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Increased GPIbα shedding from platelets treated with immune thrombocytopenia plasma

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ABSTRACT

Immune thrombocytopenia (ITP) is a heterogeneous autoimmune disease, characterized by accelerated platelet destruction/clearance or decreased platelet production. ADAM17-mediated platelet receptor GPIbα extracellular domain shedding has been shown to be involved in platelet clearance. Whether GPIbα shedding participates in the pathogenesis of ITP remains poorly understood. This study aims to investigate the role of GPIbα shedding in the development of ITP via incubating normal platelets with ITP plasma to mimic ITP in vivo environment. Plasma was isolated from ITP patients or healthy control and incubated with platelets in vitro followed by measuring GPIbα expression by flow cytometry and western blot, ADAM17 expression by western blot, ROS generation and platelet activation by flow cytometry. Compared with control plasma, ITP plasma-treated platelet displayed significantly reduced GPIbα surface expression, increased ADAM17 expression and ROS generation. However, metalloproteinase inhibitor GM6001 blocked the ITP-plasma-induced decrease in GPIbα surface expression, increase in ADAM17 expression and platelet activation. In addition, inhibitors of NADPH oxidase or mitochondria respiration significantly inhibited ROS generation from ITP plasma-treated platelets. Moreover, ROS inhibition or blocking FcγRIIa attenuated the decrease in GPIbα surface expression, platelet activation and ROS generation (for blocking FcγRIIa) in ITP plasma-treated platelets. In conclusion, ITP plasma induces platelet receptor GPIbα extracellular domain shedding, suggesting that it might participate in the pathogenesis of ITP and targeting it might be a novel approach for treating ITP.

1. Introduction

Immune thrombocytopenia (ITP) is a heterogeneous autoimmune disorder, which is characterized by increased platelet destruction and reduced platelet production, resulting in lower platelet count (thrombocytopenia), putting patients on a higher risk of bleeding [1,2]. The pathophysiology of ITP is very complicated, involving several factors. The pathogenic mechanism of ITP is thought to be mainly caused by enhanced Fc receptor (FcR)-mediated phagocytosis and macrophages-mediated destruction of autoantibodies-opsonized platelets in the reticuloendothelial system in the spleen [3,4].

In response to vascular injury, platelets are recruited to subendothelial matrix through recognition of exposed VWF/collagen in the damaged blood vessel wall by membrane surface receptors, glycoprotein (GP)VI which binds collagen, and GPIbα, the major ligand-binding subunit of GPIb-IX-V complex, which binds von Willebrand factor (VWF), leading to platelet adhesion, activation, aggregation and subsequent thrombus formation [5,6]. As one of the major platelet adhesive receptor, GPIbα plays an important role in the regulation of platelet function through several binding partners, such as coagulation factors XI and XII, thrombin, thrombospondin, and high molecular-weight kininogen, the leukocyte integrin αMβ2, and P-selectin [7–9]. In

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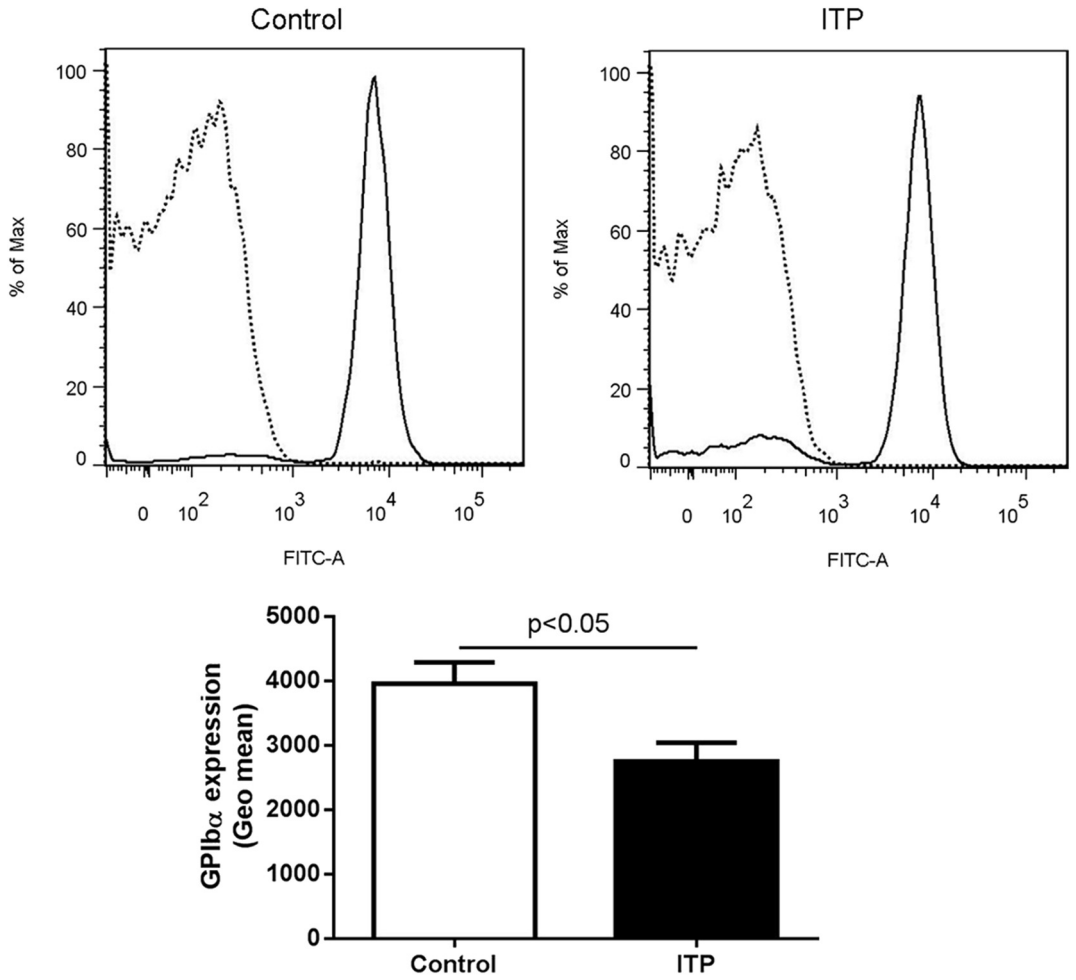

