VASCULAR BIOLOGY, ATHEROSCLEROSIS, AND ENDOTHELIUM BIOLOGY

Fc γ RIIa Ligation Induces Platelet Hypersensitivity to Thrombotic Stimuli

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Accepted for publication
September 13, 2012.

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Platelets are known for their important role in hemostasis, however their significance in other functions, including inflammation and infection, are becoming more apparent. Patients with systemic lupus erythematosus (SLE) are known to have circulating IgG complexes in their blood and are highly susceptible to thrombotic events. Because platelets express a single receptor for IgG, we tested the hypothesis that ligation of this receptor (Fc γ RIIa) induces platelet hypersensitivity to thrombotic stimuli. Platelets from SLE patients were considerably more sensitive to thrombin compared to healthy volunteers, and this correlated with elevated levels of surface IgG on SLE platelets. To test whether Fc γ RIIa ligation stimulated thrombin hypersensitivity, platelets from healthy volunteers were incubated with buffer or heat-aggregated IgG, then stimulated with increasing concentrations of thrombin. Interestingly, heat-aggregated IgG-stimulated platelets, but not buffer-treated platelets, were hypersensitive to thrombin, and hypersensitivity was blocked by an anti-Fc γ RIIa monoclonal antibody (mAb). Thrombin hypersensitivity was not due to changes in thrombin receptor expression (GPIb α or PAR1) but is dependent on activation of shared signaling molecules. These observations suggest that ligation of platelet Fc γ RIIa by IgG complexes induces a hypersensitive state whereby small changes in thrombotic stimuli may result in platelet activation and subsequent vascular complications such as transient ischemic attacks or stroke. (*Am J Pathol* 2013, 182: 244–254; <http://dx.doi.org/10.1016/j.ajpath.2012.09.005>)

Platelets have long been known for their role in maintenance of vascular homeostasis through aggregation and thrombus formation. More recently, platelets are being appreciated for a plethora of activities associated with vascular remodeling, host defense, and inflammation.¹ Interestingly, platelets have been reported to alter expression of Bcl-3 and IL-1 β upon stimulation with thrombin and fibrinogen.² Because platelets are anucleate, changes in expression are due solely to posttranscriptional modulation. These studies provide evidence that platelets are not simply “clotting factors,” but complex cells that have the potential to alter their response depending on the nature of stimulation.

Supporting a role for platelets in inflammatory diseases, Boilard and colleagues³ recently observed the presence of IL-1 β -containing platelet microparticles in synovium of patients with rheumatoid arthritis. Furthermore, in their mouse model of collagen-induced arthritis, depletion of platelets resulted in significantly reduced pathology.³ Our

recent studies measuring soluble CD40 ligand (sCD40L) and regulated on activation normal T cell expressed and secreted (RANTES) production subsequent to IgG exposure suggest that platelets may be responsible for recruiting and stimulating other inflammatory cells.^{4,5} Consistent with our observations, another recent study has shown that platelets from systemic lupus erythematosus (SLE) patients express elevated levels of CD40L, due to IgG complex binding to platelets via the fragment crystallizable (Fc) region receptor Fc γ RIIa.⁶ Importantly, immune complexes collected from

Supported by an Arthritis Investigator Award from the Arthritis foundation (R.G.W.) and by a translational research stimulation award from the University of Toledo College of Medicine and Life Sciences (R.G.W.).

Author A.J.A. is deceased.

This article is dedicated to the memory of Adam J. Antczak, M.D., who tragically passed away on June 18, 2012, only 2 weeks after receiving his M.D. degree.

the serum of SLE patients were shown to activate healthy platelets resulting in expression of CD40L.⁶ Interestingly, platelets can respond to various stimuli by exhibiting select activation profiles such as aggregation, surface expression of CD62P (P-selectin), and release of soluble molecules such as sCD40L.⁴ These observations suggest that platelets may be important mediators of inflammatory conditions such as rheumatoid arthritis, SLE, and other autoimmune disorders.

