ARTICLE

A subset of anti-HLA antibodies induces FcγRIIa-dependent platelet activation

Maaike Rijkers,[1] Anno Saris,[2] Sebastiaan Heidt,[3] Arend Mulder,[3] Leendert Porcelijn,[4] Frans H.J. Claas,[3] Ruben Bierings,[1] Frank W.G. Leebeek,[5] A.J. Gerard Jansen,[1,5] Gestur Vidarsson,[6] Jan Voorberg[1,7] and Masja de Haas[3,4,8]

[1]Department of Plasma Proteins, Sanquin-AMC Landsteiner Laboratory, Amsterdam; [2]Department of Immunopathology, Sanquin-AMC Landsteiner Laboratory, Amsterdam; [3]Department of Immunohaematology and Blood Transfusion, Leiden University Medical Center; [4]Department of Immunohaematology Diagnostics, Sanquin Diagnostic Services, Amsterdam; [5]Department of Hematology, Erasmus University Medical Center, Rotterdam; [6]Department of Experimental Immunohematology, Sanquin-AMC Landsteiner Laboratory, Amsterdam; [7]Department of Vascular Medicine, Amsterdam UMC, University of Amsterdam and [8]Center for Clinical Transfusion Research, Sanquin, Leiden, the Netherlands

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ABSTRACT

HLA antibodies are associated with refractoriness to platelet transfusion, leading to rapid platelet clearance, sometimes coinciding with clinical side effects such as fever and chills. The presence of HLA antibodies is not always manifested by clinical symptoms. It is currently unclear why refractoriness to platelet transfusion is only observed in a subset of patients. Here, we utilized the availability of a unique panel of human monoclonal antibodies to study whether these were capable of activating platelets. Three out of eight human HLA-specific monoclonal antibodies induced activation of HLA-matched platelets from healthy donors as evidenced by enhanced α-granule release, aggregation, and $\alpha_{IIb}\beta_3$ activation. The propensity of HLA monoclonal antibodies to activate platelets was independent of the HLA subtype to which they were directed, but was dependent on the recognized epitope. Activation was fully inhibited either by blocking FcγRIIa, or by blocking FcγRIIa-dependent signaling with Syk inhibitor IV. Furthermore, activation required the presence of the IgG-Fc part, as $F(ab')_2$ fragments of HLA monoclonal antibodies were unable to induce platelet activation. Mixing experiments revealed that activation of platelets occurred in an intra-platelet dependent manner. Accordingly, a proportion of sera from refractory patients with HLA antibodies induced FcγRIIa-dependent platelet activation. Our data show that a subset of HLA antibodies is capable of crosslinking HLA and FcγRIIa thereby promoting platelet activation and enhancing these cells' phagocytosis by macrophages. Based on these findings we suggest that FcγRIIa-dependent platelet activation may contribute to the decreased platelet survival in platelet-transfusion-dependent patients with HLA antibodies.

Introduction

Antibodies against human leukocyte antigen (HLA) can be induced by pregnancy, blood transfusion or transplantation.[1-3] During or after a pregnancy, 15-50% (depending on the number of pregnancies) of women develop HLA antibodies.[4-6] Platelet reactive alloantibodies commonly directed toward HLA of the donor platelets develop in 20-30% of chronic platelet transfusion recipients.[2,3,7] Although platelet refractoriness is more commonly caused by non-immune factors[2,7,8] 30-50% of platelet-transfusion-dependent recipients with HLA antibodies become refractory to platelet transfusions due to alloimmunization.[2,7] HLA antibodies in this setting are primarily composed of immunoglobulin G (IgG) and are directed toward HLA-

Correspondence:
m.rijkers@sanquin.nl

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A and B.[3] Binding of antibodies to HLA class I on donor platelets results in the formation of IgG-opsonized platelets which are rapidly cleared from the circulation. Several parameters may contribute to the efficacy of HLA antibody-induced platelet clearance. Firstly, HLA density on platelets may differ between individuals. A recent study showed that platelets from donors with consistently low HLA-B8, B12 or B35 displayed a strongly reduced antibody-mediated internalization by macrophages.[9] Furthermore, low levels of HLA antibodies were not associated with platelet refractoriness in the TRAP (Trial to Reduce Alloimmunization to Platelets) study.[10] Results from the same study revealed that high levels of anti-HLA antibodies were clearly related to refractoriness to platelet transfusion.[10] Transfusions with HLA-compatible platelets have been shown to be effective in patients with pre-existing HLA antibodies.[11] In an early clinical trial no beneficial effect of treatment with HLA-matched platelet concentrates was observed.[12] These findings indicate that platelet refractoriness is of non-immune origin in a significant number of patients and, collectively, suggest that HLA antibodies, dependent on their titer and the HLA density on donor platelets, can induce platelet refractoriness. Whether additional mechanisms contribute to the observed clinical effects of anti-HLA antibodies has not yet been clearly delineated.

Apart from the transfusion setting, a pathogenic role for platelet-specific antibodies has been described in several diseases. Patients with immune thrombocytopenia often have autoantibodies against glycoprotein (GP)Ib/IX or GPIIbIIIa, frequently coinciding with refractoriness.[13–15] Anti-GPIbα has been associated with Fcγ receptor IIa (FcγRIIa)-independent platelet activation, through loss of sialic acid and subsequent clearance via the Ashwell Morell receptor localized on hepatocytes.[16] Alternatively, in heparin-induced thrombocytopenia, antibodies directed to platelet factor 4/heparin complex induce platelet clearance and FcγRIIa-dependent platelet activation.[17,18] Previous studies have described on FcγRIIa-dependent activation of platelets by (non-physiological) crosslinking of the murine pan-HLA class I antibody W6/32.[19] Complement-dependent platelet aggregation induced by HLA antibodies has also been reported.[20] Based on these findings we hypothesized that a subset of human HLA antibodies may be able to activate platelets. To address this issue we tested a panel of well-characterized human monoclonal HLA antibodies and HLA antibody-containing sera from platelet-transfusion refractory patients for their ability to activate platelets.

Methods

HLA monoclonal antibodies and patients' sera

Human HLA-specific monoclonal antibodies, all of IgG1 isotype, were produced by hybridoma technology as described previously.[21,22] Blood samples of patients refractory to platelet transfusion were sent to the Department of Immunohematology Diagnostic Services, Sanquin, Amsterdam, the Netherlands. Leftover material was used according to the Dutch established codes of conduct for responsible use of patients' material and as approved by our institute.[23] HLA antibody specificities in patients' sera were determined by a single antigen bead assay (Luminex). Thirteen sera positive for HLA antibodies and negative for other platelet-specific antibodies were used.

Human platelets

Citrated whole blood was obtained from healthy human volunteers with known HLA type (second field) in accordance with Dutch regulations and after approval from the Sanquin Ethical Advisory Board in accordance with the Declaration of Helsinki. Written informed consent was given by all participants. Platelets were isolated and washed as described elsewhere,[24] and resuspended in platelet assay buffer (10 mM HEPES, 140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2$, 10 mM glucose and 0.5 mM $NaHCO_3$, pH 7.4).

Platelet activation

Washed platelets ($2.5x10^8$ platelets/mL) were incubated with HLA monoclonal antibodies or patients' sera (1:50) containing HLA antibodies for 1 h at room temperature. Where appropriate, platelets were pre-incubated with FcγRIIa blocking antibody IV.3, Syk inhibitor IV or intravenous immunoglobulin.

Flow cytometry

For flow cytometry measurements, platelets were fixed in 1% PFA and diluted in platelet assay buffer. Anti-CD62P, anti-PAC-1 and anti-IgG antibodies were used to stain the platelets. Analysis was performed using a FACSCanto II (Becton Dickinson) flow cytometer.