Fig. 1. GPIbα surface expression in plasma-treated platelets. Normal platelets were treated with control or ITP plasma followed by measuring GPIbα surface expression by flow cytometry. GPIbα surface expression was presented as Geo Mean of fluorescent intensity. Dash line indicates the isotype control. Comparison between control and ITP: $p < 0.05$. Data were presented as Mean ± SE (n = 15).

addition, through GPIbα, platelets also interact with activated endothelial cells (via P-selectin) or leukocytes (via αMβ2), thus participating in the regulation of inflammatory response, and coagulation factors to control localized clotting at sites of injury or disease [5]. Moreover, GPIbα has been demonstrated to regulate platelet survival in vivo as antibody or chilling-induced clustering/dimerization of GPIbα on the platelet surface causes rapid clearance of circulating platelets [10–12]. Furthermore, platelet desialylation is also reported to be capable to trigger platelet clearance [13,14].

Apart from regulating platelet activation and aggregation, engagement of GPIbα by ligands also triggers a rapid and irreversible activation of metalloproteinase ADAM17, which cleaves the ectodomain of GPIbα, resulting in the release of the 110–130 kDa soluble fragment of GPIbα (glycocalicin) [9,15]. In human body, soluble form GPIbα accounts for approximately up to two-thirds of the total GPIbα [5], suggesting GPIbα is constitutively shed from the surface of platelets in normal individuals. Even though the exact physiological role of glycocalicin remains unclear, GPIbα shedding attenuates the thrombotic propensity of platelets as GPIbα plays critical roles in thrombus formation. In addition, GPIbα shedding has also been demonstrated to be associated with platelet clearance as inhibition of metalloproteinase [16], genetic ablation of ADAM17 (responsible for GPIbα shedding) [17] or treatment with MAb 5G6 (binding specifically to GPIbα and block GPIbα shedding) [18] prevented shedding of GPIbα in CCCP-damaged platelets or during platelet storage and improved the hemostatic function as well as the survival of these platelets in vivo [19].

Considering the abnormal platelet clearance in the pathogenesis of ITP as well as the association of GPIbα shedding with platelet clearance, whether GPIbα shedding is involved in the pathogenesis or development of ITP remains to be elucidated although a few studies showed abnormal glycocaclin level in patients with ITP [20,21]. In this study, we incubated normal human platelets with ITP plasma to mimic the ITP in vivo environment to investigate the role of GPIbα shedding in ITP.

2. Materials and methods

2.1. Patients

From October 2017 to March 2018, 15 primary ITP patients (6 males and 9 females with a median age of 41, ranged from 22 to 65 years old) with a median platelet number of 21×10^9/l from Department of Hematology, the Affiliated Hospital of Xuzhou Medical University, Xuzhou China were recruited into this study. ITP was diagnosed according to the criteria from international working group [22]. Patients with diabetes, cardiovascular diseases, hypertension, and active or chronic infection were excluded. Meanwhile, 15 age and gender matched healthy individuals (7 males and 8 females with a median age of 40 ranged from 20 to 62 years old) were included as a control. This study was approved by the Medical Ethics Committee of the Affiliated Hospital of Xuzhou Medical University, Xuzhou China. Informed consent was obtained from all enrolled individuals.

