Anti-HLA class I IgG subclasses skew platelet activation mechanisms in transfusion refractoriness

Adèle Couvidou,[1*] Mathieu Wald,[2*] Catherine Angénieux,[1] Juliana Pires-Marafon,[1] Marie-Joëlle Apithy,[3] Catherine Humbrecht,[3] Luc-Matthieu Fornecker,[2] Nicolas Congy-Jolivet,[4] Arnaud Dupuis,[1] Jérôme Rollin[5,6] and Blandine Maître[1]

[1]Université de Strasbourg, INSERM, Etablissement Français du Sang (EFS) Grand Est, BPPS UMR_S 1255, Fédération de Médecine Translationnelle de Strasbourg (FMTS), Strasbourg; [2]Institut de Cancérologie de Strasbourg (ICANS), Service d'Hématologie, Strasbourg; [3]Etablissement Français du Sang (EFS) Grand Est, Strasbourg; [4]U1297 INSERM I2MC Team «Cell signaling in Response to Arterial Stress: Impact on Cardiovascular Diseases», Université Toulouse 3, Laboratoire d'Histocompatibilité, CHU de Toulouse, Toulouse; [5]Université de Tours, Institut National de la Santé et de la Recherche Médicale (INSERM) UMR 1327 ISCHEMIA, Tours and [6]Centre Hospitalier Régional Universitaire de Tours, Service d'Hématologie-Hémostase, Tours, France

**AC and MW contributed equally as first authors.*

Correspondence: B. Maître
blandine.maitre@efs.sante.fr

Received: March 5, 2025.
Accepted: September 8, 2025.
Early view: September 18, 2025.

https://doi.org/10.3324/haematol.2025.287677

Abstract

Patients requiring recurrent platelet transfusions are subject to platelet transfusion refractoriness (PTR), a therapeutic failure due to rapid clearance of transfused platelets from the circulation. One major cause of PTR is the presence in the recipient of multiple immunoglobulin (Ig)G directed against allogeneic HLA class-I (HLA-I) molecules expressed by the donor platelets. Strikingly, the presence of anti-HLA-I IgG does not necessarily correlate with PTR, thus questioning the role of the antibody properties themselves. Using blood of HLA-I allo-immunized patients with or without PTR, we identified the subclasses of their anti-HLA-I IgG. We found the distribution of these subclasses to be different in patients, with IgG1 being predominant in non-PTR patients while IgG1 in combination with IgG2 or IgG3 were detected in PTR patients. To understand the mechanisms associated with PTR, we used human chimeric pan-HLA-I IgG1, IgG2, or IgG3 antibodies and assessed the functional implications of these human IgG subclasses on platelet activation. We showed that each subclass led to platelet aggregation, P-selectin exposure and Annexin V binding. However, we found that the mechanisms of platelet activation differed between subclasses. Specifically, we discovered that pan-HLA-I hIgG2-induced platelet activation was CD32a dependent, while hIgG1- and hIgG3-induced platelet activation relied on complement recruitment. Hence, this study may have direct implications for hierarchizing pathogenic anti-HLA-I alloantibodies in highly poly-immunized patients and be a valuable aid in selecting suitable treatments, particularly by streamlining the search for functionally compatible platelet components (*clinicaltrials gov. Identifier: NCT05399693*).

Introduction

Onco-hematological disorders are often associated with treatment-induced or disease-related severe thrombocytopenia. As a result, these patients require repeated platelet transfusion support to stop or prevent bleeding. Despite continuous improvements in transfusion practices, platelet transfusion refractoriness (PTR), characterized by a therapeutic failure that results in peripheral platelet clearance, remains a serious complication in polytransfused patients. PTR is associated with an elevated risk of patient mortality, and has a significant impact on treatment costs, due to prolonged hospital stays and higher consumption of labile blood units.[1,2] PTR may occur due to non-immune factors, such as inflammatory conditions, drug interference, or disseminated intravascular coagulation. However, approximately 20% of platelet rejections are attributed to the existence of immunoglobulin (Ig)G directed against HLA class I (HLA-I) molecules, which classifies them as immune PTR.[3] Immune PTR can potentially be circumvented by using HLA-I-compatible platelets, i.e., perfectly HLA-I-matched platelets between donor and recipient, platelets carrying only antigens to which the recipient has not developed antibodies (permissive antigens) or cross-matched platelets.

However, finding a suitable donor is extremely challenging, especially for highly immunized patients.[4,5]

Interestingly, not all allo-immunized patients develop PTR following platelet transfusion, raising the question which factors are relevant for the pathogenicity of HLA-I-reactive antibodies. The TRAP (Trial to Reduce Alloimmunization to Platelets) study showed a correlation between high levels of circulating alloantibodies and the occurrence of PTR.[6] Moreover, the density of HLA-I antigens expressed on donor platelets should also be taken into account, as a study showed that platelets from donors with consistently low HLA-I antigen expression exhibited significantly reduced antibody-mediated internalization by macrophages *in vitro*.[7] In addition to these quantitative factors, the intrinsic properties of the antibodies have been correlated with their effect on platelets. Several studies have suggested that platelet activation by anti-HLA-I antibodies leads to their rapid clearance from the bloodstream in allo-immunized patients, with the type of antibody determining how this happens. Rijkers *et al*. reported that anti-HLA-I antibodies can activate platelets either through CD32a mobilization or through plasmatic complement recruitment.[8,9]

Since IgG subclasses have different functional properties, it is an important parameter to examine when studying the pathogenicity of antibodies. IgG are classified into four subclasses (IgG1, IgG2, IgG3, and IgG4) based on the amino acid sequence of their heavy chains, particularly in the hinge and CH2 domains. The subclass influences the ability of IgG to recruit complement and their affinity for the different Fc receptors (FcR).[10] Distinct IgG subclasses targeting HLA-I molecules may therefore potentially affect platelets in varying ways. In addition, a study conducted on baboons suggested that the IgG subclasses may influence the site of platelet sequestration during refractoriness: IgG1 led to platelet sequestration in the spleen while IgG2 resulted in platelets being eliminated in the liver.[11]

Full understanding of how anti-HLA-I antibodies affects platelets is crucial for developing strategies to safely transfuse platelets despite the presence of allo-antibodies in the recipient. We found that serum from poly-immunized patients against HLA-I molecules induced platelet activation *in vitro*. As several observations suggest that anti-HLA-I antibody subclasses may affect platelet elimination, we investigated in a prospective pilot clinical study the anti-HLA-I IgG profiles found in transfused patients. Interestingly, immunized patients with inadequate transfusion yield exhibited anti-HLA-I IgG1, IgG2 and IgG3, whereas IgG1 was the only subclass detected in efficiently transfused patients. Using chimeric pan-HLA-I antibodies of identical specificity, we showed that hIgG-mediated platelet activation relied on different mechanisms depending on the IgG subclass. hIgG2 led to platelet activation by mobilizing CD32a, while hIgG1/hIgG3 activated platelets by recruiting the complement.

Methods

Reagents

The murine IgG2a pan anti-human HLA-I (clone W6/32) and the isotype control were respectively purchased from InVivoMab and BioXcell. Human recombinant chimeric pan-HLA-I IgG1, IgG2, IgG3-W6/32 were generated by Proteogenix. Human recombinant chimeric pan-HLA-I IgG4-W6/32 was kindly provided by N. Congy-Jolivet. Briefly, the variable regions of the heavy and light chains of the mouse pan-HLA-I mAb W6/32 were fused to the Fc part of the human IgG1, IgG2, hIgG3 or IgG4.[12] The bacterial protease IdeS (IgG-degrading enzyme derived from *Streptococcus pyogenes*) that cleaves the hinge region of IgG was purchased from Genovis.