Internalization of opsonized platelets by macrophages

Internalization of platelets by macrophages was determined as previously described.[9] In short, PKH26-labeled platelets were opsonized with HLA monoclonal antibodies in the presence or absence of Syk inhibitor IV and incubated with monocyte-derived macrophages. Platelet internalization was quantified by imaging flow cytometry (ImageStream®X Mark II Imaging Flow Cytometer, Merck Millipore, Amsterdam, the Netherlands).

Data and statistical analysis

Flow cytometry data were analyzed using FlowJo version 10 (Ashland, OR, USA). Data are represented as either mean ± standard deviation (SD) or all data points are shown. Statistical analyses were performed using GraphPad Prism 7 version 7.02 (La Jolla, CA, USA), with the analyses used specified in the respective figure legends. Differences were considered statistically significant when P values were <0.05.

Further details on materials and methods can be found in the *Online Supplementary Data*.

Results

HLA monoclonal antibodies induced platelet α-granule release

Eight human HLA-specific monoclonal antibodies were used to study the effect of HLA antibodies on platelets from healthy donors (Table 1). These antibodies all recognize different HLA epitopes, of which some are specific for a particular HLA antigen (e.g. GV2D5 binds only HLA-A1) and others are broadly reactive (e.g. WIM8E5, binding to HLA-A1/A10(A25/A26/A34/A43/A66)/A11/A9(A23/A24)/A29/A30/A31/A33/A28(A68/A69)).[21,25] Donors were selected in such a way that platelets expressed an HLA type matching the specificity of antibodies used in each experiment. A similar level of binding of HLA monoclonal antibodies to the matched platelets was obtained in all experiments, as verified by flow cytometry (Figure 1A). The ability of HLA monoclonal antibodies to induce α-granule release was assessed by measuring CD62P expo-

sure on the platelet surface. At low concentrations (2.5 μg/mL), a subset of HLA monoclonal antibodies induced α-granule release, as shown by significantly increased CD62P membrane exposure (Figure 1B). Statistically significant enhanced CD62P exposure was observed only for the broadly reactive WIM8E5 and HLA-A2/A28-specific SN607D8, whereas GV5D1 (anti-HLA A1/A23/A24) showed a trend of enhanced CD62P exposure, but this was not statistically significant. SN230G6, with a specificity similar to that of SN607D8 and GV2D5, binding to HLA-A1 like GV5D1, did not induce platelet activation. VTM1F11, HDG8D9 and BRO11F9 also did not induce enhanced CD62P membrane exposure on platelets. Increased CD62P exposure was observed upon incubation with higher concentrations of WIM8E5, SN607D8 and GV5D1 (Figure 1C). Addition of 10 μg/mL of the other HLA monoclonal antibodies included in this study did not induce significantly increased CD62P membrane exposure (Figure 1C). Additionally, incubation of platelets with two human monoclonal anti HPA-1a antibodies did not induce CD62P exposure despite their efficient binding to platelets (*Online Supplementary Figure S1A*).To further confirm the release of α-granules from platelets, the release of von Willebrand factor (VWF) and SPARC (secreted protein acidic and rich in cysteine), both proteins residing in α-granules,[26] was measured in the supernatant of platelets incubated with HLA monoclonal antibodies. VWF (Figure 1D) and SPARC (Figure 1E, *Online Supplementary Figure S2A*) were released by platelets upon incubation with either WIM8E5 or SN607D8. Incubation with SN230G6 resulted in significant, but low levels of released VWF and SPARC. Together these results indicate that a subset of HLA monoclonal antibodies can induce α-granule release in platelets.

HLA monoclonal antibodies induce integrin $\alpha_{IIb}\beta_3$ activation and platelet agglutination

We subsequently studied the activation of GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$) using the PAC-1 antibody, which recognizes the active configuration of this integrin.[27] PAC-1 binding was increased significantly upon incubation with WIM8E5 and SN607D8, while SN230G6, an antibody not potent in inducing α-granule release, did not lead to activation of integrin $\alpha_{IIb}\beta_3$ (Figure 2A). We then performed light aggregometry to study whether platelets aggregate upon incubation with HLA monoclonal antibodies. In agreement with the results for α-granule release and integrin $\alpha_{IIb}\beta_3$ activation, WIM8E5 and SN607D8 induced dose-dependent platelet aggregation. No platelet aggregation was observed upon incubation with SN230G6 (Figure 2B). A combination of suboptimal concentrations of PAR1 activating peptide and HLA monoclonal antibodies did not induce enhanced CD62P exposure. In combination with low suboptimal concentration of PAR1 activating peptide or collagen, both activating (WIM8E5 and SN607D8) and non-activating HLA monoclonal antibodies (SN230G6) significantly enhanced platelet aggregation, which was most pronounced for WIM8E5 (*Online Supplementary Figure S1B,C*).

WIM8E5 also induced exposure of phosphatidylserine, as measured by annexin V binding. No significantly increased levels of annexin V binding were observed for SN607D8 and SN230G6, suggesting that only strongly activating HLA antibodies can induce phosphatidylserine exposure on platelets (*Online Supplementary Figure S2B*). These results indicate that the subset of HLA antibodies which induces α-granule release also stimulates integrin $\alpha_{IIb}\beta_3$ activation, platelet aggregation and phospatidylserine exposure.

Table 1. Human HLA-specific monoclonal antibodies used in this study.

Antibody name	HLA specificity
WIM8E5	A1/A10(A25/A26/A34/A43/A66)/A11/A9(A23/A24)/A29/A30/A31/A33/A28(A68/A69)
SN607D8*	A2/A28(A68/A69)
SN230G6*	A2/B57/B58
GV5D1[$]	A1/A23/A24 {not A*2403; A80 weak}
GV2D5[$]	A1
VTM1F11	B27/B7/B60
HDG8D9	B51/B35
BRO11F6	A3/A11/A24

* SN607D8 and SN230G6 originate from the same patient. [$]GV5D1 and GV2D5 originate from the same patient.

Activation of platelets by HLA monoclonal antibodies is FcγRIIa dependent

Next, we tested whether the platelet-Fc receptor, FcγRIIa,[28,29] is involved in anti-HLA mediated platelet activation. The pathway of FcγRIIa-dependent platelet activation by IgG is well described in, for instance, heparin-induced thrombocytopenia.[17] FcγRIIa-mediated platelet activation via its ITAM motif has been shown to be dependent on the tyrosine kinase Syk.[30] We tested whether HLA antibody-mediated activation of platelets required Syk,[31] employing the extensively characterized Syk inhibitor IV.[30] This inhibitor has no effect on activation via PAR1 receptor (*Online Supplementary Figure S2C*), confirming its specificity. CD62P exposure induced by WIM8E5 or SN607D8 was inhibited in a dose-dependent manner by Syk inhibitor IV (Figure 3A). Involvement of Syk was further supported by blocking experiments employing Syk inhibitor I and II (*Online Supplementary Figure S2D*). Similarly, VWF release (Figure 3B) and SPARC release (Figure 3C) were blocked by Syk inhibitor IV. Activation of integrin $\alpha_{IIb}\beta_3$ (Figure 3D) and platelet aggregation (Figure 3E) were completely abrogated following the addition of Syk inhibitor IV. These results indicate that HLA monoclonal antibody-induced platelet activation is dependent on Syk, which acts downstream of FcγRIIa. To further substantiate the involvement of FcγRIIa, blocking monoclonal anti-FcγRIIa-antibody IV.3 was used to prevent binding of the Fc tail of the HLA monoclonal antibodies to FcγRIIa.[29] Analysis of CD62P surface exposure and of VWF and SPARC secretion revealed that α-granule release was completely blocked upon pre-incubation with IV.3 (Figure 3F-H). In addition, IV.3 completely blocked WIM8E5- and SN607D8-induced platelet aggregation (Figure 3I), integrin $\alpha_{IIb}\beta_3$ activation (Figure 3J) and annexin V binding (*Online Supplementary Figure S2B*). The R131H polymorphism in FcγRIIa did not affect platelet activation by the HLA monoclonal antibodies used in this study (*Online Supplementary Figure S3*). Together these results indicate that platelet α-granule release, activation and aggregation induced by HLA monoclonal antibodies are FcγRIIa dependent.