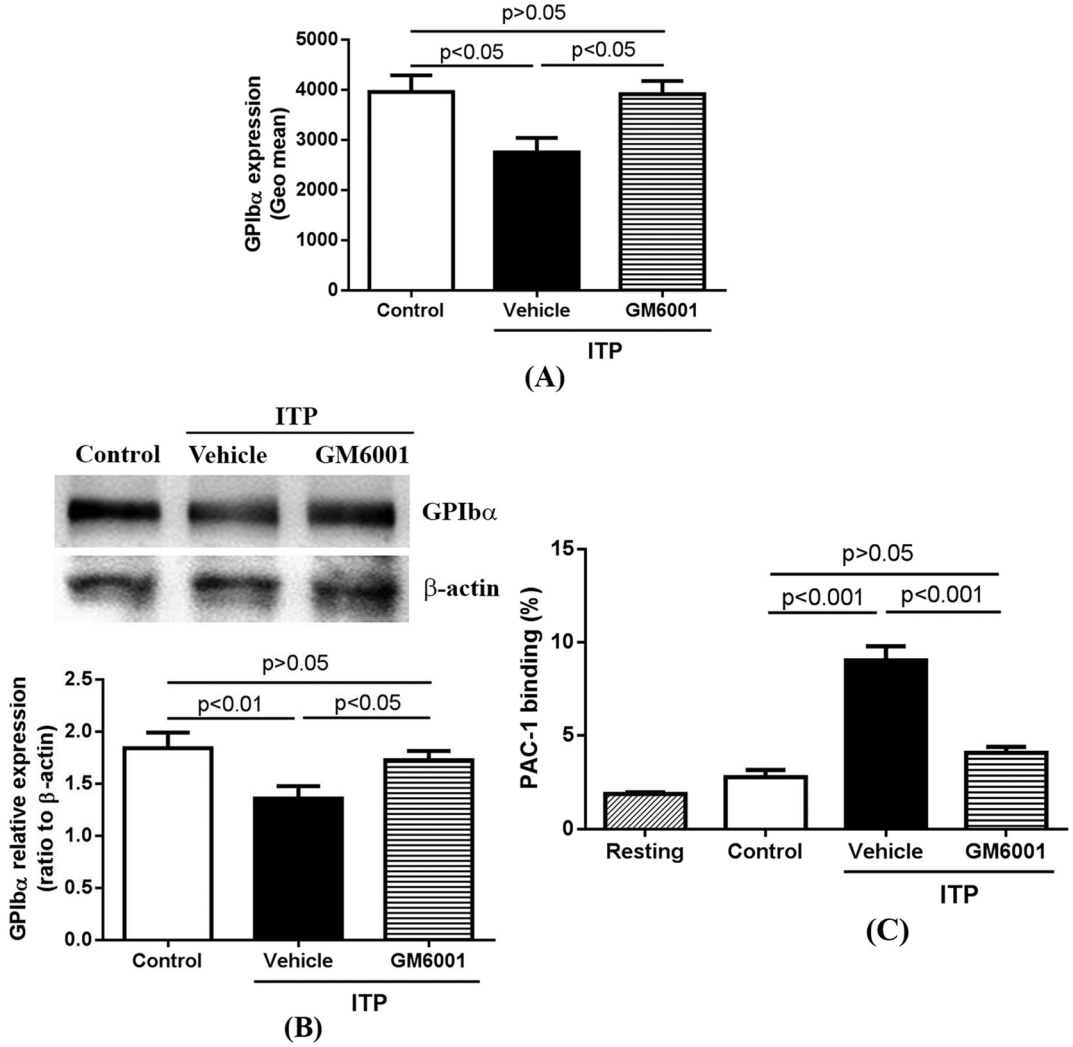

Fig. 2. GPIbα expression and platelet activation. Normal platelets were incubated with control or ITP plasma in the presence or absence of GM6001 and GPIbα expression was measured by flow cytometry (A) and western blot (B). Meanwhile, platelet activation represented as PAC-1 binding was measured by flow cytometry (C). Comparison between control and Vehicle (ITP): $p < 0.05$ for panel (A) and (B), and $p < 0.001$ for panel (C); control and GM6001 (ITP): $p > 0.05$; Vehicle (ITP) and GM6001 (ITP): $p < 0.05$ for panel (A) and (B), and $p < 0.001$ for panel (C). Data were presented as Mean ± SE (n = 15).

2.2. Plasma extraction

Peripheral blood from 15 ITP patients or 15 controls was collected into citrate-anticoagulated tubes and centrifuged at 2000 rpm for 20 min to collect plasma followed by storage at −80 °C until further analysis.

2.3. Platelets preparation

All individuals signed the informed consent before donating blood and the study was approved by the Medical Ethics Committee of the Affiliated Hospital of Xuzhou Medical College, Xuzhou, China. Platelets were prepared as previously described [23,24]. In brief, peripheral blood was collected from 15 healthy persons into a tube containing 2.5% (w/v) trisodium citrate, 2.0% (w/v) glucose and 1.5% (w/v) citric acid in distilled water (ACD) and centrifuged at 120 x g at room temperature for 20 min to collect platelet-rich plasma. Platelets were obtained through centrifuging the platelet-rich plasma at 1350 ×*g* for 15 min and resuspended at 5×10^8 platelets/ml in Tyrode's buffer (0.36 mM $NaH_2PO_4.H_2O$, 5.5 mM glucose, 138 mM NaCl, 12 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 0.49 mM $MgCl_2.6H_2O$, 2.6 mM KCl, pH 7.4). Platelets were allowed to rest at 37 °C for 1 h before use.