Ethics statement

The human studies were performed according to the Helsinki Declaration. Human whole blood was collected from volunteer blood donors who gave written informed consent and were recruited by the blood transfusion center where the research was carried out (Établissement Français du Sang-Grand Est). The clinical study was approved by the French Ethics Committee of "Sud Méditerranée IV" (ref: 21.03384.000027, number: ID-RCB: 2021-A01780-41).

Clinical study design

Blood samples were taken before and after each platelet transfusion. One transfusion per individual patient in the group of patients with antibodies and without non-immune factors was considered in this analysis and each transfusion was given to a different patient. Patients underwent sampling before the first transfusion of their aplastic episode to check for the presence of anti-HLA-I antibodies, using Luminex® technology. Platelet counts were examined 24 hours post transfusion to determine the corrected count increment (CCI).[3] The transfusion yield was considered as poor when the CCI was <7. Although PTR is typically diagnosed after two consecutive transfusion failures, for a more precise analysis of each event, any transfusion with a CCI of less than 7 was reported as insufficient CCI and considered to represent PTR in this study. Non-immune factors explaining potential platelet consumption (inflammation, bleedings, drugs interference etc.) and platelet concentrate characteristics (platelet quantity, storage duration, ABO phenotype) were collected online by completing a secured structured database (Table 1; Table 2).

Platelet activation measurement

Washed platelets (WP) or hirudinated platelet-rich plasma (PRP) from healthy voluntary donors was incubated for 10 minutes (min) at 37°C with serum from poly-immunized patient or commercial anti-HLA-I antibodies diluted in Tyrode-albumin (TA) buffer with hirudine 10 U/mL. For some tests, washed platelets were pre-incubated with serum or

Table 1. Baseline characteristics of the transfused patients. Demographic data of the patients are summarized.

Demographic data	
Age, years, median (IQR)	59.5 (46,9-64,8)
Sex: M/F, N	31/19
Length of hospital stay, days, median (IQR)	28 (22-34)
Reason for hospitalization, N (%) Acute leukemia HSCT	 27 (53) 24 (47)

IQR: interquartile range; M: male; F: female; HSCT: hematopoietic stem cell transplantation.

with serum heated at 56°C for 30 min (de-complemented serum). Incubated platelets were labeled for 20 min at 37°C using APC anti-CD62P (2 μg/mL), FITC-coupled Annexin V (AnnV) (1:20). The samples were then diluted in TA buffer with hirudine 10 U/mL and analyzed using flow cytometry. In some experiments, before incubation of PRP with chimeric antibodies, platelets were treated either with eculizumab (100 μg/mL) or with IV.3 (10 μg/mL) during 5 min at room temperature. Collagen-related peptide (CRP) at 5 μg/mL was used as a positive control.

Statistical analysis

Statistical analyses were performed using the Prism 5.02 software (GraphPad). All data are reported as the mean ± standard error of the mean and were analyzed using Mann-Whitney test, a difference with a *P* value ≤0.05 was considered as statistically significant.

Results

Serum from HLA-I poly-immunized patients activate platelets differently *in vitro*

In addition to their role as opsonins, some anti-platelet antibodies can induce platelet activation and consequently may facilitate platelet clearance during PTR.[8] We first evaluated whether serum from HLA-I immunized patients can activate platelets. PRP from healthy donors with a given HLA haplotype were incubated with the serum from HLA-I-matched immunized patients (S^+). Sera without antibodies were used as negative control (S^-). The platelet activation status was determined by P-selectin exposure (Figure 1A) and AnnV binding (Figure 1B). We observed that all sera from HLA-I-immunized patients activated platelets *in vitro*, albeit with different levels of activation. In order to understand the cause of these differences, we characterized the antibodies directed against the epitopes expressed by the donor, in terms of IgG quantity (mean fluorescence intensity [MFI]) and subclasses. Each serum contained different IgG subclasses, directed against distinct epitopes, which may explain the variation in activation levels (Figure 1C). Serum

Table 2. Transfusion and clinical data of the patients. The transfusion, clinical parameters and the antibiotics received by the patients are summarized.

Transfusional data	
N of blood products units transfused, median (IQR)	28 (7-54)
N of platelet concentrates units transfused, median (IQR)	10 (2-20)
ABO group, N A O B AB Rh(D)	 29 31 0 3 56
ABO compatible platelets, N	51
Platelet concentrates, N Buffy-coat pooled platelets Apheresis platelets Apheresis and buffy-coat pooled platelets	 51 10 2
Age of platelet concentrates, N ≤3 days ≥3 days	 5 58
Clinical and biological data	
Temperature >38°C, N (%)	21 (33)
Splenomegaly, N (%)	4 (36.5)
Bleeding complications, N (%)	23 (6.35)
CRP, mg/mL, median (IQR) <20 mg/mL, N (%) >20 mg/mL, N (%)	25(11-77) 29 (46) 34 (54)
Prothrombin time,%*, median (IQR)	88 (76-100)
D-dimers*, μmol/L, median (IQR)	975 (507-1,748)
Fibrinogen*, g/L, median (IQR)	4.2 (3.1-5.5)
Hemoglobin (H24-H0), g/dL (IQR)	-0.3 (-0.7 to - 0.3)
Leukocytes, $x10^9$/L, median (IQR)	0.2 (0-0.5)
Sepsis, N (%)	23 (36)
DIVC, N (%)	2 (3,2)
Platelet count H0, $x10^9$/L, median (IQR)	11 (8-19)
Antibiotics	
N of patients treated with antibiotics (%)	41 (65)
N of antibiotics 1 2 3	 13 23 5
Types of medications, N Amoxicillin/clavulanic acid Cephalosporins 3rd generation Ceftazidime Piperacillin/tazobactam Meropeneme Vancomycin Fluoroquinolones Amikacin Amphotericin B Posaconazole Sulfamethoxazole-trimethoprim (high dose)	 1 1 3 22 4 19 5 0 2 15 2

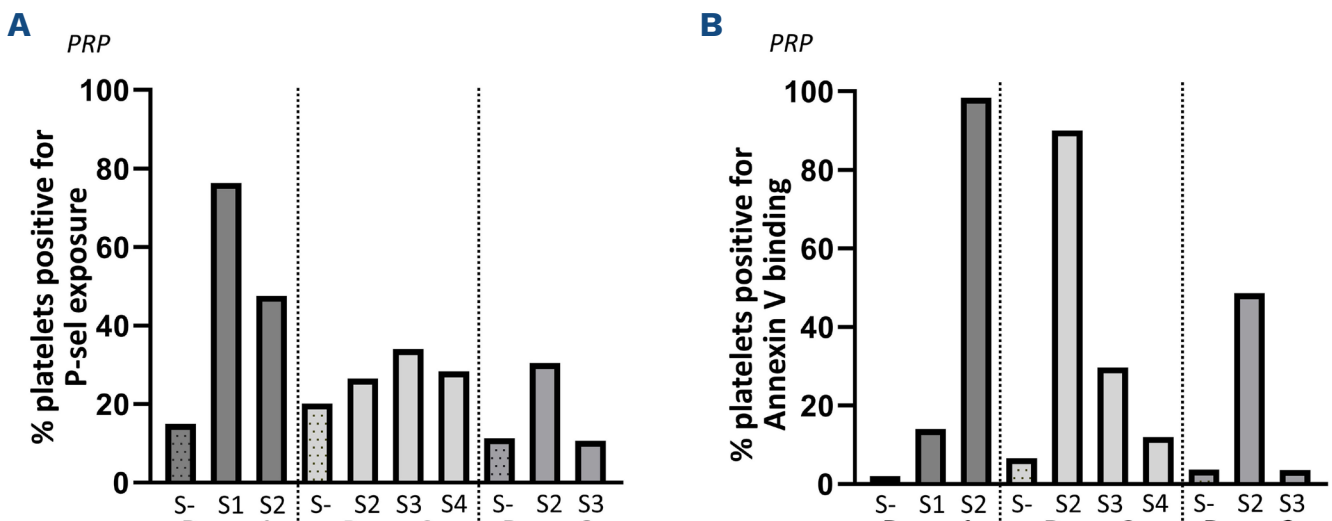
Platelet count H0: platelet count before platelet transfusion. The parameter 'hemoglobin H24-H0' refers to the difference between hemoglobin levels measured 24 hours after transfusion (H24) and those measured just before transfusion (H0). CRP: C-reactive protein; DIVC: disseminated intravascular coagulation; *Missing values. IQR: interquartile range.