It is well known that patients with autoimmune diseases are more susceptible to platelet abnormalities such as thrombocytopenia, transient ischemic attack, and stroke.^{7–13} The mechanisms behind these clinical observations are unknown and may be due to a multitude of factors including activation of vascular endothelium, interaction with leukocytes, opsonization by antiplatelet antibodies, or reaction to immune complexes. Many autoimmune disorders are characterized by the presence of IgG complexes in circulation, which could bind to platelet FcγRIIa.

It is possible that in addition to inducing expression of select activation markers, binding of IgG complexes to platelet FcγRIIa initiates signaling that results in platelets becoming more sensitive to other thrombotic stimuli. To this end, we tested the hypothesis that exposure to IgG complexes will induce platelets to become hypersensitive to thrombotic stimuli. Interestingly, platelets from SLE patients exhibit hypersensitivity to thrombin compared to platelets from healthy volunteers. Moreover, platelets from healthy volunteers pretreated with heat-aggregated IgG (aggIgG) before thrombin stimulation were hypersensitive to thrombin, resulting in increased expression of CD62P (P-selectin) compared to platelets pretreated with buffer alone.

Materials and Methods

Patients

Patients ($n = 20$) who fulfilled at least four American College of Rheumatology criteria for SLE were enrolled into this study in accordance with University of Toledo Biomedical Institutional Review Board approval. Patients on platelet-modifying therapy [aspirin, thienopyridines, or glycoprotein IIb/IIIa (GPIIb/IIIa) inhibitors] were excluded from the study. Patient profiles show demographics (Table 1), SLE Disease Activity Index criteria (Table 2), and medications (Table 3). Healthy volunteers consisted of employees of the University of Toledo Health Science Campus who were free of autoimmune disease, had no active infections, and were not on any of the platelet-modifying therapies listed above.

Platelet Preparation

Platelets were prepared as previously described.¹⁴ Briefly, blood was drawn into evacuated blood collection tubes

Table 1 Healthy Volunteer and SLE Patient Demographics

Demographics	SLE	Control
No.	20	20
Male	3	3
Female	17	17
Average age (range)	46.5 (27–59)	46 (27–55)
Ethnicity		
Hispanic	9	9
Caucasian	4	4
African American	7	7
Average disease duration in years (range)	14.83 (10–21)	NA
Mean platelet count	246,000/cm ³ *	232,000/cm ³ *

*No patients had thrombocytopenia at the time of sample collection. NA, not applicable.

containing 10% acid citrate dextrose solution (Vacutainers; BD, Franklin Lakes, NJ): 56 mmol/L sodium citrate, 65 mmol/L citric acid, and 100 mmol/L glucose. The blood was centrifuged at $200 \times g$ for 10 minutes to obtain platelet-rich plasma. Platelet-rich plasma was collected and washed in buffer containing: 2.75 g/L sodium citrate, 1.0 g/L citric acid, 3.2 g/L glucose, and 8.5 g/L sodium chloride, mixed well, and pH adjusted to 6.5. After washing, platelets were kept in the buffer and stored for no more than 30 minutes before use. For experiments, platelets were resuspended in buffer containing: 8.0 g/L sodium chloride, 0.2 g/L potassium chloride, 0.2 g/L magnesium chloride, 0.45 g/L sodium phosphate dibasic, 0.9 g/L HEPES, 3.5 g/L bovine serum albumin, 1.0 g/L glucose, and adjusted to pH 7.4. Platelet preparations are >90% pure as assessed by flow cytometry for platelet glycoprotein Ib alpha chain (GPIb α) (Supplemental Figure S1).

aggIgG Preparation

aggIgG was prepared by diluting fluorescein isothiocyanate-labeled IgG from human serum (Sigma-Aldrich, St. Louis, MO) in Dulbecco's phosphate buffered saline (Thermo Fisher Scientific, Waltham, MA) to a concentration of 10 mg/mL and heating the suspension to 62°C for 20 minutes. Large aggregates were cleared by centrifugation at $10,000 \times g$ for 10 minutes, leaving a mixture of aggIgG consisting of two to six molecules per complex as previously reported.¹⁴

Table 2 SLEDAI-Qualifying Symptoms at Time of Sample Collection

Symptom	Patients (%)	SLEDAI score
Arthritis	11 (79%)	4
dsDNA Antibodies	3 (21%)	2
Low complement C3 or C4	0 (0%)	2

Antinuclear antibodies (<1:40) in 11 patients (79%).

dsDNA, double-stranded DNA; SLEDAI, SLE Disease Activity Index.

