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

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AUTHOR CONTRIBUTIONS

A.C, M.W., C.A performed experiments, analyzed and interpreted the data. M.W. and L.-M. F. contributed to patients' selection and data collection. M.-J. A, C.H., N. C.-J., A.D. and J. P.-M. interpreted the data. J.R and B.M. designed the research and interpreted the data. B.M. supervised the study. All authors edited the manuscript and gave their final approval for submission.

Original data and protocols are available upon request from the corresponding author, Blandine Maître (blandine.maitre@efs.sante.fr).

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Key points

. Platelet activation mediated by anti-HLA-I IgG depends on IgG subclasses

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 IgG 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 alloimmunized 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 according to 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 polyimmunized patients and be a valuable aid in selecting suitable treatments, particularly by streamlining the search for functionally compatible platelet components.

INTRODUCTION

Onco-haematological 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 IgG directed against HLA Class I (HLA-I) molecules, which classifies them as immune PTR³. 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 alloimmunized patients develop PTR following platelet transfusion, raising the question of 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 PTR⁶. 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 alloimmunized 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. IgGs 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 IgGs 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 liver¹¹.

Full understanding of how anti-HLA-I antibodies affects platelets is crucial for developing strategies to safely transfuse platelets despite the presence of alloantibodies 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 IgG4¹². 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, n° 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,...) and platelet concentrate characteristics (platelet quantity, storage duration, ABO phenotype) were collected online by completing a secured structured database (Table 2).

Platelet activation measurement

Washed platelets (WP) or hirudinated platelet-rich plasma (PRP) from healthy voluntary donors was incubated for 10 minutes at 37°C with serum from polyimmunized 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 with serum heated at 56°C for 30 minutes (decomplemented serum). Incubated platelets were labelled for 20 min at 37°C using APC anti-CD62P (2µg/mL), FITC-coupled Annexin V (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 (100µg/mL) or with IV.3 (10µg/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 \pm SEM 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 PTR⁸. 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 Annexin V 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 (MFI) and subclasses. Each serum contained different IgG subclasses, directed against distinct epitopes, which may explain the variation in activation levels (Figure 1C). Serum 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 alloantibodies 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) ($CCI \geq 7$) 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 counts were collected, and the CCI value of each transfusion was calculated (Supplemental Table 1).

Each serum was analyzed by Luminex[®] technology to characterize the total IgG detected in each serum (Supplemental Figure 1A) or the different subclasses: IgG1, IgG2 or IgG3 (Figure 2B and Supplemental Figure 1B). 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 to I),

IgG1 was identified in all cases without any other IgG subclasses. Conversely, in the group with insufficient CCI value (A to E), simultaneous presence of IgG1 and IgG2, or IgG1 and IgG3 was systematically detected (Supplemental 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 (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 μ g/mL) was used as a positive control of platelet activation. At 20 μ g/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 Annexin V (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 μ g/mL to 100 μ g/mL) did not 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.

Anti-HLA-I IgG subclasses impact platelet activation

Given that serum with anti-HLA-I lead to an 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 IgGs 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 (Supplemental Figure 2). Interestingly, we observed different activation level 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 Annexin V binding (Figure 4A, right panel).

Consistently with the flow cytometry results, we observed that all subclasses (10 µg/mL) induced a 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 (an IgG-degrading enzyme derived from *Streptococcus pyogenes*), a protease that specifically cleaves IgG below the hinge region¹³. 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).

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 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 (Supplemental Figure 3). Interestingly, analysis of P-selectin exposure and Annexin V 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 (Supplemental Figure 4).

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 dig 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, Annexin V 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 (Supplemental Figure 4).

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- or hIgG3-W6/32 induced P-selectin exposure (Figure 6A, left panel), Annexin V binding (Figure 6A, right panel) and a complete aggregation of washed platelet (Figure 6B). Finally, TA buffer, serum (as a source of complement) or decompemented 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 decompementation 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 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 reported that alloimmunized 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 activation⁹. 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 plasma¹⁴. Accordingly, no effect on platelet activation was observed after incubation with hIgG4-W6/32 (Supplemental Figure 2). 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 IgG2¹⁵. It would be interesting to further characterize the importance of this polymorphism in the context of platelet transfusion refractoriness.

All these data emphasize on 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 alloantibodies to be ranked. Thus, it could dictate which should be considered in the selection of platelet concentrates, and would 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 4 of the 10 patients had an improvement in their transfusion yield following this treatment, suggesting that Eculizumab may help some patients to overcome PTR¹³. 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 Imlifidase. This is why, after an initial injection, the development of these antibodies may limit the efficacy of Imlifidase if a second injection is required¹⁶.

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.

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Table 1. Baseline characteristics of the transfused patients

Demographic data of the patients are summarized in the table. Data are expressed as n (%) or median [IQR].