2 highly activated platelets from donor 1 (48% P-sel$^+$ and 98% Ann V$^+$) but induced a moderate activation on platelets from donor 2 and 3 (26% Psel$^+$ and 90% AnnV$^+$ for donor 2; 30% Psel$^+$ and 49% AnnV$^+$ for donor 3) although the MFI of the respective allo-antibodies was similar. These observations suggest that the epitope recognized and/or the IgG subclasses involved induced different level of activation.

The distribution of IgG subclasses differs between refractory and non-refractory patients

To examine the IgG subclasses parameter in the context of platelet transfusion refractoriness we conducted a prospective pilot clinical study. We analyzed the composition of anti-HLA-I antibodies in the serum of patients requiring platelet transfusions in a context of myelosuppressive chemotherapy. We did not include transfusions that did not respect the ABO compatibility, and looked at the 51 transfusions where the blood types matched. We used the CCI value to categorize transfusions in insufficient (refractory) (CCI <7) or sufficient (non-refractory) (CCI ≥7) CCI value (Figure 2A).

Interestingly, the proportion of patients with anti-HLA-I antibodies detected was similar between the group with insufficient CCI value (56.4% of transfusions), and the group with sufficient CCI value (58.3% of transfusions) (Figure 2A). Non-immune factors, i.e., inflammation, bleedings, splenomegaly, drug-interference, can interfere with the transfusion yield. To avoid such a bias, we narrowed our analysis on patients presenting only antibodies without any other non-immune factors (N=5 patients in the group with insufficient CCI value and N=4 patients in the group with sufficient CCI value). Pre- and post-transfusion platelet



C

Haplotype donor 1	IgG	IgG1	IgG2	IgG3
A*02:01	-	-	-	-
A*24:02	**S2** 17848	14644	8042	82
B*44:02	**S1** 2465 **S2** 17459	117 9214	8 2650	- 402
B*44:05	-	-	-	-

Haplotype donor 2	IgG	IgG1	IgG2	IgG3
A*01	-	-	-	-
A*66	**S2** 19270 **S3** 21280 **S4** 20707	12341 12550 5590	5176 545 820	142 21 -
B*8	**S2** 3929 **S3** 24377 **S4** 10455	891 15331 2145	- 9081 7	- 142 -
B*41	**S2** 17002 **S3** 23633 **S4** 13097	8753 8603 2740	1769 5219 16	634 75 -

Haplotype donor 3	IgG	IgG1	IgG2	IgG3
A*11:01	**S3** 3270	118	1	-
A*29:02	**S2** 19794	10957	3605	88
B*44:03	**S2** 19774	13000	5609	884
B*51:01	**S3** 5150	306	3	14

Figure 1. HLA-I polyimmunized serum activated HLA-matched platelets *in vitro*. Hirudinated platelet-rich plasma (PRP) from 3 different donors with a known HLA haplotype were incubated with serum from HLA-I matched immunized patients (S1, S2, S3 or S4). Serum without antibodies was used as negative control (S$^-$). (A) Percent of platelets positive for P-selectin (P-sel) exposure (B) % of platelets positive for Annexin V binding. (C) Tables summarizing the HLA haplotype of each platelet donor with the corresponding MFI of each anti-HLA-I immunoglobulin (Ig)G subclasses found in the serum from the HLA-I matched immunized patients determined by Luminex®.

counts were collected, and the CCI value of each transfusion was calculated (*Online Supplementary Table S1*).

Each serum was analyzed by Luminex® technology to characterize the total IgG detected in each serum (*Online Supplementary Figure S1A*) or the different subclasses: IgG1, IgG2 or IgG3 (Figure 2B; *Online Supplementary Figure S1B*). This approach enables the quantity of each antibody to be determined, as well as its specificity (i.e., the HLA-I molecule recognized) and the type of IgG subclass. It is notable that in the group with a sufficient CCI value (F-I), IgG1 was identified in all cases without any other IgG subclasses. Conversely, in the group with insufficient CCI value (A-E), simultaneous presence of IgG1 and IgG2, or IgG1 and IgG3 was systematically detected (*Online Supplementary Figure 1B*). No IgG4 was detected in any groups (Figure 2B). Interestingly, the analysis of IgG subclasses in all the serum including the ones with associated non-immune factors revealed only IgG1 (N=6-7) in the group with a sufficient CCI value.

Altogether, these data indicate that the presence of distinct IgG subclasses could be associated with an insufficient transfusion yield.

Platelets can be activated by a pan HLA-I IgG in an Fc-dependent pathway

In order to break down the polyclonal immune response and fully investigate the effects of anti-HLA-I antibodies, we examined the impact of a murine pan anti-human HLA-I monoclonal antibody (mAb) (mW6/32) on platelet activation. mW6/32 mAb was incubated with hirudinated platelet-rich plasma (PRP) and platelet activation was assessed by flow cytometry and aggregation assay. CRP (5 μg/mL) was used as a positive control of platelet activation. At 20 μg/mL, mW6/32 induced α-granule secretion as measured by P-selectin (Psel) exposure (Figure 3A, left panel) and phosphatidylserine exposure as revealed by fluorescent AnnV binding (Figure 3A, right panel) as compared to the isotype control (mIgG2a). Furthermore, incubation of PRP with mW6/32 resulted in complete and irreversible platelet aggregation, consistent with the flow cytometry data (Figure 3B).

To assess the importance of the Fc part in mW6/32-mediated platelet activation, we generated F(ab')2 fragments from the trypsin digestion of mW6/32. Incubation of PRP with F(ab')2-W6/32 (from 10 μg/mL to 100 μg/mL) did neither result in platelet activation as shown by flow cytometry analysis (Figure 3C) nor in platelet aggregation (Figure 3D), indicating that mW632-mediated platelet activation is dependent on the Fc part.

Taken together, these results showed that platelet activation mediated by the murine W6/32 pan anti-human HLA-I monoclonal antibody is Fc-dependent.