To confirm the involvement of the Fc-tail of the HLA monoclonal antibodies in platelet activation, $F(ab')_2$ frag-

ments of WIM8E5, SN607D8 and SN230G6 were generated (*Online Supplementary Figure S4A*). The lack of an Fc-tail (*Online Supplementary Figure S4B*) and binding (*Online Supplementary Figure S4C*) of the F(ab')$_2$ fragments to HLA molecules on platelets was confirmed. Staining with anti-IgG directed to the Fc-tail of human IgG was negative for F(ab')$_2$ fragments and positive for IgG. For WIM8E5, F(ab')$_2$ binding was lower than that of the corresponding IgG, but significant binding was still observed (*Online Supplementary Figure S4C*). None of these F(ab')$_2$ fragments induced CD62P membrane exposure (*Online Supplementary Figure S4D*), indicating that crosslinking of an HLA molecule and FcγRIIa by an intact anti-HLA IgG is crucial to induce platelet activation.

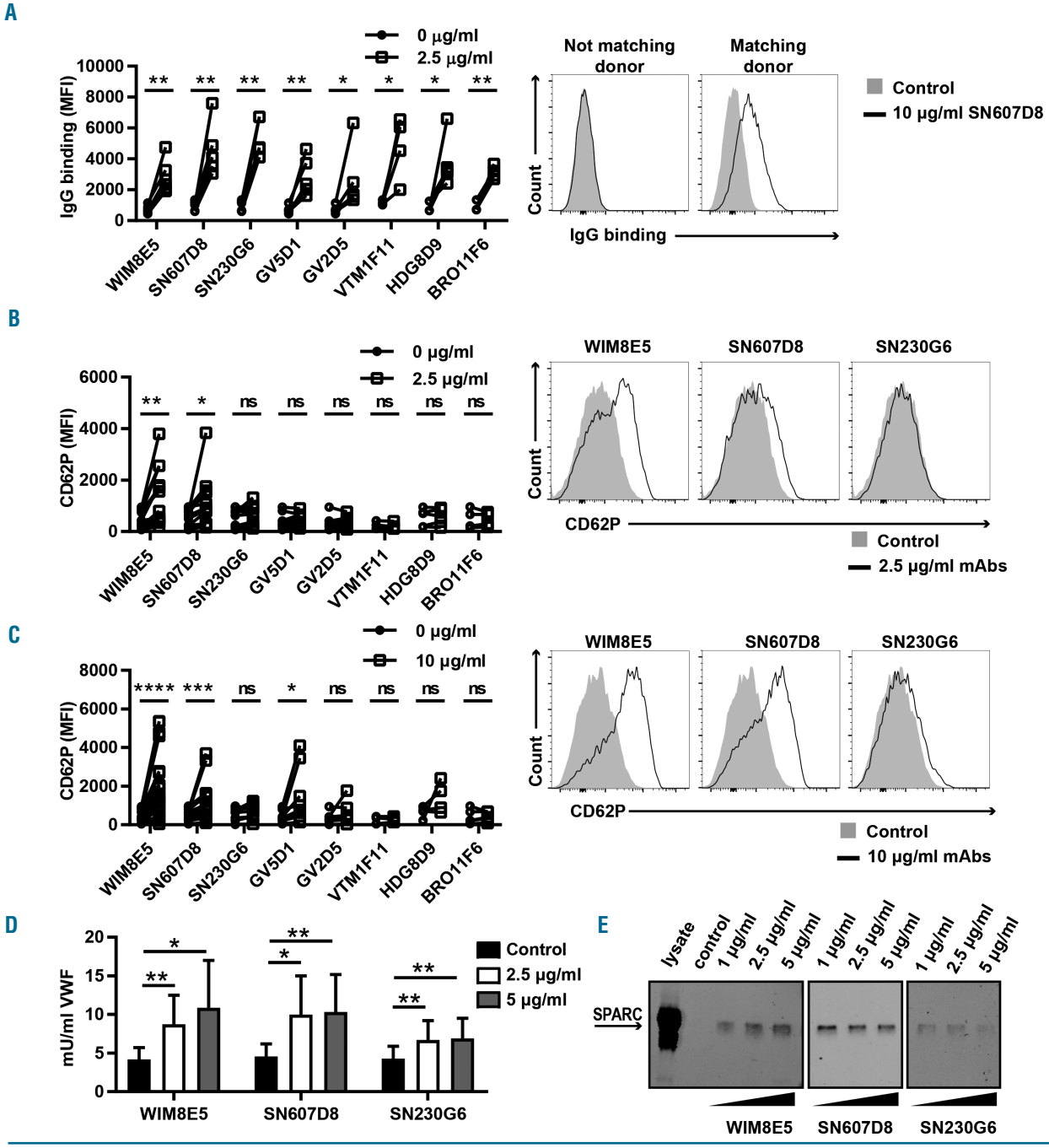

Figure 1. HLA monoclonal antibodies induce platelet α-granule release. (A) Platelets were matched for HLA type with the specificity of eight HLA monoclonal antibodies (mAbs) directed at different epitopes. Mean fluorescent intensity (MFI) upon staining with anti-human IgG was measured with flow cytometry for the control (buffer only, no HLA antibodies) and 2.5 µg/mL of the HLA mAbs. Right panel: representative flow cytometry plot of 10 µg/mL SN607D8 with a not matching donor and a matching donor. (B,C) CD62P surface expression of platelets incubated with 2.5 µg/mL (B) or 10 µg/mL (C) HLA mAbs compared to control (buffer only). Representative flow cytometry plots of WIM8E5, SN607D8 and SN230G6. (D) VWF release in platelet supernatant upon incubation with HLA mAbs WIM8E5, SN607D8 and SN230G6 measured by enzyme-linked immunosorbent assay. (E) Representative western blot of SPARC release in platelet supernatant upon incubation with HLA mAbs WIM8E5, SN607D8 and SN230G6. Paired t-tests (A, B and C) or paired ANOVA with the Tukey multiple comparison test (D). Each line represents a separate experiment with a separate donor (A, B and C). Mean ± SD (D). *P<0.05, **P<0.01, ***P<0.005, ****P<0.001.

FcγRIIa-dependent platelet activation has been firmly implicated in the pathogenesis of heparin-induced thrombocytopenia.[17] *In vitro* experiments have demonstrated that platelet activation by heparin-platelet factor 4 can be inhibited by intravenous immunoglobulin.[18] To study whether the mechanism of platelet activation by monoclonal antibodies is similar to that described for heparin-induced thrombocytopenia, platelets were pre-incubated with intravenous immunoglobulin and subsequently incubated with the HLA monoclonal antibodies WIM8E5 or SN607D8. Intravenous immunoglobulin diminished CD62P surface exposure in a dose-dependent manner and completely blocked α-granule release at a concentration of 2 mg/mL (*Online Supplementary Figure S5A*). These results suggest that high levels of IgG can compete with HLA monoclonal antibodies for binding to FcγRIIa.