Demographic data	
Age (years)	59.5 [46,9-64,8]
Gender M/F	31/19
Length of hospital stay (days)	28 [22-34]
Reason for hospitalization	
Acute Leukemia	27 (53%)
HSCT	24 (47%)

Table 2. Transfusion and clinical data of the patients

The transfusion (left panel), clinical parameters and the antibiotics received by the patients (right panel) are summarized in the tables. Data are expressed as n (%) or median [IQR].

Transfusional data	
Number of blood products units transfused	28 [7-54]
Number of platelet concentrates units transfused	10 [2-20]
ABO group	
A	29
O	31
B	0
AB	3
Rh(D)	56
ABO compatible platelets	51
Platelet concentrates	
Buffy-coat pooled platelets	51
Apheresis platelets	10
Apheresis and buffy-coat Pooled platelets	2
Age of platelet concentrates	5
≤ 3 days	58
≥3 days	

Clinical and biological data	
Temperature > 38°C	21 (33%)
Splenomegaly	4 (6,35%)
Bleeding complications	23 (36,5%)
CRP (mg/mL)	25 [11-77]
< 20	29 (46%)
> 20	34 (54%)
Prothrombin time (%)*	88 [76-100]
D-dimers* (μmol/L)	975 [507-1748]
Fibrinogen* (g/L)	4.2 [3.1-5.5]
Hemoglobin (H24-H0) (g/dL)	-0.3 [-0.7 - 0.3]
Leukocytes (10 ⁹ /L)	0.2 [0-0.5]
Sepsis	23 (36%)
DIVC	2 (3,2%)
Platelet count H0 (10 ⁹ /L)	11 [8-19]
Antibiotics	
Number of patients treated with antibiotics	41 (65%)
Number of antibiotics	
1	13
2	23
3	5
Types of medication	
Amoxicillin/clavulanic acid	1
Cephalosporins 3rd generation	1
Ceftazidime	3
Piperacillin/Tazobactam	22
Meropenem	4
Vancomycin	19
Fluoroquinolones	5
Amikacin	0
Amphotericin B	2
Posaconazole	15
Sulfamethoxazole-trimethoprim (high dose)	2

Platelet count H0: Platelet count before platelet transfusion – The parameter 'haemoglobin H24-H0' refers to the difference between haemoglobin levels measured 24 hours after

transfusion (H24) and those measured just before transfusion (H0). CRP: C-Reactive protein
; DICC: Disseminated Intravascular Coagulation.

LEGENDS

Figure 1: HLA-I polyimmunized serum activated HLA-matched platelets *in vitro*

Hirudinated PRP from three 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) % 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 IgG subclasses found in the serum from the HLA-I matched immunized patients determined by Luminex®.

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 two groups: $CCI \geq 7$ (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 IgG subclasses was analyzed for each transfusion and reported in the group with a $CCI < 7$ or ≥ 7 .

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; 20µg/mL), the corresponding isotype (mIgG2a ; 20 µg/mL) or Collagen-related peptide (CRP) (5µg/mL). After 30min 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 min 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 5μM) was used as a positive control.

Figure 4: IgG subclasses impact platelet activation

(A) Dose response to different recombinant chimeric anti-HLA-I hIgG 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 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 (IgG-degrading enzyme derived from *Streptococcus pyogenes*) 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≤0.05, ns (non significant) p >0.05.

Figure 5: IgG subclasses mediate platelet activation by different mechanisms

(A) Hirudinated platelet-rich plasma (PRP) treated or not with IV.3 (10μg/mL) was incubated with either anti-HLA-I hIgG1, hIgG2 or hIgG3 at 10 μg/mL. After 10 min of incubation,

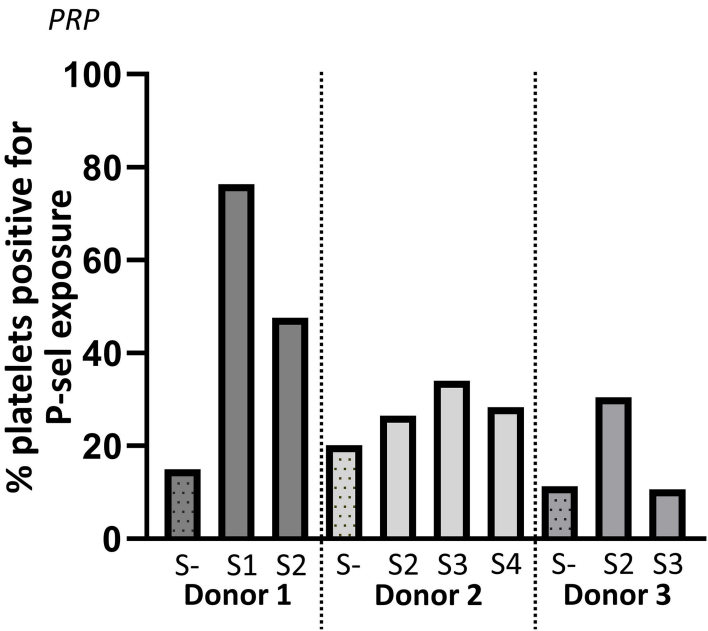
platelet activation was analyzed by flow cytometry using activation markers such as P-selectin (P-Sel) exposure (left panel) or Annexin V binding (right panel). **(B)** C3b recruitment was measured by flow cytometry after incubation of hirudinated PRP with anti-HLA-I hIgG1, hIgG2 or hIgG3 at 10 µg/mL 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 30min 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 \pm SEM; One-tailed Mann-Whitney test; * $p \leq 0.05$, ns (non significant) $p > 0.05$.