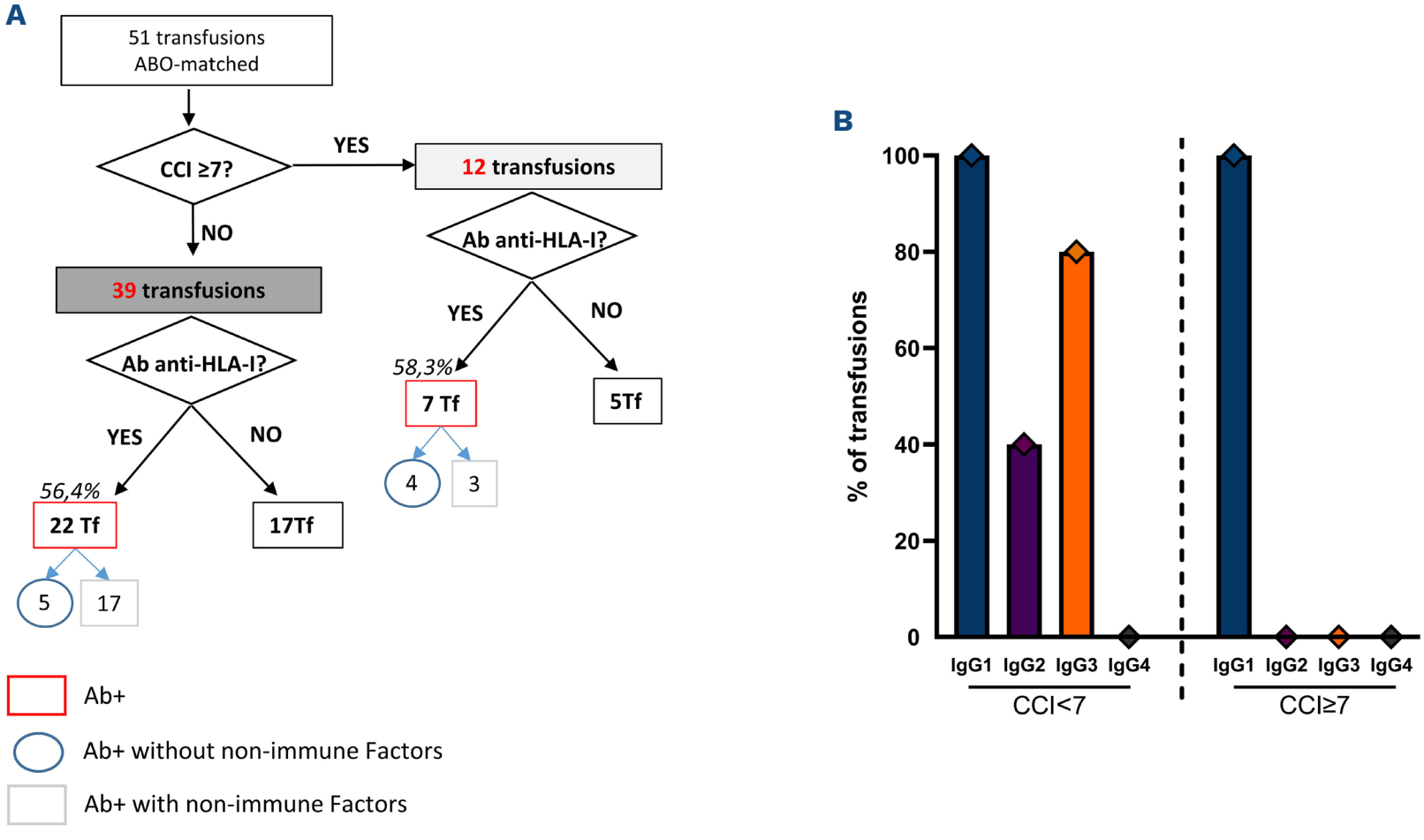


Figure 2. Anti-HLA-I IgG analysis in transfused patients enrolled in a clinical study. Flowchart of ABO-compatible transfusions (N=51) analyzed from 2022 to 2023. (A) In each category, the corrected count increment (CCI) values obtained after transfusion (Tf) were used to create 2 groups: CCI ≥7 (sufficient CCI value) and CCI <7 (insufficient CCI value). The transfusions in each group were analyzed for the presence of anti-HLA-I antibodies (Ab) (red square) and with regard to the possible association with (gray square) or without (blue circle) non-immune factors. (B) The proportion of anti-HLA-I immunoglobulin (Ig)G subclasses was analyzed for each transfusion and reported in the group with a CCI <7 or ≥7.

Anti-HLA-I IgG subclasses impact platelet activation

Given that serum with anti-HLA-I lead to a heterogeneous response, we wondered whether the type of IgG subclasses could influence platelet activation. To circumvent the different epitopes or quantity of antibodies, we used recombinant chimeric IgG consisting of the VH/VL part of the murine pan anti-human HLA-I (W6/32) antibody fused to the human Fc part of IgG1, IgG2 or IgG3. A dose-response for each recombinant chimeric antibody was performed on PRP. All subclasses induced platelet activation in a dose-dependent manner as evidenced by P-selectin exposure (Figure 4A, left panel) and AnnV binding (Figure 4A, right panel). In parallel, hIgG4-W632 was tested and showed no sign of platelet activation (*Online Supplementary Figure S2*). Interestingly, we observed different activation levels depending on the IgG subclasses. At the same concentration, hIgG2-W6/32 induced a higher P-selectin exposure than hIgG1- or hIgG3-W6/32 (Figure 4A, left panel), while hIgG1-W6/32 preferentially induced phosphatidylserine exposure as revealed by a higher AnnV binding (Figure 4A, right panel).

Consistently with the flow cytometry results, we observed that all subclasses (10 µg/mL) induced full aggregation of platelets (Figure 4B). However, the lag time between the addition of the agonist and the onset of aggregation was markedly longer for hIgG2 and hIgG3 than for hIgG1, suggesting that hIgG subclasses may lead to platelet activation through different mechanisms.

To evaluate the mechanisms of anti-HLA-I antibody-mediated activation, we incubated the different hIgG subclasses with IdeS a protease that specifically cleaves IgG below the hinge region.[13] The treatment fully abolished hIgG-1, -2 and -3-W6/32-mediated platelet activation. In this condition, the level of activation was similar to the one obtained with F(ab')2-W6/32 confirming that platelet activation mediated by anti-HLA-I antibodies was Fc-dependent (Figure 4C).