Platelet activation by HLA monoclonal antibodies occurs through intra-platelet binding to FcγRIIa

FcγRIIa-dependent platelet activation can theoretically occur in either an inter-platelet-dependent manner (the HLA molecule of one platelet is crosslinked with the FcγRIIa of another platelet) or intra-platelet-dependent manner (an HLA molecule and FcγRIIa on a single platelet are crosslinked by the antibody). Rubinstein and co-workers showed that antibodies directed to beta-2-microglobulin can bind to FcγRIIa on other platelets (inter-platelet binding) resulting in their activation.[32] Activation of platelets in patients with heparin-induced thrombocytopenia is considered to occur both in an inter- and intra-platelet manner.[18] To elucidate whether platelet activation by HLA monoclonal antibodies occurs in an inter-and/or intra-platelet manner, we studied whether platelets missing the binding epitope of the activating HLA monoclonal antibody WIM8E5 ("nonmatching") could be activated in the presence of platelets which were able to bind WIM8E5 ("matching"). In the case of inter-platelet activation, the HLA of the "matching" platelets can theoretically be crosslinked with the FcγRIIa on the "nonmatching" platelets (Figure 4A). Platelets from a donor with an HLA type not matching with WIM8E5 did not show increased levels of CD62P upon incubation with WIM8E5. When these platelets were mixed with platelets from a donor with an HLA type matched for WIM8E5, CD62P surface expression remained unaltered (Figure 4B). Platelets from a WIM8E5 "matching" donor did show enhanced CD62P surface exposure, and levels did not change when platelets were mixed with platelets from a "nonmatching" donor

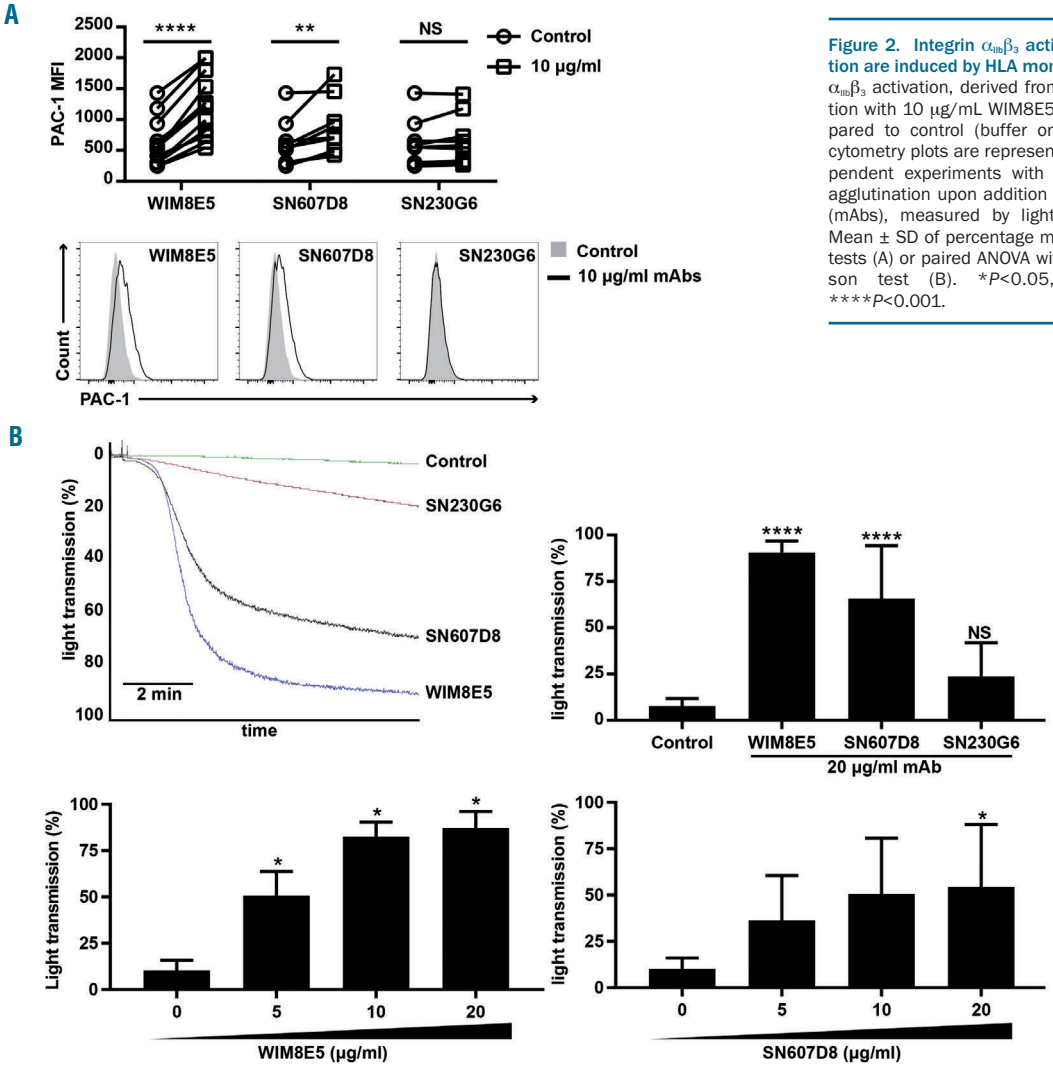

Figure 2. Integrin $\alpha_{IIb}\beta_3$ activation and platelet agglutination are induced by HLA monoclonal antibodies. A) Integrin $\alpha_{IIb}\beta_3$ activation, derived from PAC-1 binding, upon incubation with 10 µg/mL WIM8E5, SN607D8 or SN230G6 compared to control (buffer only, no HLA antibodies). Flow cytometry plots are representative of more than eight independent experiments with different donors. (B) Platelet agglutination upon addition of HLA monoclonal antibodies (mAbs), measured by light transmission aggregometry. Mean ± SD of percentage maximum aggregation. Paired *t*-tests (A) or paired ANOVA with the Tukey multiple comparison test (B). **P*<0.05, ***P*<0.01, ****P*<0.005, *****P*<0.001.