Figure 6: IgG2 antibodies induce platelet activation in washed platelets

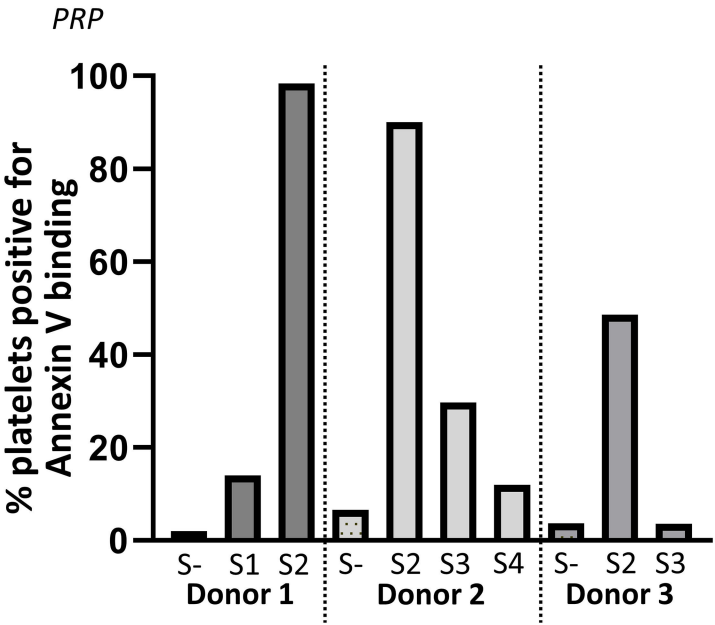
Washed platelets were incubated with either anti-HLA-I hIgG1, hIgG2 or hIgG3 at 10 µg/mL. **(A)** After incubation, platelet activation was analyzed by flow cytometry for P-selectin exposure (left panel) or Annexin V binding (right panel). **(B)** The capacity of the recombinant chimeric anti-HLA-I hIgG subclasses to induce platelet activation was also evaluated by aggregation test. **(C)** Serum decomplemented (dS) or not (S) was added to washed platelets before incubation with the anti-HLA-I hIgG subclasses. Results are presented as the mean \pm SEM; One-tailed Mann-Whitney test; * $p \leq 0.05$, ns (non significant) $p > 0.05$.

Figure 1

A



B



C

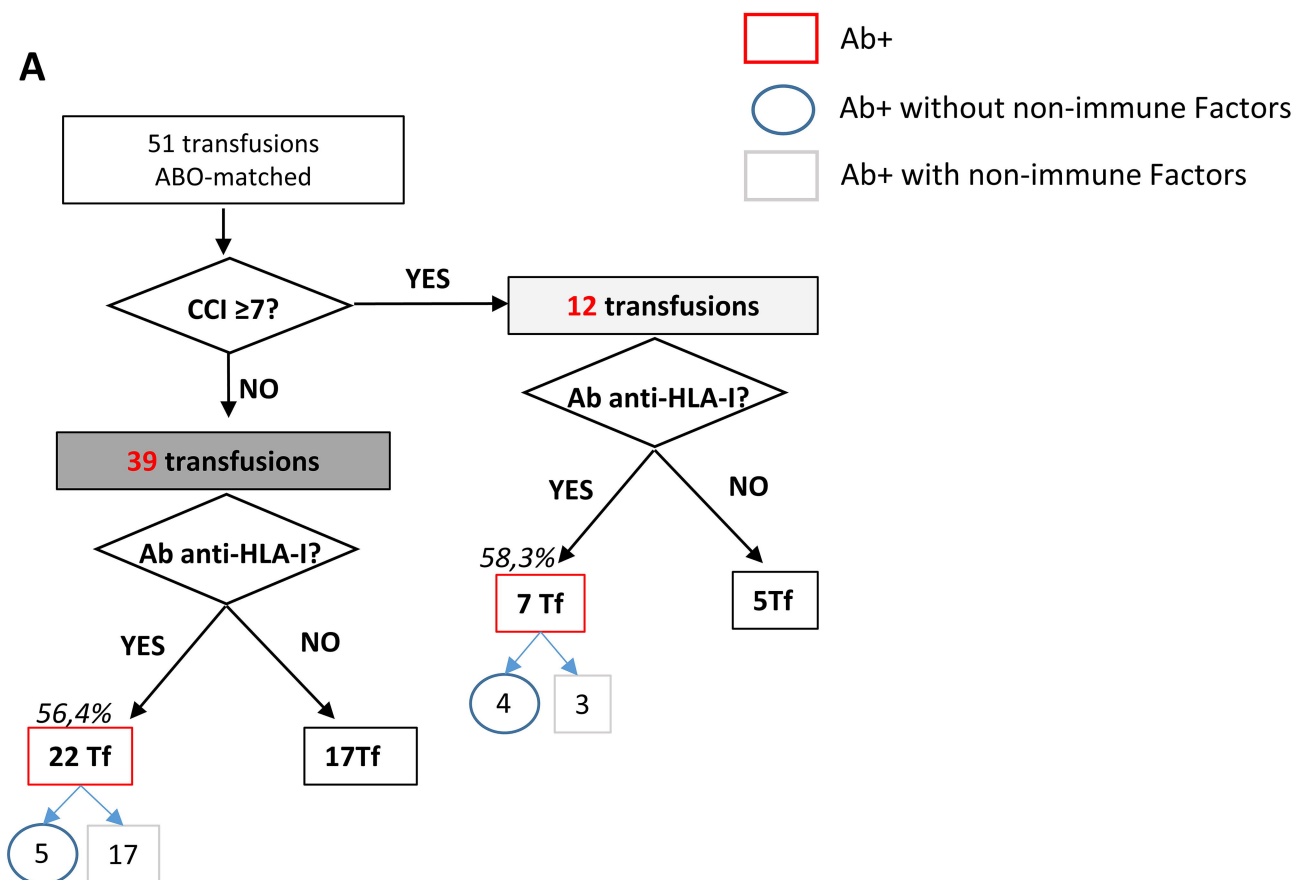
Haplotype donor 1	IgG	IgG1	IgG2	IgG3
A*02:01	-	-	-	-
A*24:02	S2 17848	14644	8042	82
B*44:02	S1 2465 S2 17459	117 9214	8 2650	- 402
B*44:05	-	-	-	-