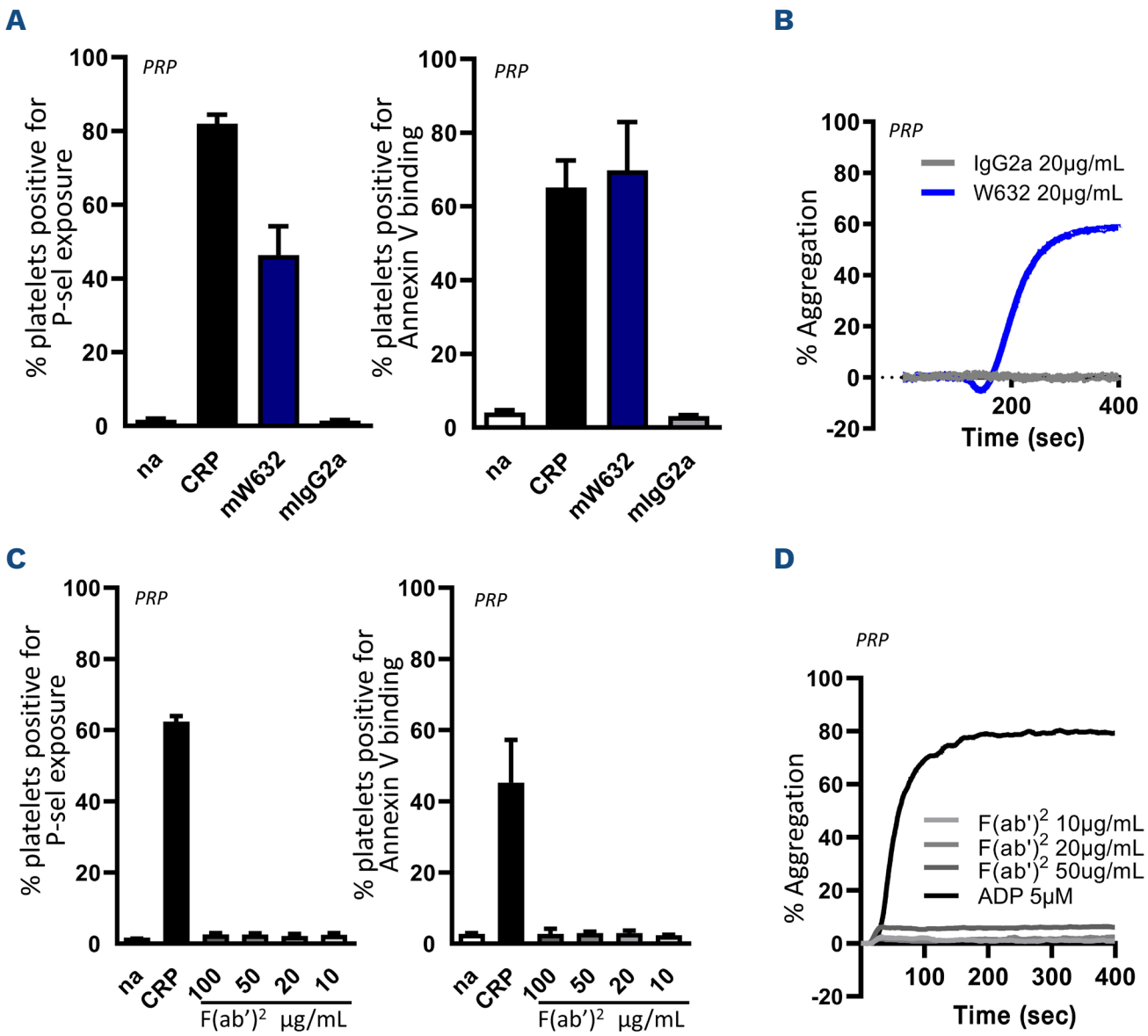


Figure 3. mW632-mediated platelet activation is Fc-dependent. Hirudinated platelet-rich plasma (PRP) was either not activated (na) or incubated with murine W6/32 (mW6/32; 20 µg/mL), the corresponding isotype (mIgG2a; 20 µg/mL) or collagen-related peptide (CRP) (5 µg/mL). After 30-minute incubation, platelet activation was analyzed by flow cytometry using activation markers such as (A) P-selectin exposure (P-sel) (left panel) and Annexin V binding (right panel). (B) The capacity of mW6/32 to induce platelet activation was also evaluated by aggregation test. Hirudinated PRP was incubated with either mW6/32 or mIgG2a. Representative trace of N=3 experiments is shown. (C) Hirudinated PRP was incubated with W6/32-F(ab')$_2$ at different concentrations. After 30-minute incubation, platelet activation was analyzed by flow cytometry using activation markers such as P-selectin exposure (P-sel) (left panel) and Annexin V binding (right panel). (D) The capacity of F(ab')$_2$ W6/32 to induce platelet activation was also evaluated by aggregation test. Adenosine di-phosphate (ADP 5 µM) was used as a positive control. sec: seconds.

hIgG1-/ hIgG3-W6/32 and hIgG2-W6/32 activate platelets through different mechanisms

The role of CD32a mobilization and the recruitment of plasma complement in the antibody-mediated activation of platelets is different depending on the type of antibody.[8,9] This raises the question of whether the difference in platelet activation between the anti-HLA-I hIgG subclasses may be attributed to varying activation mechanisms. To address