Table 3 Current Treatments at Time of Sample Collection

Treatment	Patients (%)
Mycophenolate	7 (50%)
Hydroxychloroquine	5 (36%)
Prednisone	4 (29%)
Methotrexate	2 (14%)
Celecoxib	2 (14%)
Adalimumab	1 (7%)

Platelet Activation Assay

Platelets (100-μL aliquots) at a concentration of $1 \times 10^7/\text{mL}$ were placed in a 37°C water bath for 30 minutes in the presence or absence of 0.5 mg/mL aggIgG. Buffer alone or buffer containing specified concentrations of thrombin, collagen, ADP, or arachidonic acid (Chronolog, Havertown, PA) was added at the indicated times, and the platelets were returned to the water bath for an additional 30 minutes. Following incubation, platelets were fixed in 2% para-formaldehyde for 1 hour at 4°C. Treatment with specific inhibitors was performed by pretreating platelets for 10 minutes at 37°C followed by the addition of aggIgG or buffer and allowing the activation assay to occur in the presence of the inhibitor for the entirety of the experiment. Inhibitors of Src kinase (PP2; Sigma-Aldrich)¹⁵ and spleen tyrosine kinase (Syk) (BAY61-3606; Santa Cruz Biotechnology, Santa Cruz, CA)¹⁶ were used at 10 μmol/L and 20 nmol/L, respectively, which is consistent with previous studies testing specificity. We also performed dose responses to determine the optimal concentration of BAY61-3606 to use in our system by detecting the ability to inhibit hypersensitivity (Supplemental Figure S2).¹⁷

Activation Marker Labeling and Flow Cytometry

Following fixation on ice, the platelets were labeled for analysis by flow cytometry. For gating purposes, an allophycocyanin-conjugated anti-GPIbα (clone HIP1) monoclonal antibody (mAb) (BD Biosciences, San Jose, CA) was used to verify the platelet population. Other antibodies used to measure receptor expression include anti-FcγRII (clone 3D3), anti-protease activated receptor 1 (PAR1) (clone ATAP2), anti-P-selectin (CD62P) (clone AK-4), or anti-GPIIb (clone HIP8) mAbs. Isotype-matched control IgG was used as labeling controls to set negative populations for each mAb. Association of aggIgG with platelets was assessed by measuring fluorescein isothiocyanate–aggIgG fluorescence on GPIbα⁺ platelets. Platelets were analyzed on a FACSCalibur flow cytometer (BD Biosciences) and data were interpreted using Cell Quest (BD Biosciences) and FloJo software (Tree Star, Ashland, OR). The effective concentration of thrombin required to elicit 50% of the maximum response (EC₅₀) was calculated using Prism 5 software (GraphPad, La Jolla, CA).

Western Blotting

Platelets (100-μL aliquots) at a concentration of $4 \times 10^7/\text{mL}$ were treated as described above with PP2 (10 μmol/L), BAY61-3606 (20 nmol/L), or buffer followed by aggIgG (0.5 mg/mL). Platelets were then incubated for an hour in 40 μL of a lysis buffer containing: 0.88 g/L sodium chloride, 1.19 g/L HEPES, 0.02 g/L sodium azide, 1% triton X-100, and 1% protease inhibitor. Samples were separated by SDS-PAGE and then transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA). Membranes were blocked in a 3% bovine serum albumin TBS-T solution and then blotted for 2 hours with anti-Syk (Santa Cruz Biotechnology), anti-FcγRIIa (Proteintech Group, Chicago, IL), antiphosphotyrosine (Millipore), or antiglyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Ambion, Foster City, CA). The membranes were then developed using the Western Breeze kit per the manufacturer’s instructions (Invitrogen). Films were then imaged using an Omega Molecular Imaging and Analysis System instrument (UltraLum, Claremont, CA). Absorbance was calculated using UltraQuant version 6.0 software (UltraLum).

