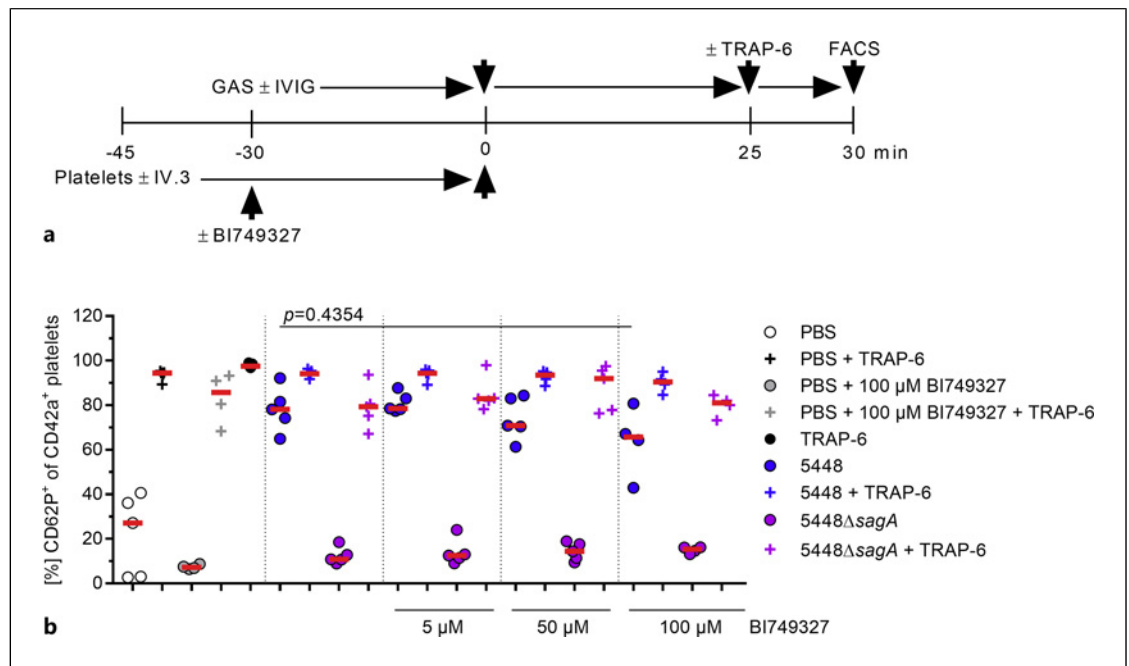


Fig. 6. Inhibition of TRPC6 does not affect platelet activation in GAS infections. **a** Schematic overview of the experimental setup. **b** Platelet activation was measured via flow cytometry using PE-Cy5-labeled CD62P antibody. TRAP-6 (20 μM) and PBS were used as positive and negative controls, respectively. Alternatively,

5 min prior to the end of infection, TRAP-6 (20 μM) was added. Each dot in **b** represents one independent experiment with cells from one donor ($n \geq 4$). Horizontal lines depict median values. The level of significance was determined using the Kruskal-Wallis test with Dunn's posttest.

killing of platelets in 5448ΔsagA infections, suggesting that other streptococcal potentially non-immunogenic factors or even host factors might still induce a partial killing [53, 54]. In general, IVIG administration, combined with antibiotic treatment, has been shown to increase platelet counts in patients, thus positively impacting the outcomes [55, 56]. Furthermore, studies using murine models have also indicated potential benefits, particularly in NSTIs and STSS [52].