2.4. Treatment of platelets with plasma

Isolated platelets (1×10^8/ml) were treated with equal volume of plasma from either ITP or control in the presence or absence of broad spectrum metalloproteinase GM6001 (final concentration: 100 μM), NAC (final concentration: 5 mM) or IV.3 antibody (final concentration: 10 μg/ml) at room temperature for 1 h followed by relevant analysis as described previously [24].

2.5. Flow cytometry analysis

The surface expression of platelet receptor GPIbα and platelet activation were measured by flow cytometry as described previously [23,24]. After plasma treatment, platelet receptor GPIbα surface expression was measured using FITC-conjugated anti-CD42b antibody and platelet activation was measured using activation-dependent monoclonal antibody (FITC-conjugated PAC-1 antibody) (Becton Dickinson, San Jose, CA, USA) in accordance with the manufacturer's instructions.

2.6. Western blot

Whole proteins were extracted from plasma-treated platelets and separated on 10% SDS-PAGE. After transferred to an NC membrane, the

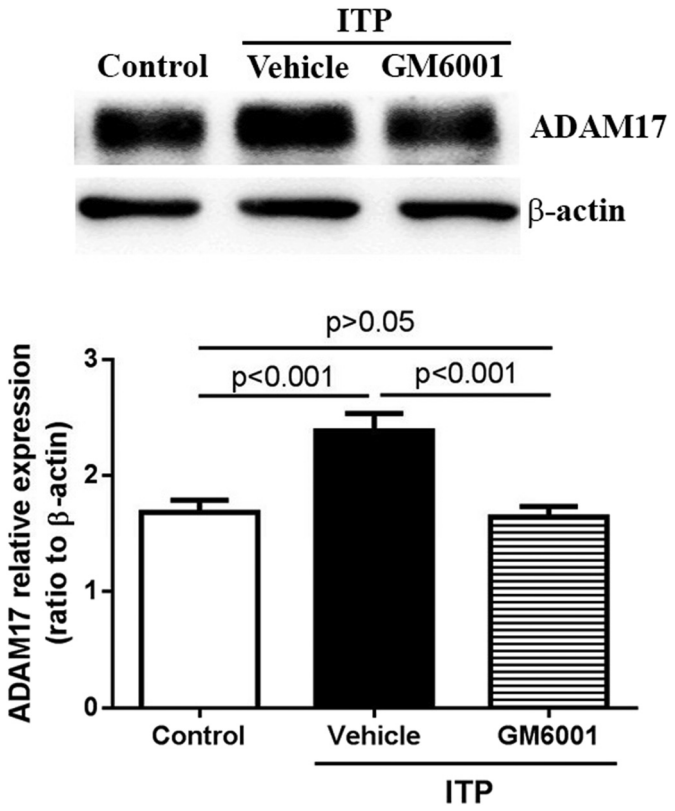

Fig. 3. ADAM17 expression in platelets after plasma treatment. After plasma treatment, ADAM17 expression in platelets was measured by western blot. ADAM17 expression was presented as a ratio relative to β-actin. Comparison between control and Vehicle (ITP): $p < 0.001$; control and GM6001 (ITP): $p > 0.05$; Vehicle (ITP) and GM6001 (ITP): $p < 0.001$. Data were presented as Mean ± SE (n = 15).

membranes were incubated with antibody against GPIbα (SZ2) (Santa Cruz Biotechnology, Dallas, Texas, USA), ADAM17 (Bioworld Technology, St. Louis Park, MN, USA) or β-actin, followed by incubation with HRP-conjugated secondary antibody. Protein band was visualized after addition of enhanced chemiluminescence. Protein expression was quantified using Image J software and presented as a ratio to β-actin.

2.7. Detection of intracellular ROS

Platelet intracellular ROS generation was detected by flow cytometry using 2′,7′-dichlorofluorescein (H2DCF-DA) as described previously [25]. Briefly, after plasma treatment, platelets were incubated with H2DCF-DA (final concentration: 10 μM) at 37 °C for 30 min followed by measuring the DCF-positive platelets by flow cytometry. For some experiments, platelets were incubated with Nox inhibitor apocynin (final concentration: 1 mM), inhibitors of mitochondrial respiration (rotenone, 50 μM), xanthine oxidase (allopurinol, 50 μM) or ROS scavenger *N*-acetyl-L-cysteine (NAC, 5 mM) before treatment with plasma.

2.8. Statistical analysis

Data were processed using GraphPad Prism software and displayed as mean ± standard error (SE). Unpaired student *t*-test was performed for comparison of the difference between two groups and one-way ANOVA was conducted for comparison among multiple groups. $p < 0.05$ indicated a statistical significance.

3. Results

3.1. Reduced GPIbα surface expression in ITP plasma-treated platelets

To test whether ITP plasma affects platelet receptor GPIbα expression, we incubated normal platelets with ITP plasma or control plasma and measured the surface expression of GPIbα by flow cytometry. As seen in Fig. 1, GPIbα surface expression was significantly reduced in platelets treated with ITP plasma (Geo Mean: 3248 ± 235.4) compared with that in platelets treated with control plasma (Geo Mean: 4165 ± 223.4) ($p < 0.05$), suggesting ITP plasma reduces platelet GPIbα surface expression.