Statistics

All statistics were calculated using Prism 5 software. Statistical significance for thrombin hypersensitivity in healthy control platelets was determined using a two-tailed, equal variance Student’s *t*-test. Statistical significance comparing SLE patients with healthy volunteers was determined using a two-tailed, paired Student’s *t*-test.

Results

Characterization of Healthy and SLE Platelets

The ability of platelets to recognize and respond to IgG complexes depends on the expression of FcγRIIa. Therefore, we determined whether FcγRIIa expression was different on platelets obtained from SLE patients (diagnosed with SLE according to American College of Rheumatology criteria as stated in *Materials and Methods*) versus platelets from healthy volunteers. Platelets from SLE patients and healthy volunteers (matched for age, sex, and ethnicity) were treated identically on the same day and using the same reagents. Purified platelets were analyzed by flow cytometry for continuity of size by forward versus side scatter and the exclusion of microparticles, as determined by comparing the platelet gate to beads of 0.5, 1, and 2 μm. Platelets appear as a pure population that is larger than 1 μm, constitutively express GPIbα (CD42b), and express P-selectin (CD62P) only after stimulation (Figure 1A). As shown in Figure 1B, platelet FcγRIIa expression shows considerable person-to-person variability, but no significant differences were noticed comparing samples from healthy volunteers and

Figure 1 Characterization of platelets from SLE patients and healthy volunteers. **A:** Flow cytometry strategy of purified platelets including: scatter plot with size gate (**left**); platelets mixed with latex beads (**middle left**); GPIb α expression (solid line) versus control IgG (dotted line) of events in gated scatter plot (**middle right**); CD62P expression of unstimulated platelets (shaded histogram) and platelets stimulated with thrombin at 0.01 U/mL (dotted line), 0.05 U/mL (dashed line), or 0.1 U/mL (solid line) (**right**). **B–D:** Washed platelets from SLE patients or healthy volunteers were fixed and stained with mAbs directed at select surface receptors. **E:** Platelets described above were stained with anti-human IgG. **F:** Pearson correlation comparing CD62P expression and levels of surface IgG. Statistical comparisons were performed by paired Student's *t*-test between age-, sex-, and ethnicity-matched patients and healthy volunteers and performed on the same day. MFI, mean fluorescence intensity. **P* < 0.05.

SLE platelets. To assess platelet phenotypes from healthy volunteers and SLE patients, we measured GPIb α expression in freshly isolated samples and found no significant differences (**Figure 1C**). Because previous reports showed elevated CD62P expression on the surface of SLE platelets, we assessed CD62P expression in our samples, which confirmed the previous reports (**Figure 1D**).⁶ We then stained platelets from SLE patients or healthy volunteers with PE-conjugated anti-IgG F(ab')₂ fragments to detect surface-bound IgG. Consistent with previous observations, platelets from SLE patients exhibited elevated levels of surface-associated IgG compared to platelets from healthy individuals (**Figure 1E**). Interestingly, CD62P levels correlated with surface IgG (**Figure 1F**), suggesting that IgG may be involved in activating human platelets.

Platelets from SLE Patients Are Hypersensitive to Thrombotic Stimuli

Because patients with SLE have a higher incidence of thrombotic disorders, we next wanted to determine whether SLE platelets exhibit hypersensitivity to thrombotic stimuli. To accomplish this, platelets from SLE patients and healthy volunteers were treated with the indicated doses of thrombin, fixed, and then stained with anti-GPIb α , as a platelet marker, and anti-CD62P, as a marker of platelet activation. CD62P is known to be stored in platelet α -granules and expressed on the surface of the granules, fusing with the plasma membrane after stimulation. As shown in **Figure 2A**, platelets isolated from SLE patients displayed a higher sensitivity to thrombin (as determined by