Platelet activation is achieved through calcium mobilization. Upon activation, calcium is released from intracellular stores and/or enters the cell via receptor-operated calcium entry and SOCE [44]. Furthermore, direct stimulation of ligand-gated ion channels and consequent Ca^{2+} influx result in platelet activation, which can be further amplified [57]. SLS of *S. anginosus* was recently shown to induce Ca^{2+} influx in HSC-2 cells [58]. Given the partially conserved nature of SLS on the streptococcal genome, we hypothesized that SLS might have similar activating effects on platelets. We demonstrate that SLS deficiency resulted in reduced Ca^{2+} influx during platelet infection. Inhibition of Ca^{2+} influx with chelators significantly decreased SLS-dependent effects in wild-

type infections, consistent with the findings in *S. anginosus* infections of HSC-2 cells [58]. It is reported that staphylococcal α -hemolysin (Hla) also induces Ca^{2+} influx and thereby activates platelets. In this case, the influx of Ca^{2+} is linked to the formation of small pores in the platelet membrane, which subsequently leads to cell death [59]. In contrast, we show that platelet activation mediated by SLS is not linked to lysis. Currently, only a limited number of SLS receptors on human cells are known. These include the anion transporters Band 3 on erythrocytes, NBCn1 on keratinocytes, and nociceptors on neurons [9, 10, 60]. In addition to these interactions, we have identified a family of SLS-interacting partners belonging to the P2R family. The general P2R antagonist, suramin, as well as the $\text{P}_2\text{X}_1\text{R}$ -specific antagonist NF449 significantly reduced SLS-mediated platelet activation in GAS infections. However, NF449 is a high-affinity antagonist for $\text{P}_2\text{X}_1\text{R}$ that generally acts within the picomolar range [46]. In this study, micromolar concentration of NF449, which also antagonizes $\text{P}_2\text{Y}_1\text{R}$ as well as $\text{P}_2\text{Y}_{12}\text{R}$ [61], reduced platelet activation in wild-type infections. Thus, SLS-mediated platelet activation cannot be specifically linked to