3.2. Increased GPIbα shedding from ITP plasma-treated platelets

As metalloproteinase-mediated GPIbα ectodomain shedding is the main mechanism to downregulated GPIbα surface expression [5,15], we then investigated whether reduced platelet GPIbα surface expression results from metalloproteinase-modulated shedding using broad spectrum metalloproteinase inhibitor GM6001. As showed in Fig. 2, GM6001 treatment significantly inhibited ITP plasma-induced decrease in GPIbα surface expression compared with vehicle in the presence of ITP plasma ($p < 0.05$) and there was no difference compared with control ($p > 0.05$) as demonstrated by flow cytometry (Fig. 2A) and western blot (Fig. 2B). Furthermore, we also found GM6001 treatment significantly inhibited platelet activation which was induced by ITP plasma as demonstrated by significantly reduced PAC-1 binding compared with vehicle treatment (Fig. 2C).

3.3. Elevated ADAM17 expression in platelets after ITP plasma treatment

Several studies have demonstrated that metalloproteinase ADAM17 is the main sheddase to induce GPIbα ectodomain shedding [26,27]. We therefore measured ADAM17 expression in platelet treated with control or ITP plasma and found ADAM17 expression was significantly higher in platelets treated with ITP plasma (2.38 ± 0.09) than that in platelets treated with control plasma (1.68 ± 0.06) ($p < 0.05$) (Fig. 3). However, GM6001 treatment significantly attenuated ITP plasma-induced upregulation of ADAM17 expression (1.64 ± 0.05) and there was no difference compared with control plasma treatment ($p > 0.05$).

3.4. Increased ROS generation in ITP plasma-treated platelets

Previous studies demonstrated that ROS is involved in regulating ADAM17 activity through oxidation or modification of ADAM17 [28,29] plus the role of oxidative stress in the pathogenesis of ITP [30]. We next measured intracellular ROS production in ITP plasma-treated platelets by flow cytometry using the fluorescent dye, H2DCF-DA and showed that ITP plasma treatment significantly increased ROS production (21.0 ± 3.8) compared with control plasma (3.2 ± 0.6) ($p < 0.05$) (Fig. 4). The main cellular source of ROS is demonstrated to be derived from NADPH oxidase or mitochondria. To assess the origin of ROS generation, we treated platelets with inhibitors of NADPH oxidase (apocynin), mitochondrial respiration (rotenone) or xanthine oxidase (allopurinol) as well as ROS scavenger (NAC) in the presence of ITP plasma. As seen in Fig. 4, all these inhibitors could inhibit the ROS generation from ITP plasma-treated platelets with relatively more inhibition after NAC treatment, suggesting ITP plasma-induced ROS generation is derived from NADPH oxidase and mitochondria. In addition, we also evaluated the effect of GM6001 on ROS generation considering the inhibition of GM6001 on ADAM17 and found GM6001 did not affect ROS generation from ITP plasma-treated platelets (data not shown).