Figure 2 Thrombin hypersensitivity of SLE platelets. **A:** Washed platelets from SLE patients (gray) or healthy volunteers (black) were incubated with the indicated concentrations of thrombin for 30 minutes at 37°C, then fixed and stained with PE-Cy5–conjugated anti-CD62P and allophycocyanin-conjugated anti-GPIIb/IIIa. CD62P expression on GPIIb/IIIa⁺ platelets was assessed by flow cytometry. **B:** EC₅₀ values for thrombin-induced activation were calculated based on data from A. **C:** EC₅₀ values for platelets stimulated with ADP (0.01 μmol/L to 10 μmol/L), collagen (0.001 μg/mL to 1 μg/mL), or arachidonic acid (0.01 mmol/L to 10 mmol/L) as calculated in B. H, healthy; L, SLE; MFI, mean fluorescence. ***P* < 0.01, *****P* < 0.0001.

activation at lower concentrations of thrombin). Data from each thrombin curve were then used to calculate the thrombin EC₅₀ values (Figure 2B). Of note, it took a >2.5-fold increase in thrombin to activate platelets from healthy volunteers than those from SLE patients. To determine whether hypersensitivity is limited to thrombin or common to many agonists, platelets from healthy volunteers or SLE patients were exposed to ADP, collagen, or arachidonic

acid. Interestingly, a slight degree of hypersensitivity was observed for ADP, but not for collagen or arachidonic acid (Figure 2C). Therefore, we used thrombin as the agonist in subsequent experiments.

Healthy Platelets Become Hypersensitive on Exposure to IgG Complexes

We next determined whether FcγRIIa ligation could induce thrombin hypersensitivity of platelets from healthy volunteers. To do this, we exposed healthy platelets to aggIgG before stimulation with thrombin. As shown previously and in Figure 3A, aggIgG does not result in significant CD62P expression during short incubation periods of 30 minutes at 37°C.⁴ Buffer alone or buffer containing thrombin at indicated concentrations was then added to platelet samples and incubated for another 30 minutes at 37°C. We noted that platelets pretreated with aggIgG responded to lower concentrations of thrombin compared to platelets pretreated with buffer alone (Figure 3A). When EC₅₀ values for thrombin sensitivity were calculated, we found that aggIgG induced a significant decrease in the thrombin EC₅₀ (Figure 3B). In similar experiments, SLE platelets were treated with buffer or aggIgG and then measured for thrombin hypersensitivity. We found that exposure to aggIgG had only marginal effects on SLE platelets, presumably because platelets from SLE patients already had IgG on their surface (Figure 1E), and subsequent addition of aggIgG had only a minimal effect (Figure 3B). As a control to determine whether hypersensitivity is simply due to exposure to two different stimuli, platelets were pretreated with thrombin followed by aggIgG to determine whether increased levels of CD62P are induced on platelet surfaces. Of note, there was no appreciable difference in CD62P expression on platelets preincubated with the indicated doses of thrombin for 30 minutes at 37°C followed by addition of buffer alone or buffer containing aggIgG (Figure 3C). These data suggest that aggIgG induces platelets to become hypersensitive to thrombin.

Because platelets from SLE patients showed hypersensitivity to thrombin, we next sought to determine whether serum from SLE patients could convert healthy platelets into a hypersensitive state. To do this, healthy platelets were incubated with SLE serum for 30 minutes at 37°C then stimulated with thrombin and EC₅₀ values were calculated. SLE serum induced healthy platelets to become hypersensitive to thrombin (Figure 3D); however, the magnitude of hypersensitivity is not the same as that elicited by aggIgG. Of note, healthy serum did not alter the EC₅₀ of SLE platelets (Figure 3D).

Given that platelets express FcγRIIa as their only IgG-binding receptor, we next tested whether the hypersensitivity is dependent on FcγRIIa. Thrombin hypersensitivity mediated by aggIgG was blocked by incubating platelets with the FcγRIIa-blocking mAb IV.3, but not control IgG, before exposing platelets to aggIgG (Figure 4A). IV.3 also inhibited aggIgG binding (Figure 4B), whereas control antibody did not.