Haplotype donor 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

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

Figure 2

A



B

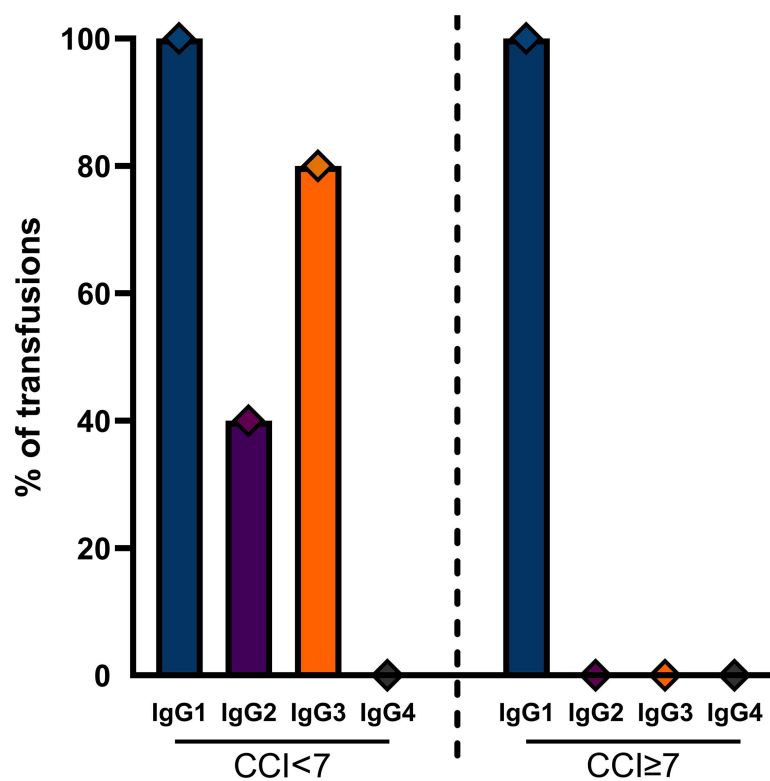
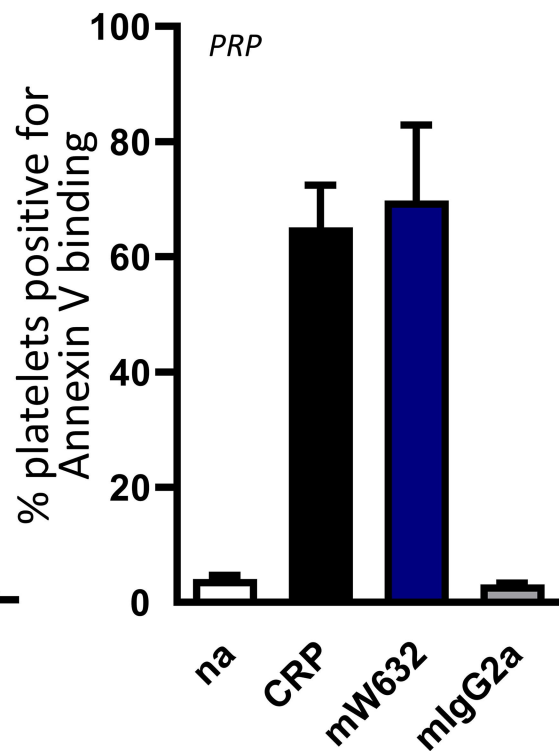
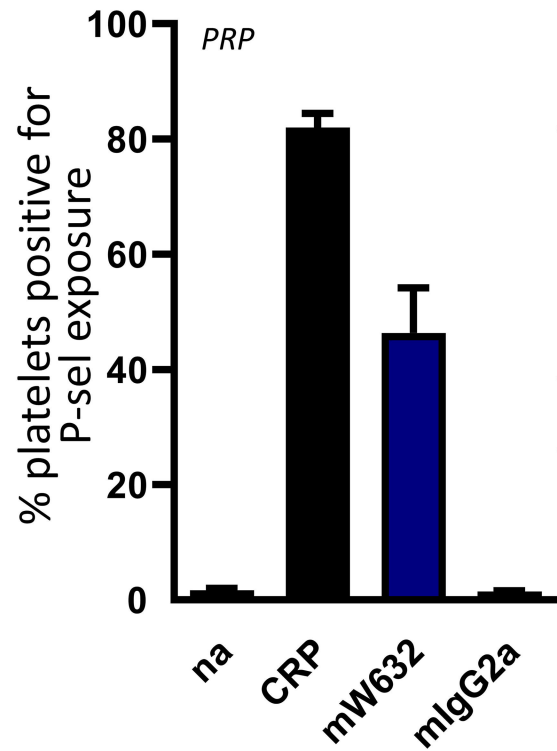
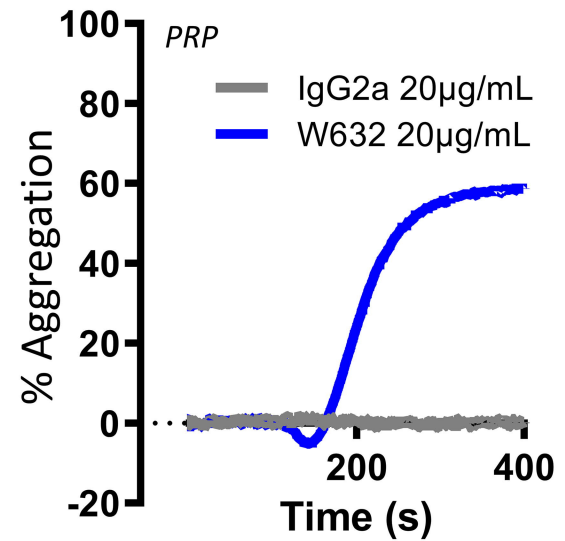