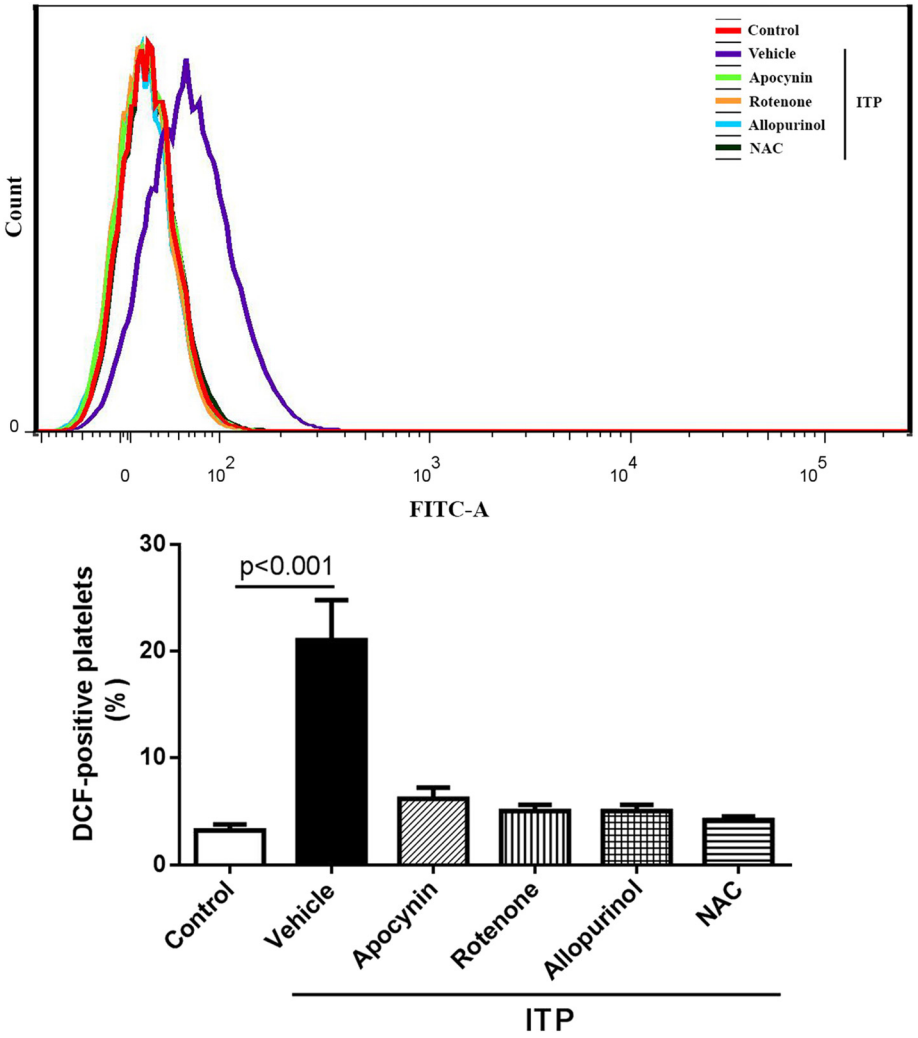

Fig. 4. ROS generation from plasma-treated platelets. After plasma treatment in the presence or absence of apocynin (final concentration: 1 mM), rotenone (50 μM), allopurinol (50 μM) or NAC (5 mM), platelet intracellular ROS was measured by flow cytometry. ROS generation was quantified as DCF-positive platelets (%). Comparison between control and Vehicle (ITP): $p < 0.001$. Data were presented as Mean ± SE (n = 15).

3.5. ROS inhibition reduces ITP plasma-induced GPIbα shedding and platelet activation

As ROS has been shown to regulate GPIbα extracellular domain shedding [31,32], we assessed whether ROS plays a role in ITP plasma-induced GPIbα shedding and found inhibition of ROS generation after NAC treatment significantly reduced the decrease in GPIbα surface expression in ITP plasma-treated platelets (3813.0 ± 308.7) compared with vehicle (2749.0 ± 289.4) ($p < 0.05$) (Fig. 5A). Since GPIbα shedding is observed during platelet activation [33], we also measured the status of platelet activation in ITP plasma-treated platelets by the activation-dependent binding of PAC-1 and showed increased PAC-1 binding in platelets after ITP plasma treatment (9.7 ± 1.2%) compared with control treatment (2.3 ± 0.3) (Fig. 5B). However, inhibition of ROS by NAC significantly reduced PAC-1 binding in ITP plasma-treated platelets (4.6 ± 0.5), suggesting that ROS might play a role in ITP plasma-induced platelet activation (Fig. 5B).

3.6. FcγRIIa inhibition reduces ITP plasma-induced GPIbα shedding, ROS generation and platelet activation

ITP plasma contains antiplatelet autoantibodies and we hypothesized that antiplatelet autoantibodies might play a role in GPIbα shedding induced by ITP plasma through engagement of Fc receptor FcγRIIa on the surface of platelets. To test this hypothesis, we incubated normal platelets with ITP plasma in the presence of FcγRIIa blocking antibody IV.3 and showed that IV.3 treatment significantly inhibited ITP plasma-induced GPIbα shedding (Fig. 6A), ROS generation (Fig. 6B) as well as platelet activation (Fig. 6C), indicating that crosslinking of FcγRIIa on the surface of normal platelets induced by antiplatelet antibodies in the ITP plasma might be involved in the initiation of GPIbα shedding.

4. Discussion

Immune thrombocytopenia (ITP) is an autoimmune disorder with lower platelet count [1,2]. Fc receptor (FcR)-mediated phagocytosis and clearance of autoantibodies-opsonized platelets have been demonstrated to play critical roles in the pathogenesis of ITP [3,4]. Platelet surface receptor GPIbα not only plays an important role in platelet adhesion, activation, aggregation as well as thrombus formation [34,35], but also is involved in the regulation of platelet survival or clearance [10–14]. Considering the abnormal platelet clearance in ITP, whether GPIbα is involved or plays a role in ITP remains unclear. In the present study, through incubating normal platelets with ITP plasma to mimic the ITP in vivo microenvironment, we showed significantly reduced surface expression of platelet receptor GPIbα in ITP plasma-treated platelets, indicating a role of GPIbα in the pathogenesis of ITP.