Mechanism of Platelet Hypersensitivity

Platelet activation by thrombin takes place by the tethering of free thrombin to GPIb α , which brings thrombin in close proximity to PAR1 and PAR4. Thrombin then cleaves PAR1 or PAR4 leading to activation of a G-protein-related signaling pathway. To determine the mechanism behind thrombin hypersensitivity, we first measured GPIb α and PAR1 expression after exposure to aggIgG. Interestingly, no difference in expression of either GPIb α (Figure 5A) or PAR1 (Figure 5B) was observed. Because FcγRIIa has been shown to signal through Syk, we examined the amounts of Syk in platelets from healthy volunteers or SLE patients using Western blot analysis. No difference in Syk expression was observed in platelets from healthy volunteers or SLE patients (Figure 5C).

We next examined intracellular signaling pathways. Platelets with prothrombotic phenotypes have been observed in certain diseases such as essential thrombocythemia and polycythemia vera.^{18,19} In both of these diseases, constitutive activation of Src kinase has been reported. Src is also used by FcγRIIa. Therefore, we incubated platelets with PP2, a potent inhibitor of Src.^{15,20,21} Not surprisingly, pretreatment of platelets with PP2 inhibited both thrombin-induced activation and hypersensitivity (Figure 6A). Because FcγRIIa has also been shown to be dependent on signaling via Syk, we pretreated platelets with BAY61-3606 to inhibit Syk activity and then incubated with aggIgG followed with thrombin. Inhibition of Syk signaling completely abolished aggIgG-induced thrombin hypersensitivity but had no effect on thrombin activation (Figure 6B). These data suggest that ligation of FcγRIIa with aggIgG initiates a Syk-dependent

Figure 5 aggIgG-induced thrombin hypersensitivity is not dependent on protein expression levels. **A:** Platelets isolated from healthy volunteers were treated with buffer or aggIgG, fixed, and then assessed for expression of thrombin receptor GPIIb/IIIa by flow cytometry. **B:** Platelets isolated and treated as in A were assessed for PAR1 expression. **C:** Platelets isolated from healthy volunteers and SLE patients were lysed, separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and blotted with anti-Syk. Quantitation was performed using anti-GAPDH as a loading control. MFI, mean fluorescence intensity.

signaling cascade that is able to potentiate subsequent signals initiated by thrombin.

Finally, as inhibition of Syk was able to abolish FcγRIIa-induced hypersensitivity, we assessed the levels of tyrosine phosphorylation of platelet lysates from healthy volunteers stimulated with buffer or aggIgG in the absence or presence of PP2 or Bay61-3606. We observed increased tyrosine

phosphorylation of proteins isolated from platelets stimulated with aggIgG compared to buffer-treated platelets (Figure 6C, lane 1 vs 4). We also observed that increased tyrosine phosphorylation was diminished on pretreatment with either inhibitor (Figure 6C, lane 4 vs 5 and 6).

Based on the observation that aggIgG-stimulated healthy platelets show increased tyrosine phosphorylation, we tested SLE platelet lysates for increased phosphotyrosine levels (Figure 6D). We observed that platelets from SLE patients had significantly increased levels of tyrosine phosphorylation of multiple proteins. We then tested the Syk inhibitor on SLE platelets, and we found moderate inhibition of thrombin sensitivity (Figure 6E). This modest level of inhibition may be because FcγRIIa signaling pathways (including Syk) in SLE patients are already activated due to chronic exposure to IgG complexes, which minimizes the inhibitory activity of BAY61-3606. Taken together, these data indicate that aggIgG-induced hypersensitivity is due to downstream signaling initiated by FcγRIIa and that excessive amounts of IgG complexes present in SLE serum may contribute to the thrombotic pathology observed in these patients.