Figure 3

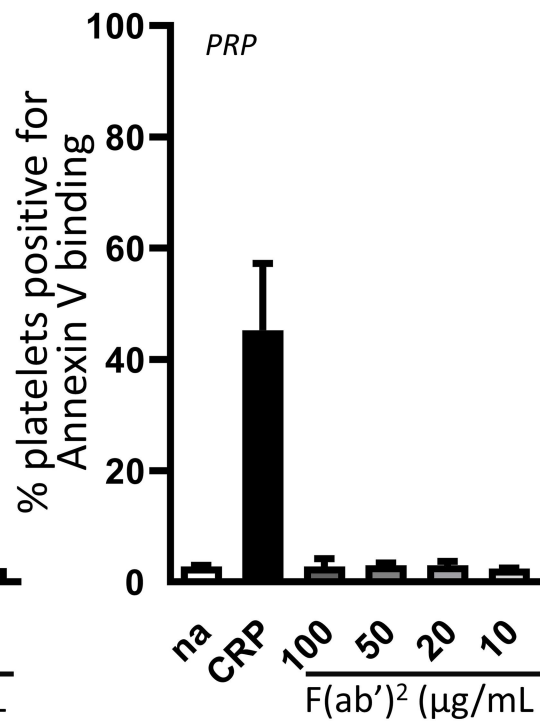
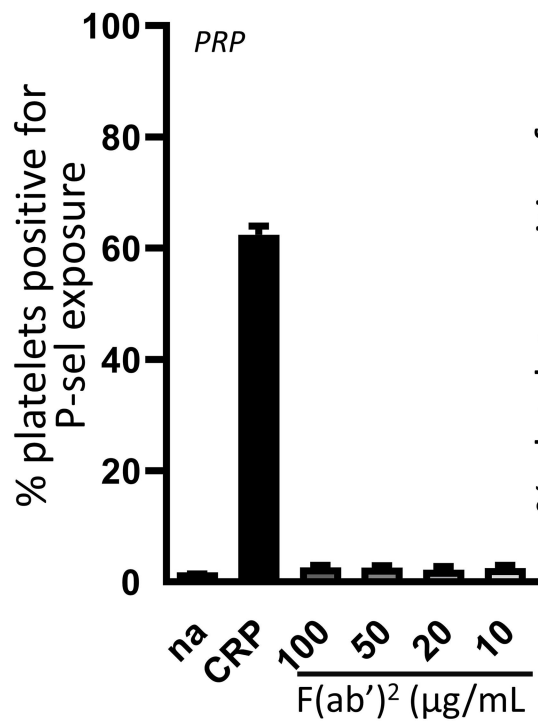
A