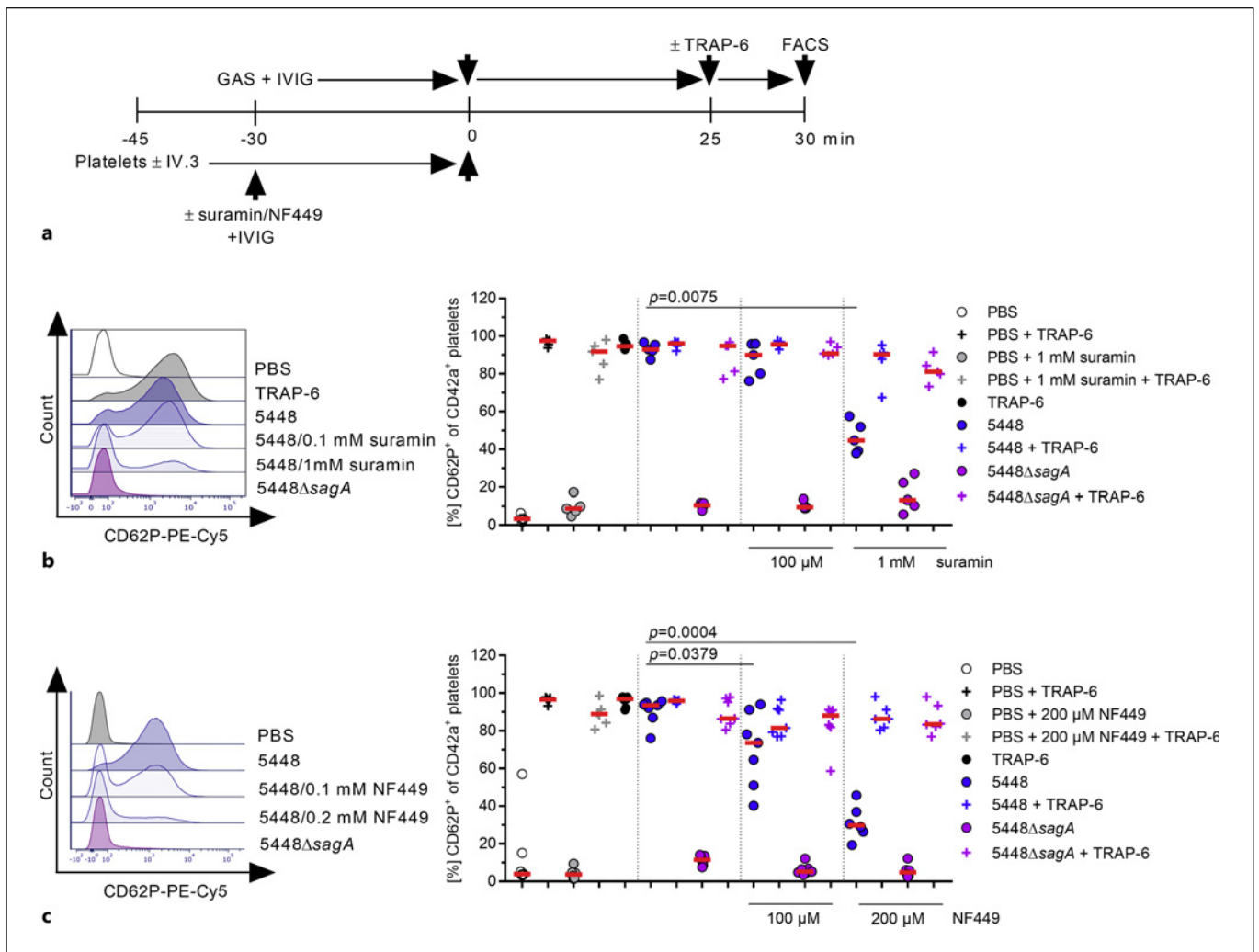


Fig. 7. Inhibition of P2R leads to reduced SLS-mediated platelet activation. **a** Schematic overview of the experimental setup. Suramin-**(b)** or NF449-treated **(c)** platelets were infected with bacteria. Platelet activation was measured via flow cytometry using PE-Cy5-labeled CD62P antibody. TRAP-6 (20 μ M) and PBS were used as positive and negative controls, respectively. Alternatively, 5 min prior to the end of

infection, TRAP-6 (20 μ M) was added. Left panels and right panels in **b**, **c** show representative histograms and summary of the data, respectively. Each dot in **b**, **c** represents one independent experiment with cells from one donor (**b**, $n = 6$; **c**, $n = 7$). Horizontal lines depict median values. The level of significance was determined using the Kruskal-Wallis test with Dunn's posttest.

one particular receptor but rather to the P2R family. The non-immunogenic nature, size, as well as natural posttranslational processing of the peptide, limited the ability to conduct further studies on SLS-P2R interactions.

In conclusion, this study highlights that GAS-mediated platelet activation is a multifactorial process. By examining platelet activation beyond the well-studied M-protein-fibrinogen-IgG interaction, we show that SLS triggers calcium influx through P2 family receptors. However, the contribution of the different P2Rs to SLS-mediated platelet activation remains to be elucidated.

Acknowledgment

We would like to thank Constantin Klein for expert technical assistance.

Statement of Ethics

The use of whole blood and washed platelets from healthy adults was approved by the Ethics Committee of the University Medicine Greifswald (BB 044/18). Buffy coats obtained from healthy blood donors were anonymously provided by the blood bank at the University Medicine Greifswald. The use of PBMCs was approved by the Ethical Research Committee at the University Medicine Greifswald.

(Ref. No. BB 014/14). All volunteers gave written informed consent in accordance with the Declaration of Helsinki. All experiments were carried out in accordance with the approved guidelines.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This research is supported by the German Research Foundation (DFG; Grant 492903360 to N.S.). The funder had no role in the design, data collection, data analysis, and reporting of this study.

Author Contributions

Conceptualization: A.R., J.W., and N.S. Experimental work: A.R. and K.J. Data analysis: A.R., K.J., and N.S. Volunteer inclusion: J.W. and T.T. Project administration: N.S. Supervision: J.W., T.T., and N.S. Writing – original draft: A.R. and N.S. Writing – review and editing: all authors.

Data Availability Statement

All data associated with this study are presented in the manuscript and supplementary material. Further inquiries can be directed to the corresponding author.

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