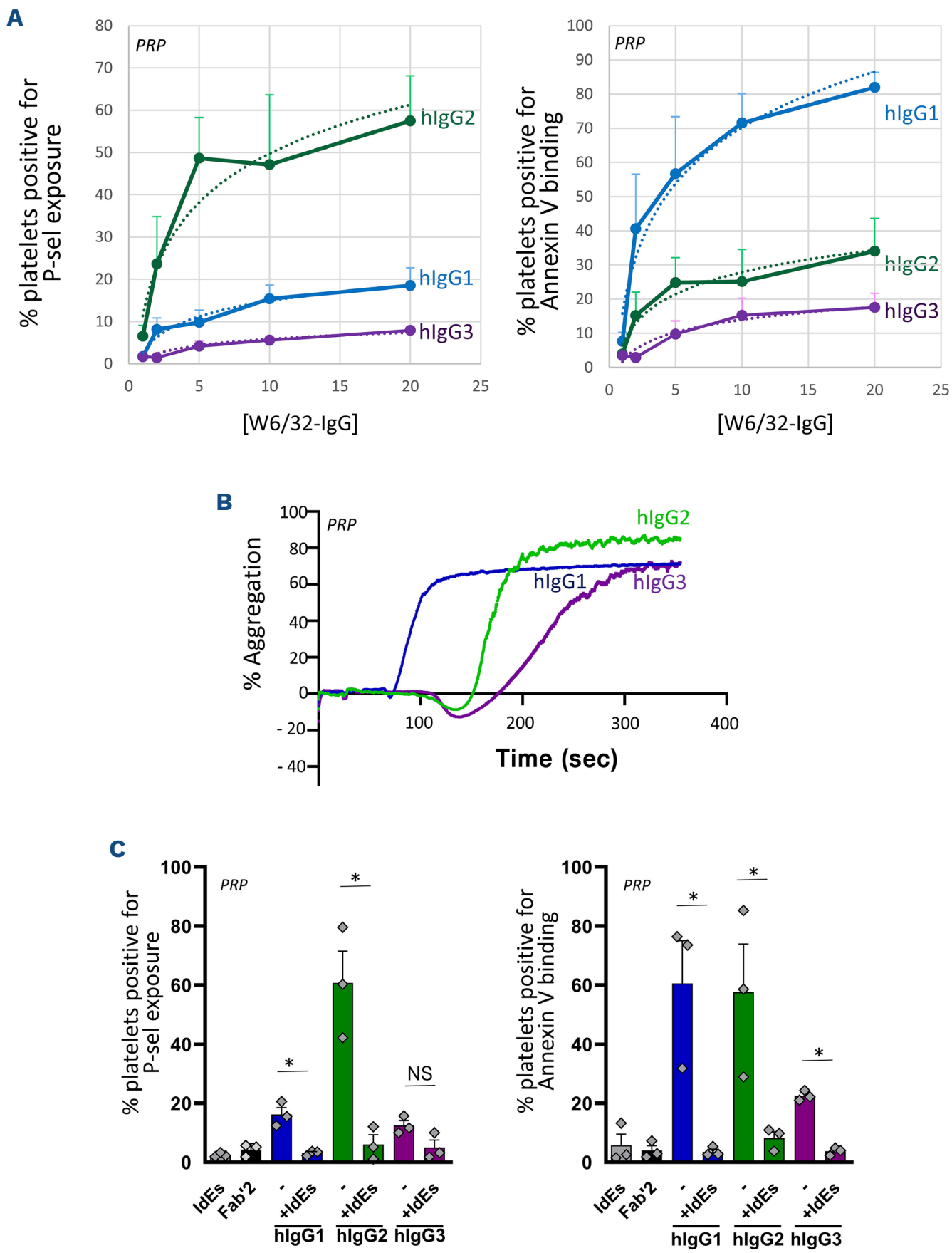


Figure 4. IgG subclasses impact platelet activation. (A) Dose response to different recombinant chimeric anti-HLA-I human immunogobulin (hIg)G subclasses (hIgG1, hIgG2 and hIgG3) for P-selectin (P-Sel) exposure (left panel) or Annexin V binding (right panel) was evaluated using flow cytometry. Exposure of platelet activation markers is indicated as percentage of positive platelets. The dots indicate the mean value (N=3) and standard error of the mean (SEM) and the dotted lines show the logarithmic trend lines. (B) The capacity of recombinant chimeric anti-HLA-I hIgG subclasses to induce platelet activation was also evaluated by aggregation test. Hirudinated platelet-rich plasma (PRP) was incubated with either anti-HLA-I hIgG1, hIgG2 or hIgG3 (10 μg/mL). Representative trace of N=3 experiments is shown. (C) Hirudinated PRP treated (+IdEs) or not (-) with IdEs was incubated with the different recombinant chimeric anti-HLA-I hIgG subclasses, or with F(ab')2 W6/32. P-selectin exposure (left panel) or Annexin V binding (right panel) was evaluated by flow cytometry. Results are presented as the mean ± SEM; one-tailed Mann-Whitney test; *$P \leq 0.05$; not significant (NS) $P > 0.05$. IdEs: IgG-degrading enzyme derived from *Streptococcus pyogenes*.

this, we assessed the importance of the two pathways for each subclass by inhibiting either the CD32a pathway or the complement pathway.

We first assessed the CD32a mobilization by using IV.3, a monoclonal antibody directed against CD32a. As a positive control of IV.3 efficacy, platelets were stimulated with anti-CD9 antibodies, reported to strongly activate platelets in a CD32a-dependent pathway. Anti-CD9 induced platelet activation was totally prevented in presence of IV.3 (*Online Supplementary Figure S3*). Interestingly, analysis of P-selectin exposure and AnnV binding by flow cytometry showed that hIgG1- or hIgG3-W6/32-mediated platelet activation was unaffected by the addition of IV.3, whereas hIgG2-W6/32-induced activation was completely prevented (Figure 5A). Similarly, platelet aggregation induced by hIgG2 was completely prevented by the addition of IV.3, whereas no effect or only a slight effect was observed with hIgG1 or hIgG3 respectively (*Online Supplementary Figure S4*).

We then assessed the role of complement by measuring the C3b recruitment at the platelet cell surface induced by different antibodies. Interestingly, hIgG1 and hIgG3 induced a recruitment of C3b as compared to hIgG2 (Figure 5B). We further dug into the role of the complement in platelet activation mediated by antibodies by adding eculizumab an antibody that targets the complement protein C5. Under these conditions, AnnV binding mediated by hIgG1 or hIgG3-W6/32 was significantly decreased by eculizumab (Figure 5C, right panel), while that mediated by hIgG2-W632 was not. However, the effect of eculizumab on P-selectin exposure induced by the different subclasses was less pronounced, with a significant effect on hIgG3 but not on hIgG1 and hIgG2 (Figure 5C, left panel). Eculizumab impacted significantly the aggregation induced by hIgG3. Regarding the hIgG1/hIgG2 induced aggregation, a variable effect of eculizumab was observed (*Online Supplementary Figure S4*).

To confirm the involvement of plasma components in the platelet activation mediated by hIgG1 and hIgG3 subclasses, we repeated the same experiments with washed platelets, i.e., platelets free of plasma components. As shown in Figure 6, only hIgG2-W6/32 but neither hIgG1- nor hIgG3-W6/32

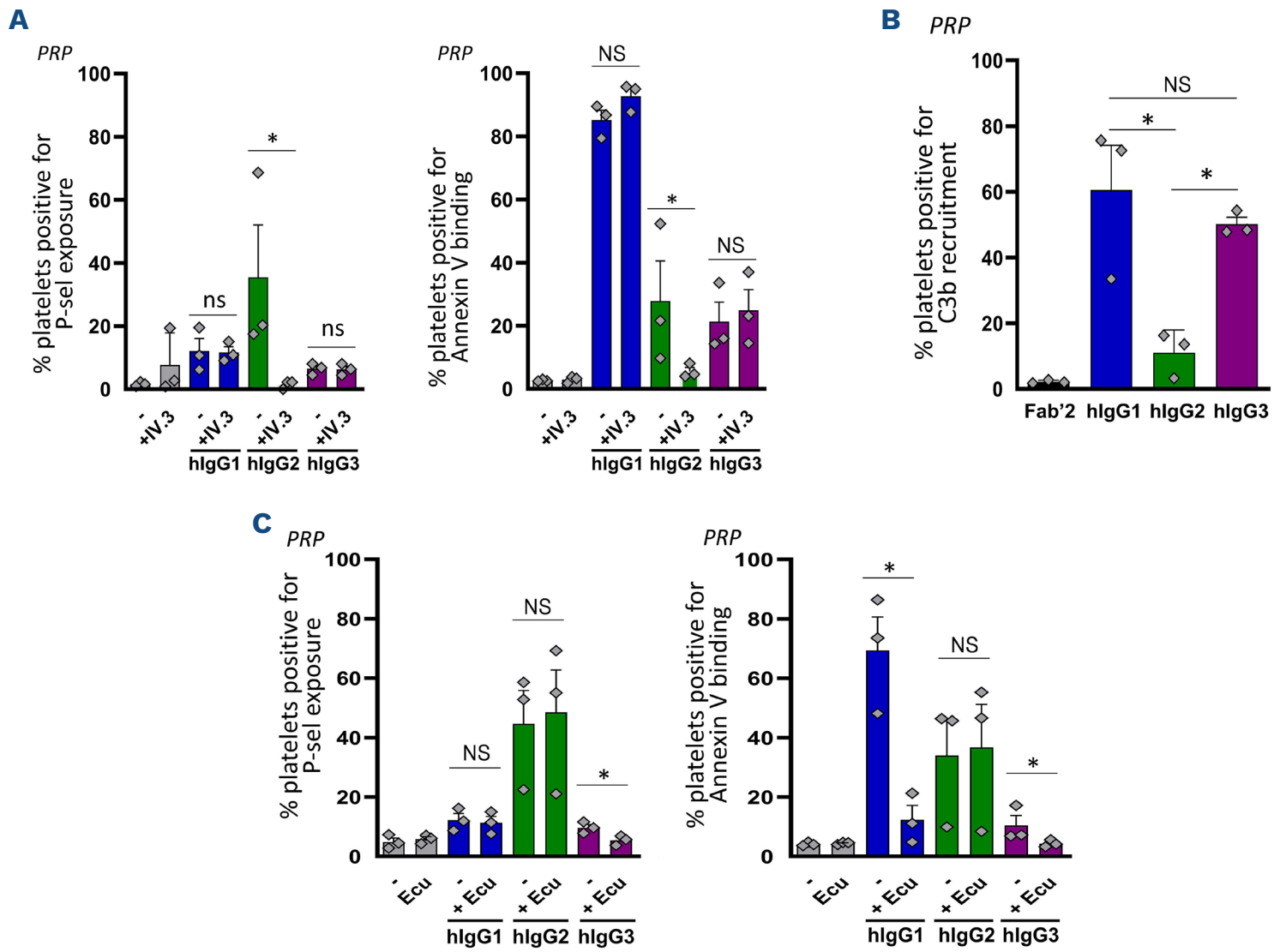


Figure 5. IgG subclasses mediate platelet activation by different mechanisms. (A) Hirudinated platelet-rich plasma (PRP) treated or not with IV.3 (10 μg/mL) was incubated with either anti-HLA-I human immunogobulin (hIg)G1, hIgG2 or hIgG3 at 10 μg/mL. After 10 minutes of incubation, platelet activation was analyzed by flow cytometry using activation markers such as P-selectin (P-Sel) exposure (left panel) or Annexin V binding (right panel). (B) C3b recruitment was measured by flow cytometry after incubation of hirudinated PRP with anti-HLA-I hIgG1, hIgG2 or hIgG3 at 10 μg/mL or corresponding Fab'2. (C) Hirudinated PRP treated or not with eculizumab (Ecu) (100μg/mL) was incubated with either anti-HLA-I hIgG1, hIgG2 or hIgG3 at 10 μg/mL. After 30 minutes of incubation, platelet activation was analyzed by flow cytometry using activation markers such as P-selectin exposure (left panel) or Annexin V binding (right panel). Results are presented as the mean ± standard error of the mean; one-tailed Mann-Whitney test; *$P \leq 0.05$; not significant (NS) $P > 0.05$.