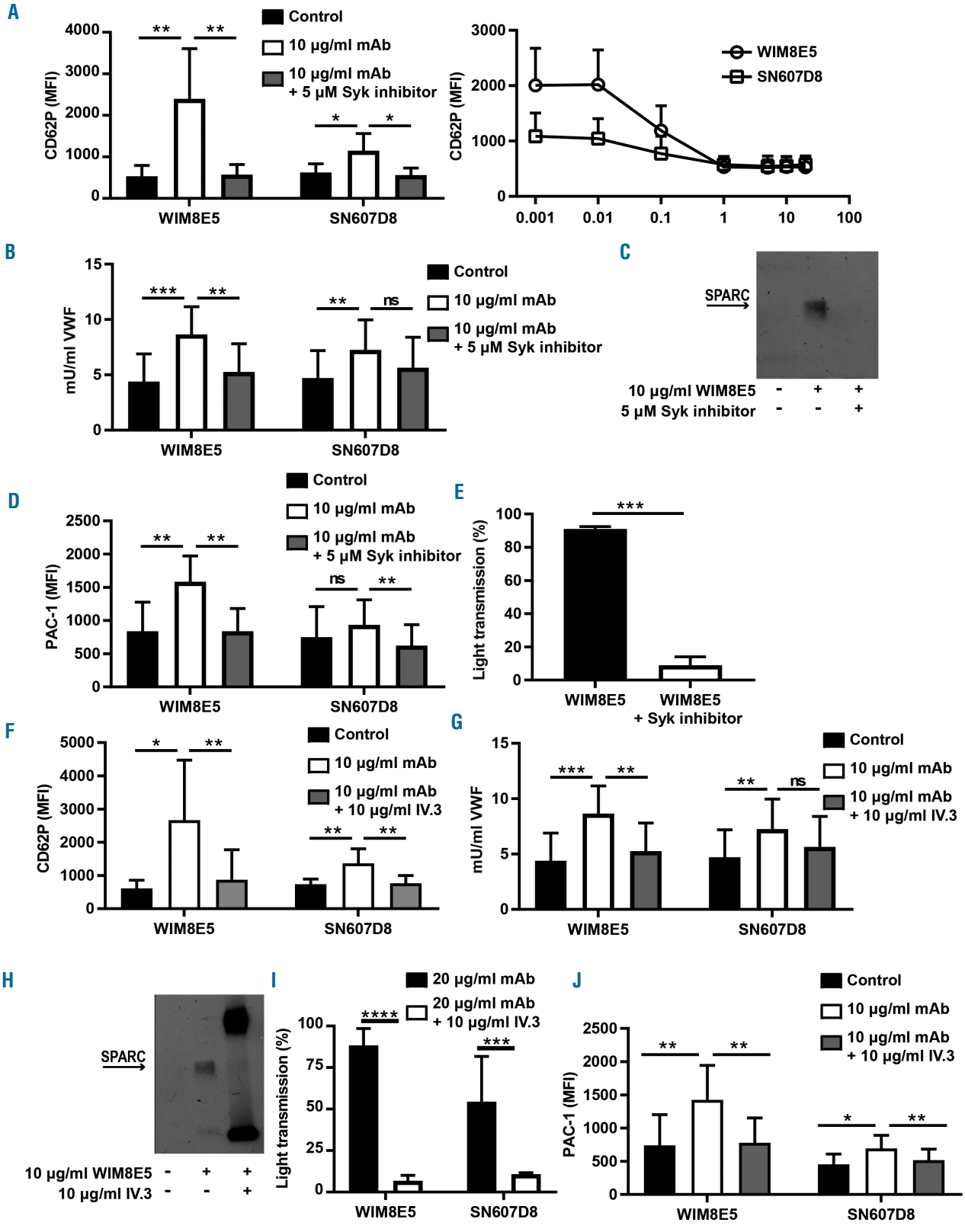

Figure 3. HLA monoclonal antibodies induce FcγRIIa-dependent platelet activation. (A) CD62P exposure upon incubation with WIM8E5 and SN607D8, inhibited by pre-incubation with Syk inhibitor IV compared to control (buffer only, no HLA antibodies). (B) VWF release, measured by enzyme-linked immunosorbent assay, induced by WIM8E5 and SN607D8, inhibited by Syk inhibitor IV. (C) SPARC release in platelet supernatant induced by WIM8E5 and SN607D8, inhibited by Syk inhibitor IV. (D) Integrin $\alpha_{IIb}\beta_3$ activation (as measured by PAC-1 binding) induced by WIM8E5 and SN607D8, inhibited by Syk inhibitor IV. (E) Agglutination induced by WIM8E5, inhibited by Syk inhibitor IV. (F) CD62P exposure induced by WIM8E5 and SN607D8, blocked by the FcγRIIa blocking antibody IV.3. (G) VWF release induced by WIM8E5 and SN607D8, inhibited by IV.3 (H) Release of SPARC in platelet supernatant inhibited by IV.3. (I) Agglutination induced by WIM8E5 and SN607D8 inhibited by pre-incubation with IV.3. (J) PAC-1 binding induced upon incubation with WIM8E5 and SN607D8 inhibited by IV.3. Data are given as mean ± SD. Paired ANOVA with the Tukey multiple comparison test. *P<0.05, **P<0.01, ***P<0.005, ****P<0.001.

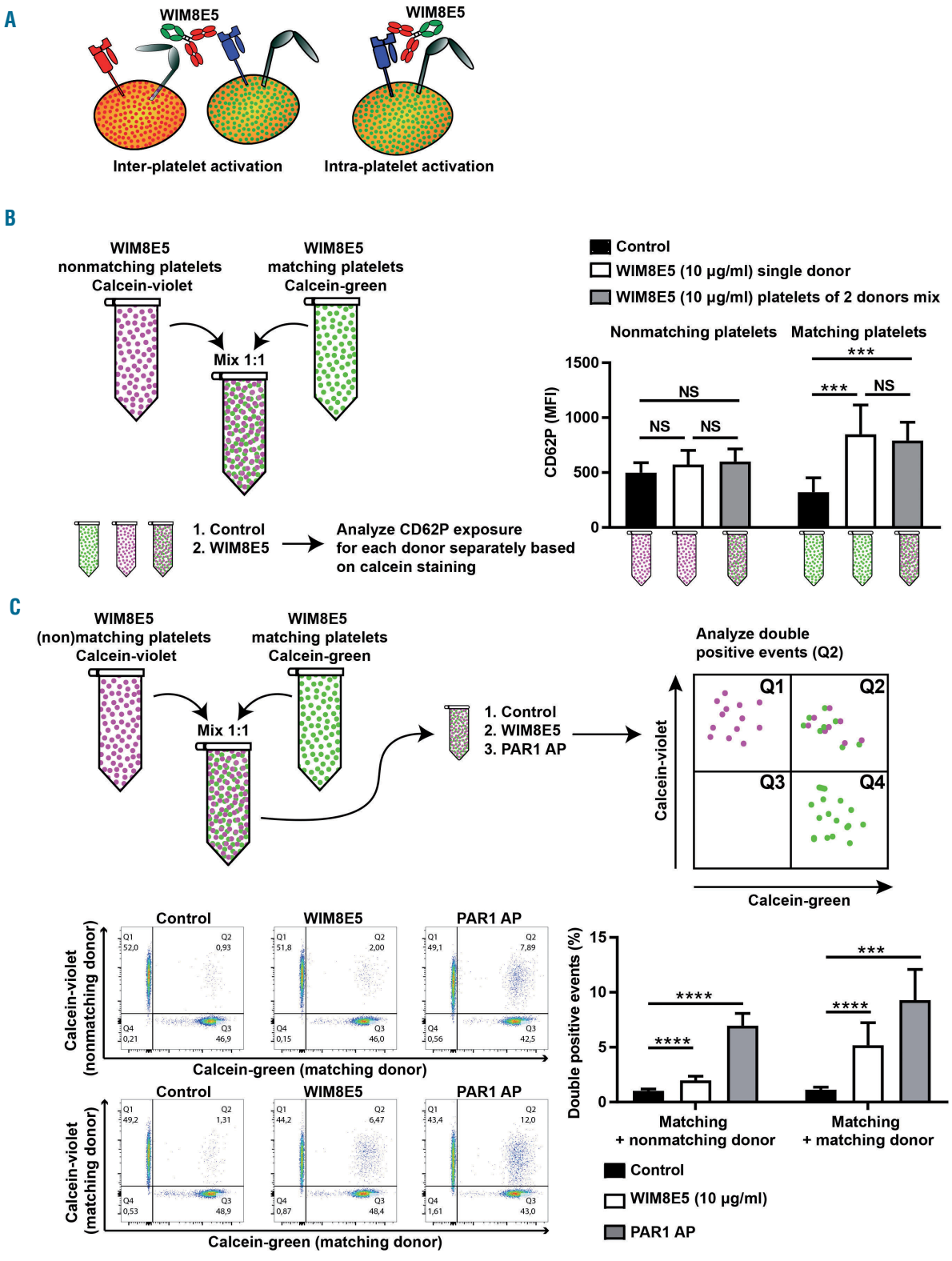

Figure 4. HLA monoclonal antibodies activate platelets in an intra-platelet-dependent manner. (A) Schematic representation of theoretically possible inter-platelet activation and intra-platelet activation induced by the HLA monoclonal antibody WIM8E5. (B) Platelet donors were selected as either "WIM8E5 matching" or "WIM8E5 nonmatching" and their platelets were stained with calcein-green or calcein-violet, respectively. Platelets from two donors were mixed in a 1:1 ratio. Platelets from a single donor or the mixed platelets were incubated with control (buffer only, no HLA antibodies) or WIM8E5. By gating for calcein-green or calcein-violet, CD62P exposure was determined for "WIM8E5 matching" and "WIM8E5 nonmatching" platelets. (C) Platelets from a "WIM8E5 matching" donor (stained with calcein green) were mixed 1:1 with platelets from a donor with either nonmatching WIM8E5 or matching WIM8E5 (stained with calcein violet). Samples were incubated with control (buffer only, no HLA antibodies), WIM8E5 or PAR1 activating peptide (PAR1 AP). Percentage double-positive events of calcein-green/calcein-violet-stained platelets are given, representing the ability of HLA antibodies to crosslink the HLA molecule with the FcγRIIa in an inter- or intra-platelet manner. Representative flow cytometry plots are shown for a mix of WIM8E5 matching + WIM8E5 nonmatching platelets and platelets from two different donors with both matching HLA typing. Paired ANOVA with the Tukey multiple comparison test. *P<0.05, **P<0.01, ***P<0.005, ****P<0.001.