B



C



D

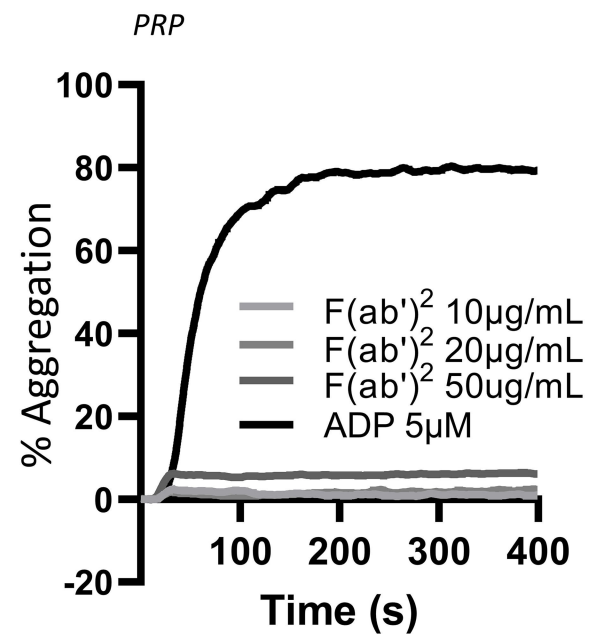
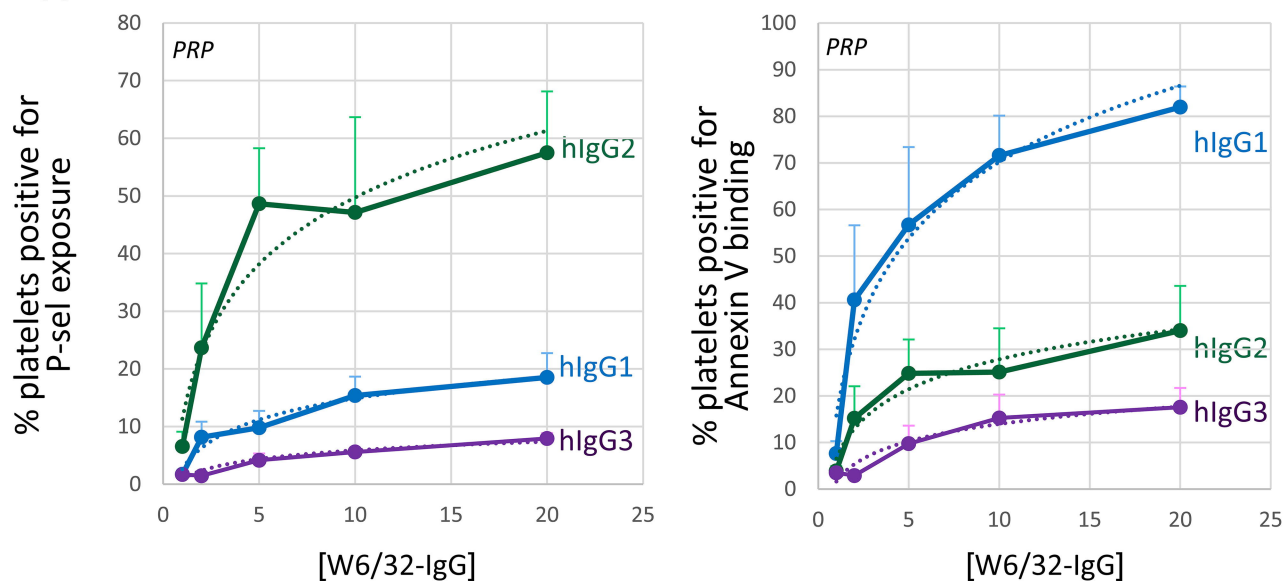
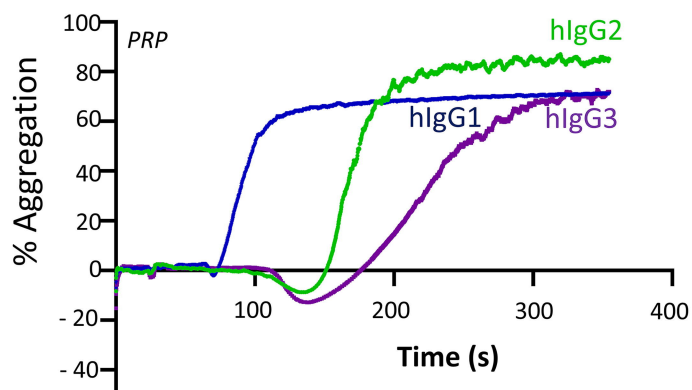