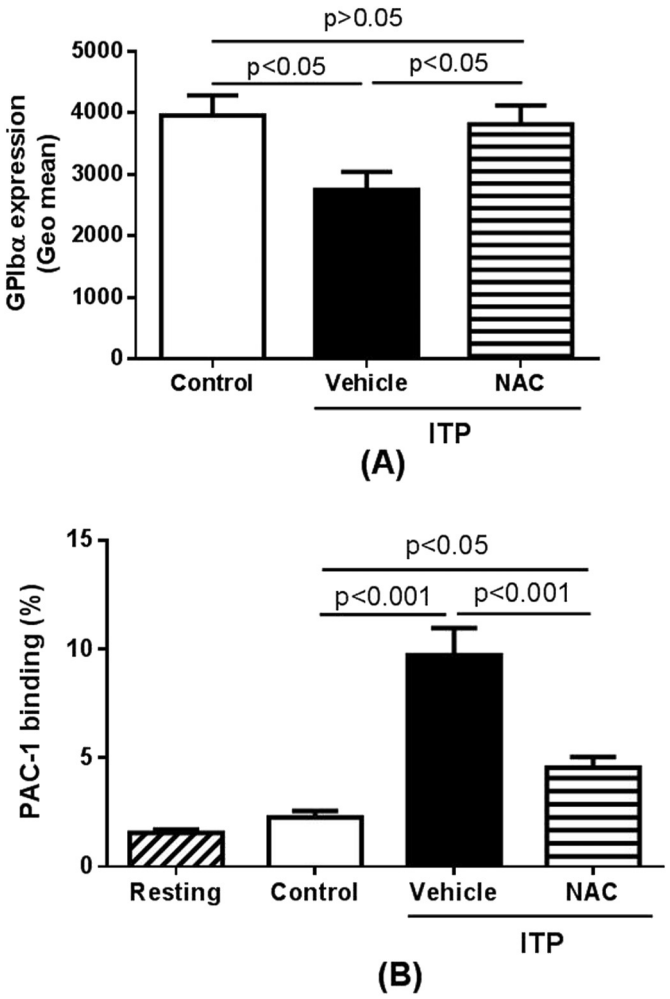

Fig. 5. GPIbα surface expression and platelet activation after ROS inhibition. Normal platelets were incubated with control or ITP plasma in the presence or absence of NAC followed by detection of GPIbα surface expression (A) and platelet activation (B) by flow cytometry. Comparison between control and Vehicle (ITP): $p < 0.05$; control and NAC (ITP): $p > 0.05$; Vehicle (ITP) and NAC (ITP): $p < 0.05$. Data were presented as Mean ± SE ($n = 15$).

Platelet receptor GPIbα is a major ligand-binding subunit of the GPIb-IX-V complex and regulates platelet adhesion through binding to VWF, leading to activation of intracellular signaling pathway, platelet activation and thrombus formation [5]. Abnormal expression or function of GPIbα caused by mutations has been demonstrated to be associated with several platelet disorders [36], such as Bernard-Soulier Syndrome or platelet-type von Willebrand disease. Beyond involvement of platelet function and thrombosis, ligand binding is also reported to trigger the activation of metalloproteinases (predominantly ADAM17), which cleaves the extracellular domain of GPIbα, releasing a soluble fragment into the plasma (glycocaclicin), a process called GPIbα shedding, which is the main downregulation mechanism of the expression and function of GPIbα [9,15]. One consequence of GPIbα shedding is the attenuation of platelet reactivity and another one is shown to be associated with platelet clearance in vivo as demonstrated by improved hemostatic function and survival of platelets after inhibition of metalloproteinases or ADAM17 [16–19]. In accordance with the role of GPIbα shedding in platelet clearance, in this study, we showed that significantly decreased GPIbα surface expression was found in ITP plasma-treated platelets compared with control plasma-treated platelets and metalloproteinase inhibitor reduced the decrease in GPIbα surface expression, suggesting that ITP plasma could induce platelet GPIbα extracellular domain shedding, implying GPIbα shedding might be involved in platelet clearance in ITP, consistent with a previous study showing increased glycocalcin level in patients with ITP [20,21]. Meanwhile, in this study, we also found that ADAM17 expression was significantly increased in platelets after ITP plasma treatment, which was restored into normal level after metalloproteinase inhibitor treatment, indicating that increased GPIbα shedding in ITP plasma-treated platelets might result from increased expression of ADAM17.

As natural by-products of aerobic metabolism, reactive oxygen species (ROS) consist of radical and non-radical oxygen species which are partially formed by reduced oxygen, such as superoxide anion ($O_2\cdot^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO\cdot$) [37]. Several studies have demonstrated that ROS participates in several physiological and pathological processes [38,39] via oxidation of cysteine (Cys) residues on signaling proteins [40]. In recent years, oxidative stress has been demonstrated to play a role in ITP as showed by significantly lower ratio of reduced to oxidized glutathione (GSH/GSSG) in patients with ITP compared with healthy individuals [41]. Moreover, the ratio of reduced to oxidized glutathione was even lower in chronic ITP patients than that in acute ITP. Consistent with the role of oxidative stress in ITP, in this study, we found increased intracellular ROS generation in ITP plasma-treated platelets compared with control plasma. To further identify the origin source of ROS, we used inhibitors of NADPH oxidase, mitochondria respiration and xanthine oxidase and showed that they all inhibited ITP plasma-induced ROS generation, indicating the involvement of NADPH oxidase, mitochondria respiration and xanthine oxidase in ROS production.