(Figure 4B). To further study interactions between platelets from "matching" and "nonmatching" donors, platelets from the "matching" donor were stained with calcein-green and the platelets of the "nonmatching" donor with calcein-violet (or *vice versa)*, and double-positive events were measured. Upon incubation with WIM8E5, double-positive events, although significant, barely increased from 1 to 2%, while activation induced by PAR1 activating peptide (used as the control activating agonist) led to 8% positive events (Figure 4C). When platelets from two "matching" donors were combined, which were both activated upon incubation with WIM8E5, double-positive events increased from 1 to 6.5% (Figure 4C). As no enhanced CD62P exposure was observed on WIM8E5 "nonmatching" platelets and WIM8E5 had a very minor effect on the percentage of double-positive events when "matching" and "nonmatching" platelets were mixed, these results suggest that activation of platelets is dependent on the ability of HLA antibodies to interact in *cis* (intra-platelet) with FcγRIIa.

HLA antibodies from patients' sera can activate platelets

To establish whether the findings observed for human HLA monoclonal antibodies were physiologically relevant, we tested whether sera from patients, refractory to

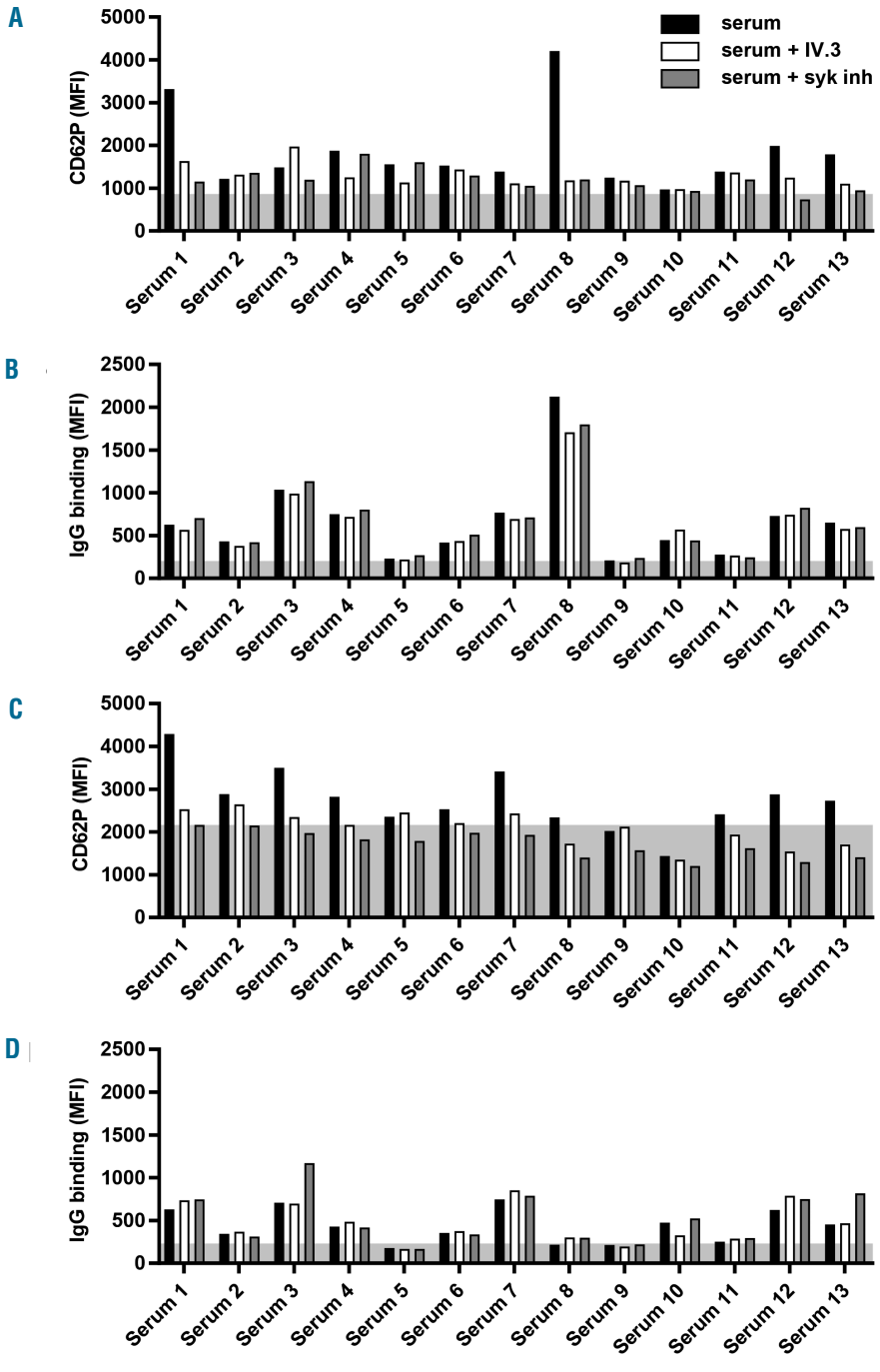

Figure 5. Patients' sera with HLA antibodies induce FcγRIIa-dependent activation of platelets from a subset of donors. Thirteen sera containing HLA alloantibodies were incubated with platelets from two different donors. AB serum tested negative for HLA and other specific platelet antibodies was used as a control. Background CD62P and IgG binding are indicated by the gray background. Activation was inhibited by pre-incubation with 10 μg/mL IV.3 or 5 μM Syk inhibitor IV. (A) CD62P exposure on platelets from donor 1 (expressing HLA A1 A2 B51). (B) IgG binding to platelets from donor 1. (C) CD62P exposure on platelets from donor 2 (expressing HLA A1 A2 B35 B62). (D) IgG binding to platelets from donor 2. Antibody specificities of the sera are reported in the *Online Supplementary Data*.

platelet transfusions and containing HLA antibodies, were capable of activating platelets (*Online Supplementary Table S1*). Thirteen sera were tested with platelets from two donors. In each serum HLA antibodies matching the HLA type of the donor platelets were present. Addition of control serum lacking HLA antibodies induced low levels of activation in both experiments (Figure 5A,C). Four of the 13 sera induced CD62P exposure on platelets from donor 1. Activation of platelets could be inhibited by IV.3 and Syk inhibitor to background levels as observed for platelets incubated with control serum (Figure 5A,B). When tested with platelets from donor 2, pronounced activation was observed for sera 1, 3, 7, 12 and 13 (Figure 5C,D). Sera 1, 12 and 13 were capable of activating platelets of both donor 1 and 2; serum 8 exclusively activated platelets of donor 1 and sera 3 and 7 only activated platelets from donor 2. Levels of IgG binding were relatively higher for sera which induced enhanced CD62P exposure, although some sera induced significant activation despite relatively low levels of IgG binding. Together, these results suggest that HLA antibodies in sera from refractory patients can induce FcγRIIa-dependent platelet activation.