Discussion

It is well known that patients with autoimmune disease are at increased risk of thrombosis.^{9,22–24} However, the underlying reasons for these observations are unknown. Human platelets express FcγRIIa as their sole receptor for IgG.^{25,26} Due to their enormous abundance in blood, we have calculated that platelets express >90% of the total circulating FcγRIIa. Because patients with SLE are known to have circulating IgG complexes and clinically exhibit increased incidence of transient ischemic attack, stroke, and thrombocytopenia, we tested the hypothesis that exposure of platelets to IgG complexes induces a state of hypersensitivity toward thrombin (a potent and well-characterized platelet agonist).

Changes in platelet sensitivity to thrombin in SLE patients could be due to constant exposure to IgG complexes. In fact, platelets from SLE patients have been reported to have high levels of complexed IgG bound to their surface compared to platelets from healthy volunteers.^{22,27} Although these reports suggest that surface IgG is in the form of antiphospholipid or anticardiolipin antibodies bound directly to platelets via the fragment antigen-binding (Fab) region, they most likely activate FcγRIIa via their Fc regions. Consistent with previous reports, we observed that platelets from SLE patients show significantly higher levels of surface-bound IgG compared to platelets from healthy volunteers (Figure 1E).²⁷ One possibility for this is the existence of two alleles of FcγRIIa, identified by the presence of Arg or His at position 131. FcγRIIa-R131 results in a low-responder form with decreased affinity for IgG of certain classes (IgG2) compared to the FcγRIIa-H131.^{28,29}

Figure 6 aggIgG-induced thrombin hypersensitivity is provoked by FcγRIIa signaling **A**: Platelets isolated from healthy volunteers were treated with buffer (closed symbols) or an inhibitor of Src kinase activity (open symbols) (PP2). Platelets were then incubated with buffer (black) or aggIgG (gray), and then stimulated with indicated doses of thrombin. After fixation, CD62P expression was detected on GPIb α ⁺ population by flow cytometry. **B**: Platelets were incubated with buffer (closed symbols) or BAY61-3606 (Syk inhibitor) (open symbols) followed by incubation with buffer (black) or aggIgG (gray) then stimulated with thrombin. Platelets were fixed and stained as in **A** ($n = 3$). **C**: Platelets isolated from healthy volunteers were incubated with 10 μ mol/L PP2 or 20 nmol/L BAY61-3606, then stimulated with aggIgG for 30 minutes. The samples were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed for phosphotyrosine. Anti-GAPDH blots were used as loading controls ($n = 3$). **D**: Platelets isolated from healthy volunteers and SLE patients were treated with buffer then probed for phosphotyrosine via Western blot. FcγRIIa blots were used as a loading control. The relative absorbance for SLE compared to healthy platelets is reported as the sum of absorbance of every band in the entire lane ($n = 4$). **E**: SLE and healthy platelets were incubated with Syk inhibitor (dotted lines) or buffer (solid lines) then stimulated with thrombin for 30 minutes. Platelets were fixed and stained as in **A** ($n = 3$). * $P < 0.01$, ** $P < 0.001$. MFI, mean fluorescence intensity.

Individuals homozygous for R131 have been reported to be at increased risk for various infections; however, the link between polymorphism and autoimmune diseases is mostly unconvincing.^{30–36} In our studies, we employ pooled IgG containing all subclasses, which minimizes the influence of the H131R polymorphism, and therefore, we did not perform genetic analysis of our SLE patients or volunteers.

However, when tested for thrombin sensitivity, platelets from SLE patients were more sensitive to thrombin stimulation than platelets from healthy volunteers (Figure 2, A and B). Together, these data indicate that exposure to complexed IgG induces a change in platelets whereby they become more sensitive to low doses of thrombin. Altered thrombin sensitivity has been reported in various pathological conditions

such as diabetes mellitus, essential thrombocythemia, and polycythemia vera.^{18,19} GPVI, the platelet receptor for collagen, requires the Fc receptor γ -chain and utilizes the same signaling pathway as FcγRIIa.^{37,38} It would be interesting to determine whether pretreating platelets with collagen induces thrombin hypersensitivity, which could be applicable to diseases such as collagen-induced rheumatoid arthritis and systemic sclerosis, which also result in thrombotic complications.^{39,40}