Recently, ROS has been reported to be capable to activate ADAM17 and induce extracellular shedding of its targets, GPIbα and GPV, in murine and human platelets [29]. Meanwhile, 12-HpETE, which is synthesized in the platelet lipoxygenase pathway, induced TACE-mediated receptor cleavage. In addition, ROS participates in agonists-induced GPIbα ectodomain shedding [32]. Moreover, mitochondrial ROS release from opened mitochondrial permeability transition pore (MPTP) is demonstrated to participate in regulating ADAM17-mediated GPIbα shedding in A23187-treated platelets [31]. Consistent with the role of ROS in GPIbα shedding, we showed that inhibition of ROS generation in ITP plasma-treated platelets significantly reduced the decrease in GPIbα surface expression, consistent with previous studies revealing blockage of GPIbα shedding after treatment of ROS inhibitor. Beyond regulating GPIbα shedding, ROS also involves in the modulation of platelet activation and thrombus formation [6,42–44] as shown by the beneficial use of antioxidants in preventing several thrombotic or cardiovascular diseases [45,46]. Consistent with this, we demonstrated that inhibition of ROS significantly blocked ITP plasma-induced platelet activation.

In conclusion, our results show increased GPIbα shedding, ADAM17 expression and ROS generation in ITP plasma-treated platelets, indicating that they might play a role in the development and pathogenesis of ITP and therapeutic targeting them might be a new strategy for treating ITP.

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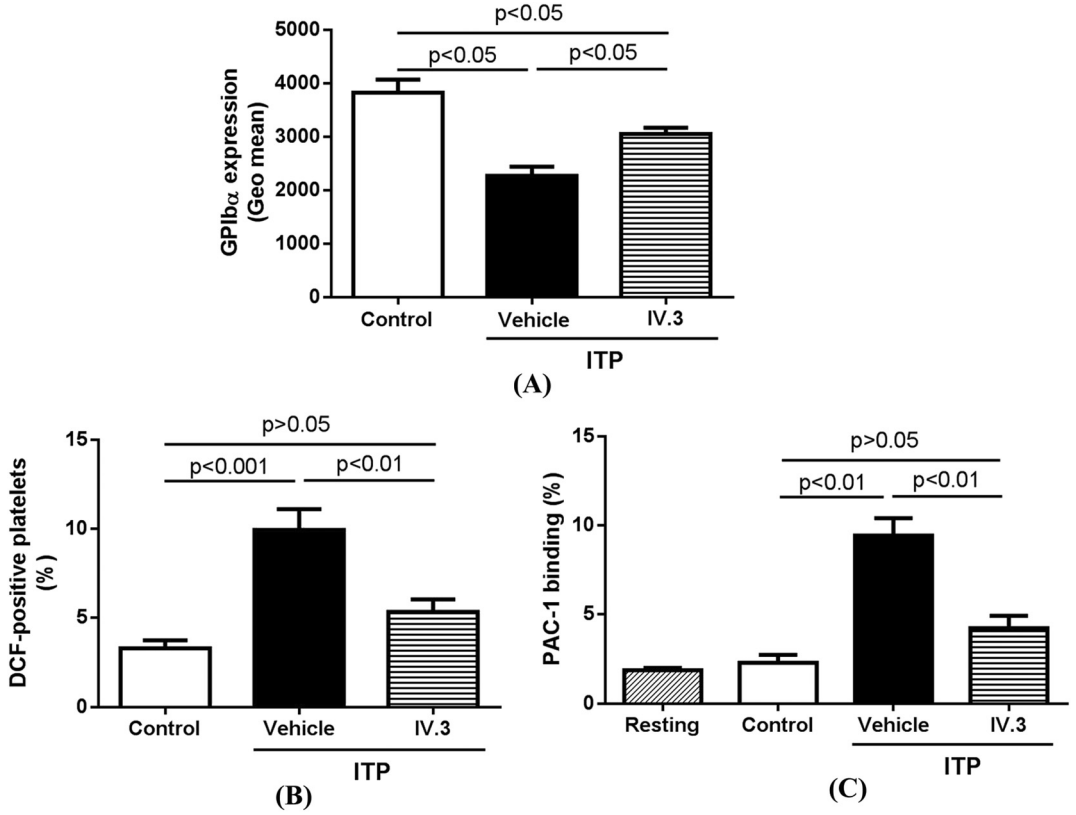

Fig. 6. Effect of FcγRIIa inhibition on GPIbα surface expression, ROS generation and platelet activation. Platelets were incubated with control or ITP plasma in the presence or absence of IV.3 and GPIbα surface expression (A), ROS generation (B) and platelet activation (C) were measured by flow cytometry. Comparison between control and Vehicle (ITP): $p < 0.05$ for panel (A), $p < 0.001$ for panel (B), $p < 0.01$ for panel (C); control and IV.3 (ITP): $p < 0.05$ for panel (A), $p > 0.05$ for panel (B and C); Vehicle (ITP) and IV.3 (ITP): $p < 0.05$ for panel (A), $p < 0.01$ for panel (B and C). Data were presented as Mean ± SE (n = 10).

Conflict of interest

All authors have no conflict of interest to declare.

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