HLA monoclonal antibodies induce phagocytosis by macrophages

To study the effect of FcγRIIa-dependent platelet activation on platelet clearance, monocyte-derived macrophages were incubated with platelets opsonized with either WIM8E5, SN607D8, SN230G6 or anti-HPA-1a antibody (as a positive control) and phagocytosis was studied employing imaging flow cytometry (Figure 6A-C). Opsonization by WIM8E5 significantly enhanced phagocytosis of platelets as shown by the increase in PKH-labeled platelets that were internalized by macrophages (Figure 6B). Incubation with Syk inhibitor IV significantly reduced phagocytosis of platelets opsonized by WIM8E5 (Figure 6C). Quantitative assessment of the effect of activating (WIM8E5 and SN607D8) and non-activating (SN230G6) revealed that enhanced phagocytosis of

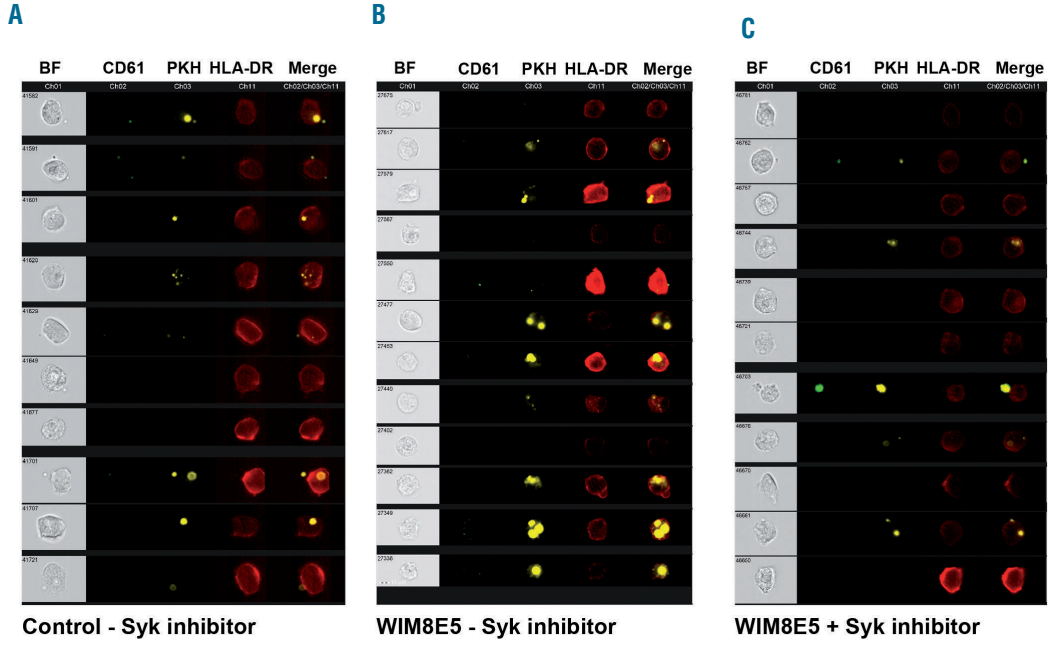

Figure 6. Phagocytosis of platelets opsonized by HLA monoclonal antibodies and the effect of FcγRIIa-dependent signaling. Platelets were incubated with 10 μg/mL WIM8E5, SN607D8 or SN230G6 in the presence or absence of 5 μM Syk inhibitor IV. Opsonized platelets were incubated for 1 h with monocyte-derived macrophages and internalization was analyzed through the use of imaging flow cytometry. (A-C) Representative images of imaging flow cytometry. BF: bright field; CD61: extracellular platelet staining; PKH: platelet staining; HLA-DR: macrophage staining. (A) Control without Syk inhibitor, (B) WIM8E5 without Syk inhibitor, (C) WIM8E5 with Syk inhibitor. (D) Intracellular platelet (PKH) fluorescence quantifies the amount of platelets taken up by macrophages. Data are given as mean ± SD, *P<0.05, **P<0.01. Control: buffer only, no HLA antibodies added. MF: macrophage.

WIM8E5 and SN607D8 was significantly decreased in the presence of Syk inhibitor IV (Figure 6D). The results obtained were derived from three independent experiments employing platelets from different donors (*Online Supplementary Figure S7*). Together, these results show that FcγRIIa-dependent activation of a subset of HLA antibodies promotes uptake of platelets by macrophages.

Discussion

The presence of HLA antibodies in patients receiving platelet transfusions is in some cases associated with rapid platelet clearance, which may be accompanied by transfusion reactions, including chills and fever.[2,7] Here, we studied whether HLA antibodies can activate platelets. We showed that a subset of human HLA monoclonal antibodies induced platelet α-granule release, integrin $\alpha_{IIb}\beta_3$ activation and platelet aggregation. All these effects could be fully inhibited by blocking FcγRIIa-dependent signaling in platelets. This indicates that activation by HLA antibodies is induced upon crosslinking of HLA molecules with the platelet Fc receptor FcγRIIa. Employing sera from HLA-antibody positive and refractory patients we confirmed that FcγRIIa-dependent platelet activation can be induced on donor platelets. We also showed that FcγRIIa-dependent activation enhances phagocytosis of platelets by macrophages.

Although all the monoclonal antibodies tested in this study bound efficiently to HLA-matched platelets, only three of them significantly induced CD62P surface exposure. It has been reported that high levels of HLA antibodies in patients correlate with a higher risk of refractoriness,[10] however, we did not observe a correlation between the level of IgG bound to platelets and the ability of HLA monoclonal antibodies or patients' sera to induce platelet activation. No correlation between platelet activation and binding of HLA monoclonal antibodies to specific HLA alleles was found. Antibodies WIM8E5, GV5D1 and GV2D5 all bind to HLA-A1, but only WIM8E5 and GV5D1 induce significantly increased CD62P membrane exposure on platelets. Similarly, whereas SN607D8 and SN230G6 both bind to HLA-A2, only SN607D8 induces platelet activation. Recently the affinities of SN607D8 and SN230G6 for HLA-A2 were reported: SN607D8 has a K_D of $1.2x10^{-8}$ M and SN230G6 has a K_D of $5.9x10^{-10}$ M.[33] It is likely that the affinity of HLA antibodies affects their ability to activate platelets to some extent. However, as the non-activating antibody SN230G6 has a significantly higher affinity than that of the activating antibody SN607D8, the propensity of HLA monoclonal antibodies to induce FcγRIIa-dependent platelet activation is not exclusively dependent on their affinity. For SN607D8 and SN230G6, residues critical for their binding to HLA have been determined (149A and 152V[25], and 62GE[34] respectively). These residues are located on opposite sides of the peptide-binding groove. We, therefore, hypothesize that the location of the binding site on HLA determines whether the Fc tail of an HLA antibody can bind and crosslink with FcγRIIa, causing activation through Syk.

We showed that FcγRIIa-dependent signaling enhanced platelet phagocytosis by macrophages. The degree of Syk-dependent phagocytosis correlated with the ability of the HLA monoclonal antibodies to activate and induce phosphatidylserine exposure on the platelets (*Online Supplementary Figure S7*). Similarly, differences in platelet clearance via activating and non-activating antibodies have been described in human FcγRIIa transgenic mice, in which an anti-CD9 antibody inducing FcγRIIa-platelet activation led to more rapid development of thrombocytopenia compared to a non-activating platelet specific antibody.[35] Besides, shock and thrombosis were observed only in the presence of platelet-activating antibodies and not with non-activating antibodies.[35] The activating potential of HLA antibodies has not been correlated with a risk of adverse effects, possibly because HLA-incompatible platelets are usually given to thrombocytopenic patients with low platelet counts, reducing the risk of thrombosis.