induced P-selectin exposure (Figure 6A, left panel), AnnV binding (Figure 6A, right panel) and a complete aggregation of washed platelet (Figure 6B). Finally, TA buffer, serum (as a source of complement) or de-complemented serum were added to the washed platelets before incubation with the antibodies. We observed that hIgG1- and hIgG3- W6/32, but not hIgG2- W6/32, led to platelet activation only with intact serum whereas de-complementation prevented any sign of activation (Figure 6C).

Altogether, these results showed that anti-HLA-I hIgG subclasses mediated platelet activation by different mechanisms.

Discussion

Platelet transfusion refractoriness remains a major complication for patients with onco-hematological disorders. Currently, there is no satisfactory solution for managing the multiple transfusions required to treat these patients. Understanding the mechanisms leading to the elimination of transfused platelets from the bloodstream is the first essential step to design future therapeutic strategies. In the present work, we showed that serum from HLA-I poly-immunized patients were able to induce platelet activation and that the recognized epitope seems to modulate the level of activation. Using

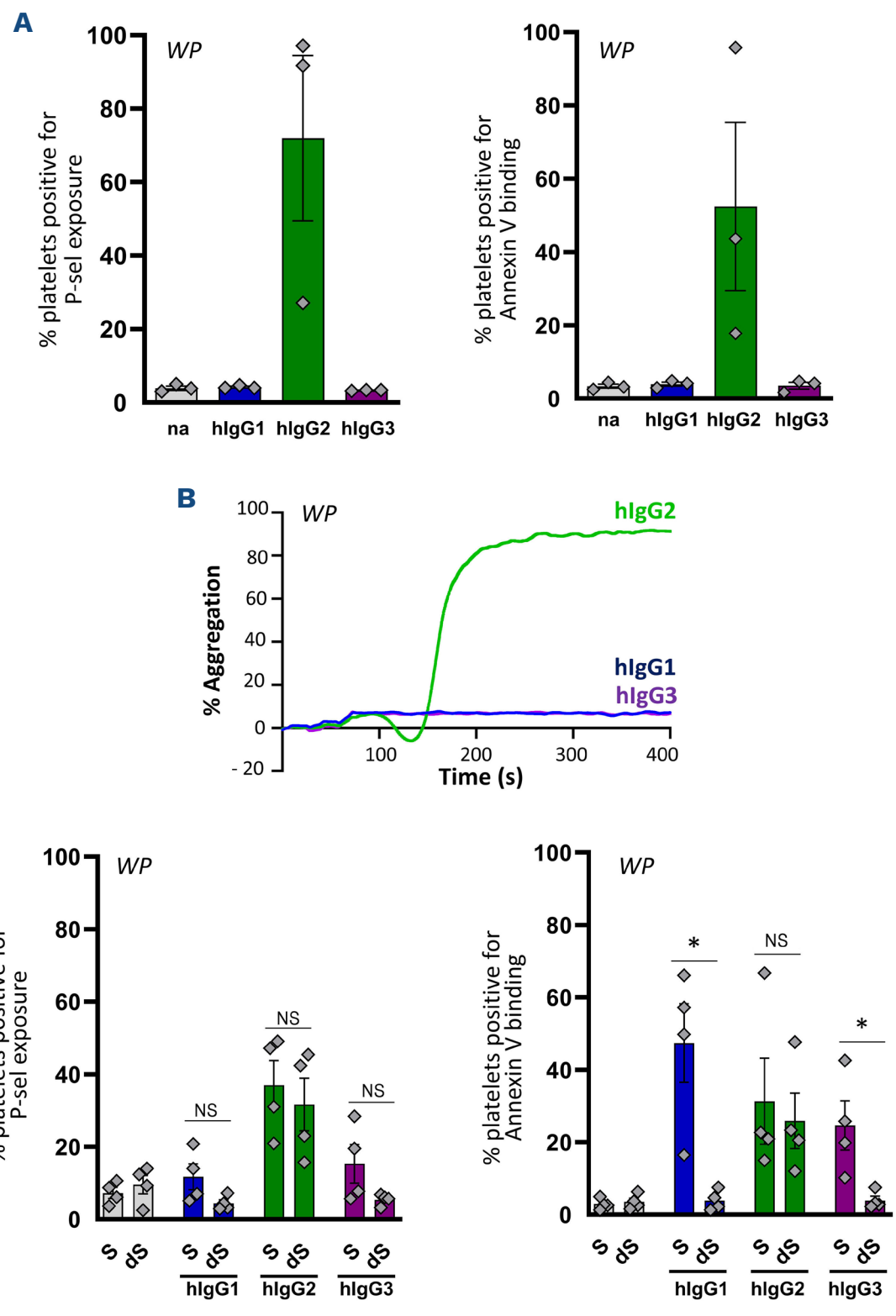


Figure 6. IgG2 antibodies induce platelet activation in washed platelets. Washed platelets were incubated with either anti-HLA-I human immunogobulin (hIg)G1, hIgG2 or hIgG3 at 10 μg/mL. (A) After incubation, platelet activation was analyzed by flow cytometry for P-selectin exposure (left panel) or Annexin V binding (right panel). (B) The capacity of the recombinant chimeric anti-HLA-I hIgG subclasses to induce platelet activation was also evaluated by aggregation test. (C) Serum de-complemented (dS) or not (S) was added to washed platelets before incubation with the anti-HLA-I hIgG subclasses. Results are presented as the mean ± standard error of the mean; one-tailed Mann-Whitney test; *$P \leq 0.05$; not significant (NS) $P > 0.05$.

anti-pan HLA-I antibodies, we demonstrated that the Fc region of the antibody was essential. Interestingly, different mechanisms of platelet activation were involved according to the IgG subclass, with CD32a-dependent activation for IgG2 and complement-dependent activation for both IgG1 and IgG3. Finally, we reported a difference in the distribution of anti-HLA-I IgG subclasses in patients who developed immune PTR and those who did not.

Although the number of multi-transfused patients analyzed deserves to be increased to reach more robust statistical significance, our results indicate that the distribution of anti-HLA-I-IgG subclasses differs between patients who received effective and ineffective transfusions. Increasing the number of patients should allow us to find a correlation between the IgG subclasses distribution and the probability of developing or not an immune PTR.