Thrombin sensitivity has been suggested to be due to either changes in the surface expression of thrombin receptors, or altered intracellular signaling pathways.^{18,19,41,42} Thrombin binding to platelet surfaces takes place through GPIb α .^{43,44} On binding to GPIb α , thrombin is then brought into close

proximity to PARs, which are responsible for transmission of signals resulting in platelet activation. We did not observe any changes in GPIIb/IIIa expression in SLE platelets, nor were any changes in GPIIb/IIIa noted after treatment of healthy platelets with aggIgG (Figures 1C and 5A). PAR1 and 4 are the two isoforms found on platelets.^{45,46} PAR1 is the predominant isoform for signaling in the presence of low concentrations of thrombin (<0.5 units/mL), whereas PAR4 is most active at high thrombin concentrations.⁴⁶ In our experiments, we did not observe any changes in expression of PAR1 on SLE platelets or healthy platelets treated with aggIgG.

The second mechanism by which platelets may become more sensitive to thrombin after incubation with aggIgG is by “priming” signaling pathways. To test this hypothesis, platelets were treated with inhibitors of known signaling molecules activated by FcγRIIIa. We demonstrate that inhibition of Src kinase results in reduction of hypersensitivity but also decreases thrombin-induced platelet activation. These results are not surprising because Src has been shown to be involved in PAR1-mediated platelet activation.^{19,47,48} By contrast, inhibition of Syk abolished thrombin hypersensitivity without altering normal responses to thrombin. Additionally, we observed that binding of aggIgG to platelets resulted in tyrosine phosphorylation and that platelets from SLE patients showed increased tyrosine phosphorylation. These observations support other reports showing the involvement of Syk in platelet activation through FcγRIIIa and may support the use of Syk inhibitors in SLE patients similar to the proposed use in such other thrombotic disorders as heparin-induced thrombocytopenia.^{49,50} Interestingly, BAY61-3606 only partially inhibited thrombin hypersensitivity in SLE platelets, as compared to complete inhibition of hypersensitivity in healthy platelets incubated with aggIgG (Figure 6, B versus E). One explanation for this observation is that BAY61-3606 binds irreversibly to the ATP-binding pocket of Syk, thus inhibiting ATP binding and, therefore, the kinase activity.¹⁶ Because SLE platelets have been chronically exposed to IgG complexes, Syk activation has already taken place, leading to activated signaling cascades. Addition of BAY61-3606 is only capable of preventing subsequent phosphorylation events but is not able to diminish previously activated pathways.

Platelet disorders are a major contributor to mortality and morbidity in such autoimmune diseases as SLE.^{13,51,52} Previous reports have shown that, on stimulation, platelets release vast amounts of such inflammatory molecules as sCD40L, RANTES, and IL-1β.^{4,6,53} Due to their abundant number in circulation, their ability to recognize and respond to immunological molecules, and their capacity to produce inflammatory molecules, platelets should be considered major participants in inflammatory reactions. For example, recent studies have demonstrated the potent capacity of platelet-derived microparticles in mediating the clinical symptoms of rheumatoid arthritis through their ability to activate fibroblasts via IL-1β.³ Further studies have implicated platelet CD40L in triggering interferon-α secretion by

dendritic cells, which contributes to SLE pathogenesis.⁶ In both of these reports, administration of antiplatelet therapies resulted in significantly reduced pathology.

The findings in this article show that stimulation with IgG complexes primes platelets into a hypersensitive state in which only slight changes in thrombotic stimuli induce robust platelet activation, which could result in accelerated vascular complications *in vivo*. We have previously shown that stimulation of platelets with aggIgG induces potent release of sCD40L and RANTES. Combined, these findings suggest that immune complex–stimulated platelets may play significant roles in disease pathogenesis (thrombosis) and in promoting inflammation (cytokine release). Therefore, further insight into platelet activity in autoimmune diseases is needed.

Acknowledgments

We thank the patients and volunteers who unselfishly agreed to participate in this research study and the Jacobsen Center for Clinical and Translational Research at the University of Toledo for assistance in institutional review board preparation and statistical support.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2012.09.005>.

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