Previous studies on the effect of HLA antibodies on platelets have suggested that they are either inert[36] or induce platelet activation through complement activation.[20] Our experiments were performed with washed platelets in the absence of complement, so we cannot rule out that complement activation plays a role as well. Rubinstein *et al.* showed that crosslinking of a murine pan-anti-HLA antibody induced platelet activation.[19] However, the effect of the anti-HLA mouse monoclonal antibody that they used was only observed by artificial crosslinking of the HLA antibodies with goat anti-mouse IgG. Employing a mouse model, Waterman *et al.* showed that Fcγ receptors were critical for mice to develop platelet refractoriness induced by major histocompatibility complex alloantibodies.[37] Previous studies have shown that both murine and human HLA antibodies can induce release of Weibel-Palade bodies and subsequent CD62P exposure on endothelial cells, inducing enhanced monocyte adherence to endothelial cells via both CD62P binding and crosslinking of endothelial HLA with monocyte Fc receptors.[38] It has been described previously that platelets can be activated by monoclonal antibodies directed to β2-microglobulin in an FcγRIIa-dependent manner.[32] Similar to our observations, Rubinstein *et al.* reported differences in the platelet-activating abilities of several monoclonal antibodies directed towards different epitopes on β2-microglobulin.[32] However, activation by anti-β2-microglobulin monoclonal antibodies was proposed to occur in an inter-platelet fashion, while our data provide evidence for intra-platelet activation by HLA monoclonal antibodies. The mechanism of platelet activation by HLA monoclonal antibodies is similar to that of the platelet activation described in heparin-induced thrombocytopenia, in which antibodies directed to the platelet factor 4/ heparin complexes develop and induce FcγRIIa-dependent platelet activation.[17] Intravenous immunoglobulin has been shown to have a beneficial effect in patients with heparin-induced thrombocytopenia;[39,40] here we have shown that intravenous immunoglobulin inhibits HLA monoclonal antibody-induced FcγRIIa-dependent platelet activation *in vitro*. Based on our observations it may be worth exploring whether intravenous immunoglobulin can be used to reduce the rapid clearance of transfused platelets in a subset of refractory patients with anti-platelet antibodies.

Li *et al.* described a mechanism in which platelets are activated by GPIbα antibodies in an FcγRIIa-independent way.[16] Recently, Quach *et al.* showed that this activation was dependent on mechanomolecular signaling.[41] Similar to our data, Li *et al.* and Quach *et al.* documented enhanced CD62P exposure on platelets. In addition, their data suggested that activation of platelets coincides with

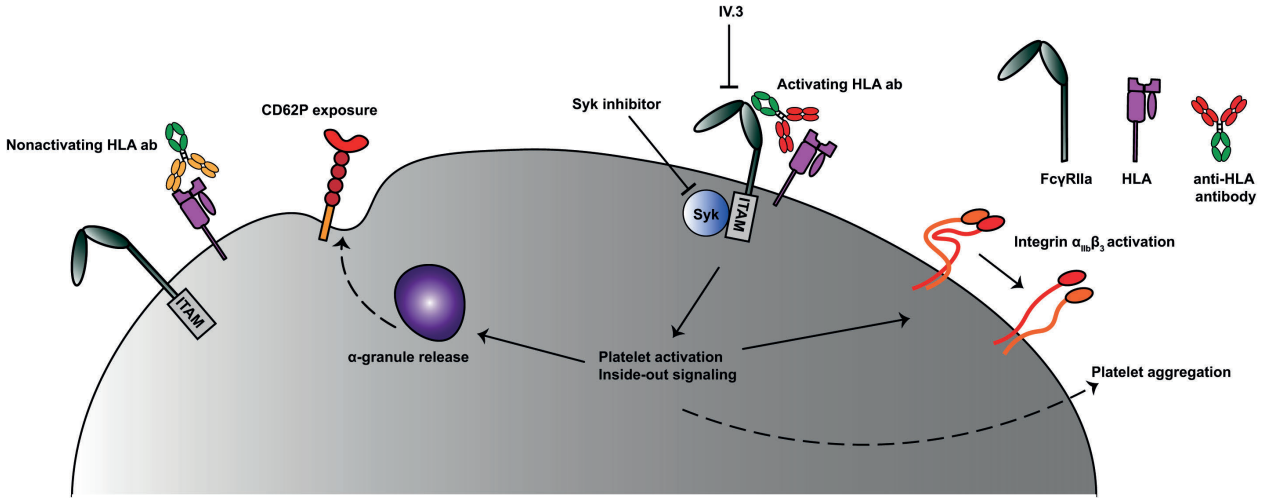

Figure 7. Proposed mechanism of platelet activation. When an activating HLA antibody (ab) binds to HLA on the platelet surface it crosslinks with FcγRIIa. This induces FcγRIIa-dependent signaling leading to the activation of Syk via the ITAM motif on FcγRIIa. Downstream signaling leads to platelet activation: α-granules are released (followed by e.g. CD62P exposure), integrin $\alpha_{IIb}\beta_3$ is activated and platelets start to aggregate. This activation pathway can be inhibited by IV.3 (which blocks crosslinking with FcγRIIa) or Syk inhibitor IV (which blocks signaling via Syk). HLA antibodies that bind to an epitope on HLA preventing interaction with FcγRIIa do not induce platelet activation. FcγRIIa-dependent signaling also leads to phosphatidylserine exposure and induces enhanced phagocytosis by macrophages.

the release of neuraminidase which cleaves sialic acid from platelet surface receptors thereby enhancing clearance of platelets through the Ashwell Morell receptors expressed by hepatocytes.[42] We show that HLA antibodies activate platelets in an FcγRIIa-dependent manner. Whether this also results in release of sialidase and subsequent clearance of platelets in patients with HLA antibodies has not been studied. However, our macrophage internalization experiments show that platelet activation induced by HLA alloantibodies may have an impact on platelet survival.

Incubation of healthy donor platelets with patients' sera containing HLA antibodies revealed that FcγRIIa-dependent platelet activation could be induced by polyclonal HLA antibodies from approximately one third of the tested sera from refractory patients. Some sera, such as sera 7 and 8, only induced activation of platelets in one of the two donors. This suggests that only a subset of antibodies present in the polyclonal sera is responsible for FcγRIIa-dependent platelet activation. Apparently, these antibodies only matched HLA antigens present on one of the two donors. HLA antibodies present in sera which did not induce FcγRIIa-dependent activation with the donor platelets tested might potentially have a different effect when tested with platelets derived from a larger panel of donors. As observed for the panel of monoclonal HLA antibodies, the results obtained for the patients' sera indicate that only a subset of HLA antibodies is capable of inducing FcγRIIa-dependent platelet activation.

Prediction models based on the three-dimensional structure of HLA epitopes (eplets), rather than the HLA antigens itself, have been developed as a basis for matched platelet transfusions.[34,43] If the eplets targeted by platelet-activating HLA antibodies were known, it is possible that incorporation of this knowledge in the selection of HLA-matching donor platelets could help to further reduce side effects of platelet transfusions in refractory patients.

In conclusion, we have shown that platelets are activated in an FcγRIIa-dependent manner by a subset of HLA antibodies (Figure 7). This mechanism may contribute to the enhanced clearance of platelets in refractory patients, as suggested by the increased phagocytosis of platelets opsonized by a subset of activating HLA antibodies. This suggests that testing the capacity of patients' sera to induce platelet activation could be used to further stratify patients with HLA antibodies who need platelet transfusions.

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