The distinct subclasses of anti-HLA-I IgG found in patients could differentially modulate platelet activation. Precisely, using chimeric antibodies recognizing the same HLA-I epitope, we found that each hIgG subclass activated platelets to different extents with distinct activation mechanisms. hIgG1- and hIgG3-induced activation depend on complement recruitment, whereas IgG2-activated platelets by mobilizing CD32a. It is well documented that allo-immunized patients have a polyclonal immune response toward various recognized epitopes, therefore these results need to be completed by chimeric antibodies directed against different epitopes to confirm the specificity of IgG2 action, as compared to IgG1 and IgG3. In a previous study, Rijkers *et al*. described that anti-HLA-I antibodies act synergistically to recruit plasmatic complement factors which lead to platelet activation.[9] Here we showed that complement-dependent platelet activation is possible with only one epitope recognized. Our results do not exclude the fact that a polyclonal response favors a synergistic effect on platelet activation, but they do highlight the importance of considering the IgG subclasses, since depending on their affinities, complement-mediated activation would be possible without any synergistic effect. At this stage, it is unlikely that anti-HLA-I IgG4 is important in PTR due to its unique structural properties, such as functional monovalence or bi-specific form, and the low abundance of this subclass in plasma.[14] Accordingly, no effect on platelet activation was observed after incubation with hIgG4-W6/32 (*Online Supplementary Figure S2*). The affinity of IgG1, 2 and 3 for the receptor CD32a could be significantly influence by its polymorphism. The CD32a receptor has two allotypic variants, Histidine (H) or arginine (R) at position 131, interfering with the affinity of IgG subclasses, in particular with IgG2.[15] It would be interesting to further characterize the importance of this polymorphism in the context of platelet transfusion refractoriness.

All these data emphasize the interest of knowing the characteristics of the antibodies found in multi-transfused patients sera. Determining the hierarchy of all antibodies' features which lead to a PTR would allow the transfusion impact of allo-antibodies to be ranked. Thus, it could dictate which allo-antibodies should be considered in the selection of platelet concentrates, and could become an essential resource in the design of future therapeutic strategies. Furthermore, a full understanding of the mechanisms of action of anti-HLA-I IgGs is essential to develop novel approaches to prevent or better manage PTR. We could consider targeting the complement pathway or Fc receptors, depending on the predominant subclass found. A pilot study has already been carried out to assess the efficacy of eculizumab in this context, in which four of the ten patients had an improvement in their transfusion yield following this treatment, suggesting that eculizumab may help some patients to overcome PTR.[13] According to our results, investigating the IgG subclasses found in these patients would help to explain the response, or lack of response to this treatment. On the other hand, targeting the FcR could be the appropriate strategy when IgG2 are the most abundant IgG found in the patients.

Finally, we showed that IdEs (Imlifidase) was efficient on all IgG subclasses. This innovative approach could be the universal solution to overcome immune refractoriness. However, the main problem to date has been the restriction to a single injection, given the risk of immunization against this protease. As idEs is derived from *Streptococcus pyogenes*, endogenous anti-IdeS antibodies are frequent and can be reinforced by the injection of imlidifase. This is why, after an initial injection, the development of these antibodies may limit the efficacy of imlifidase if a second injection is required.[16] A clinical trial with a larger cohort should provide definitive conclusions concerning these different approaches. However, it is important to consider the high cost of these treatments (imlifidase, eculizumab) today. They will not allow to treat all PTR, even if they prove to be effective, but only a few transfusion deadlocks.

In summary, this work identified IgG subclasses as a new factor to consider in the management of PTR patients. These observations may pave the way for new pharmacological tactics to alleviate PTR and may lead to innovation in the practice of personalized transfusion medicine.

Disclosure

No conflicts of interest to disclose.

Contributions

AC, MW and CA performed experiments, analyzed and interpreted the data. MW and L-MF contributed to patients' selection and data collection. M-JA, CH, NC-J, AD and JP-M interpreted the data. JR and BM designed the research and interpreted the data. BM supervised the study. All authors edited the manuscript and gave their final approval for submission.

Acknowledgments

We thank Nathalie Brouard from the CytoTriCS flow cytometry platform. We would also like to thank the staff of the

EFS Grand-Est, and particularly the HLA Department, for technical support throughout our studies.

Funding

This study was supported by EFS, INSERM, SFTS ("Société Francophone de la Transfusion Sanguine") and ART ("Association pour la Recherche en Transfusion"). MW was supported by ICANS.

Data-sharing statement

Original data and protocols are available upon request from the corresponding author.

References

1. Meehan KR, Matias CO, Rathore SS, et al. Platelet transfusions: utilization and associated costs in a tertiary care hospital. Am J Hematol. 2000;64(4):251-256.
2. Toor AA, Choo SY, Little JA. Bleeding risk and platelet transfusion refractoriness in patients with acute myelogenous leukemia who undergo autologous stem cell transplantation. Bone Marrow Transplant. 2000;26(3):315-320.
3. Couvidou A, Rojas-Jimenez G, Dupuis A, Maitre B. Anti-HLA class I alloantibodies in platelet transfusion refractoriness: from mechanisms and determinants to therapeutic prospects. Front Immunol. 2023;14:1125367.
4. Cohn CS. Platelet transfusion refractoriness: how do I diagnose and manage? Hematology Am Soc Hematol Educ Program. 2020;2020(1):527-532.
5. Elhence P, Chaudhary RK, Nityanand S. Cross-match-compatible platelets improve corrected count increments in patients who are refractory to randomly selected platelets. Blood Transfus. 2014;12(2):180-186.
6. Trial to Reduce Alloimmunization to Platelets Study G. Leukocyte reduction and ultraviolet B irradiation of platelets to prevent alloimmunization and refractoriness to platelet transfusions. N Engl J Med. 1997;337(26):1861-1869.
7. Saris A, Tomson B, Brand A, et al. Platelets from donors with consistently low HLA-B8, -B12, or -B35 expression do not undergo antibody-mediated internalization. Blood. 2018;131(1):144-152.
8. Rijkers M, Saris A, Heidt S, et al. A subset of anti-HLA antibodies induces FcgammaRIIa-dependent platelet activation. Haematologica. 2018;103(10):1741-1752.
9. Rijkers M, Schmidt D, Lu N, et al. Anti-HLA antibodies with complementary and synergistic interaction geometries promote classical complement activation on platelets. Haematologica. 2019;104(2):403-416.
10. Bruhns P, Jonsson F. Mouse and human FcR effector functions. Immunol Rev. 2015;268(1):25-51.
11. Coetzee LM, Pieters H, van Wyk V, et al. The effect of monoclonal anti-human-platelet antibodies on platelet kinetics in a baboon model: IgG subclass dependency. Thromb Haemost. 2000;83(1):148-156.
12. Congy-Jolivet N, Drocourt D, Portet S, Tiraby G, Blancher A. Production and characterization of chimeric anti-HLA monoclonal antibodies targeting public epitopes as tools for standardizations of the anti-HLA antibody detection. J Immunol Methods. 2013;390(1-2):41-51.
13. Jordan SC, Lorant T, Choi J, et al. IgG endopeptidase in highly sensitized patients undergoing transplantation. N Engl J Med. 2017;377(5):442-453.
14. Rispens T, Huijbers MG. The unique properties of IgG4 and its roles in health and disease. Nat Rev Immunol. 2023;23(11):763-778.
15. Shashidharamurthy R, Zhang F, Amano A, et al. Dynamics of the interaction of human IgG subtype immune complexes with cells expressing R and H allelic forms of a low-affinity Fc gamma receptor CD32A. J Immunol. 2009;183(12):8216-8224.
16. Huang E, Maldonado AQ, Kjellman C, Jordan SC. Imlifidase for the treatment of anti-HLA antibody-mediated processes in kidney transplantation. Am J Transplant. 2022;22(3):691-697.