Figure 4

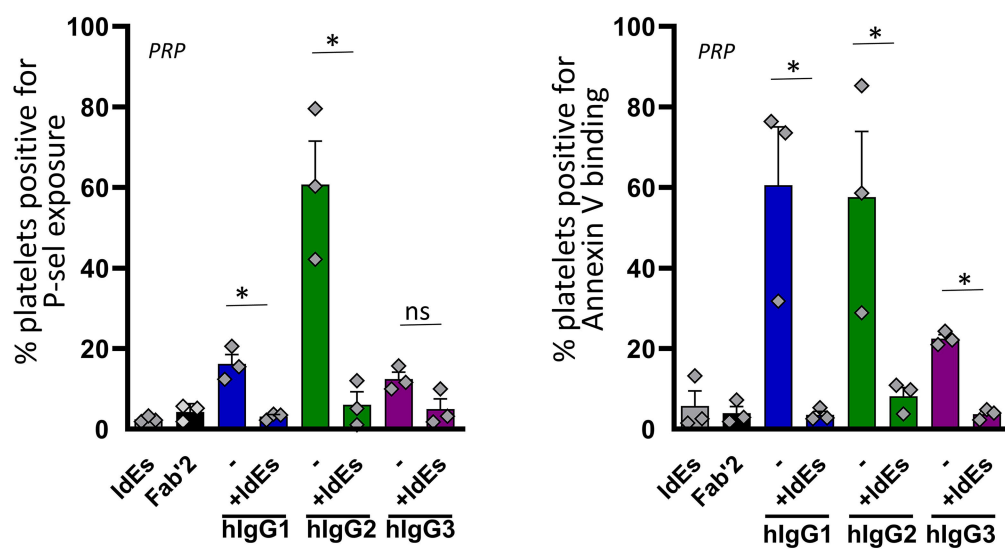
A



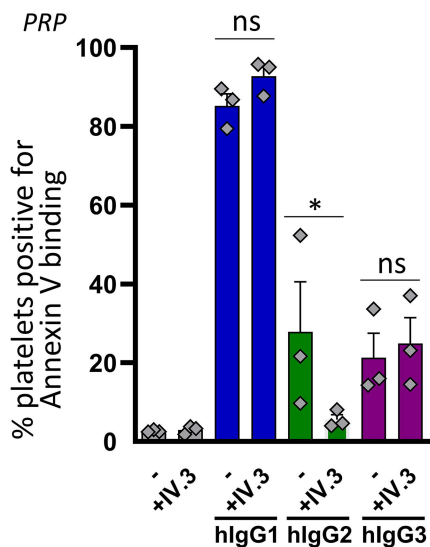
B



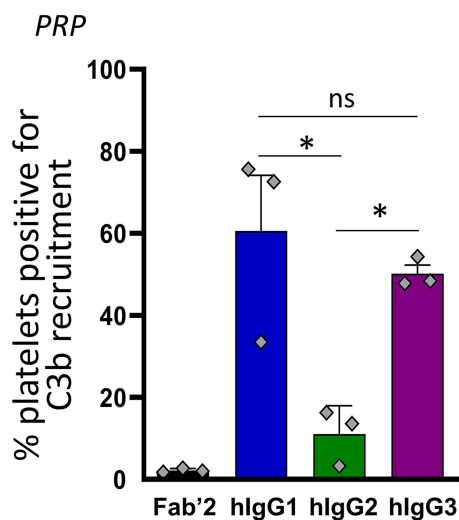
C



A



B



C

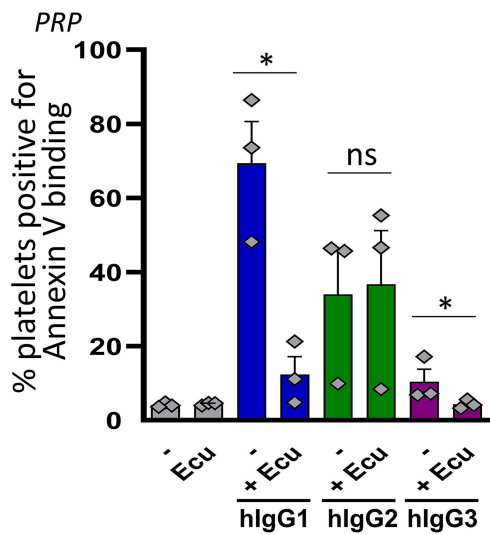
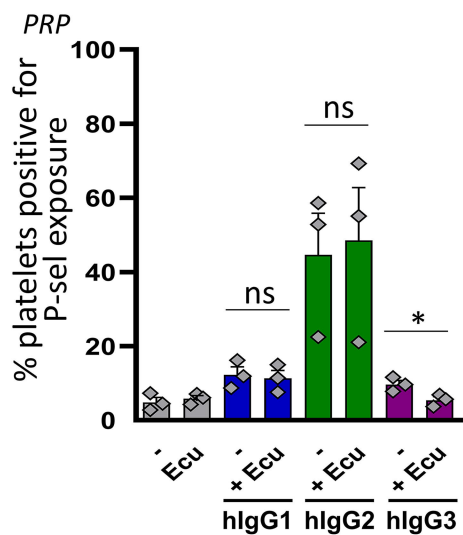
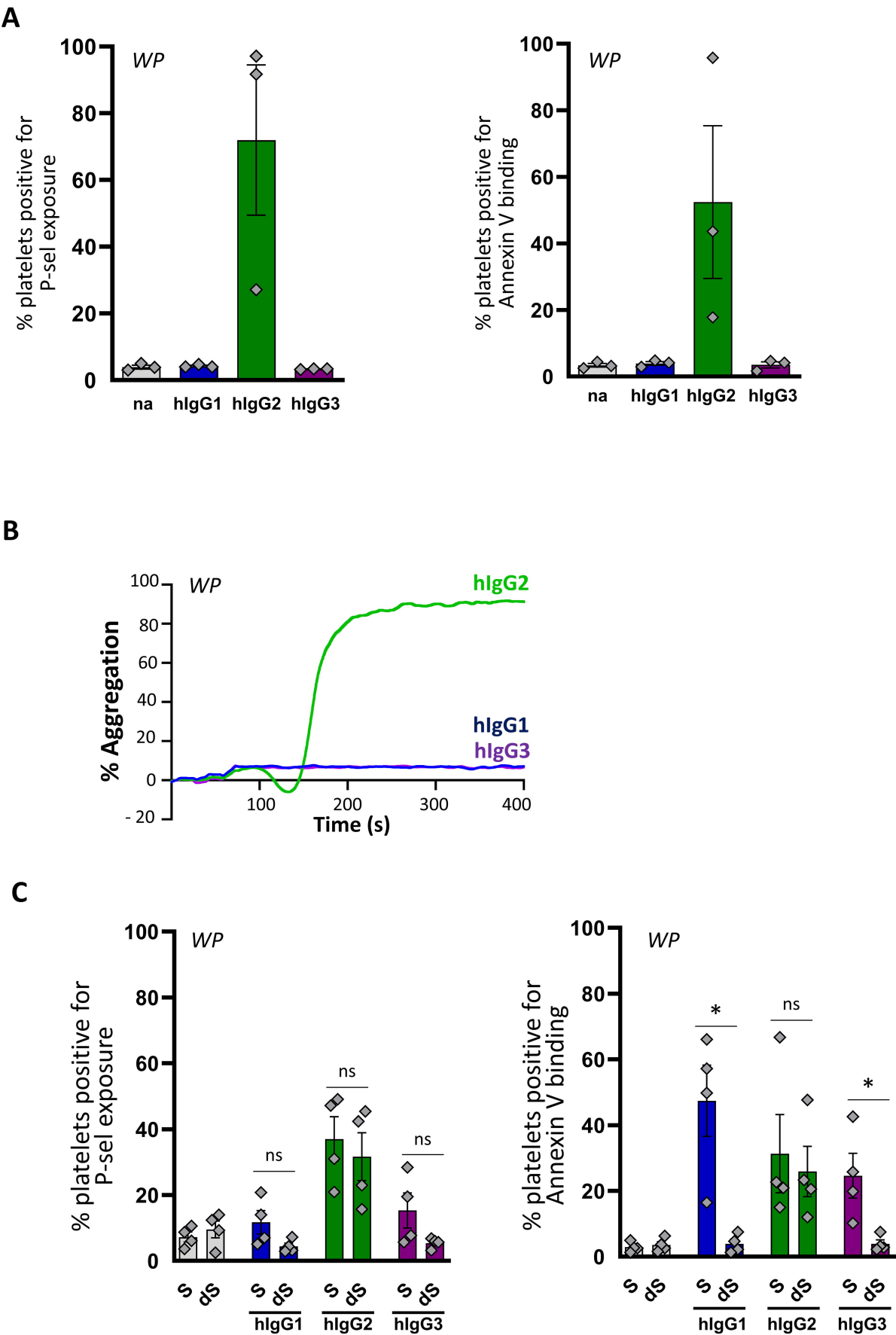


Figure 6



Supplemental Materials

APC anti-human CD62P (Clone AK4) antibodies were purchased from Biolegend. FITC-coupled Annexin V was purchased from Roche. As previously reported, CD9 antibody (ALMA-12) and Tyrode's albumin (TA) buffer (5mM Hepes, pH 7.35, 0.35% human serum albumin) were prepared in our laboratory. Anti-CD32a antibody (clone IV.3) was purchased from StemCell. Eculizumab was provided by the University Hospital of Strasbourg. Subclass-specific secondary antibodies coupled to phycoerythrin were purchased from LifeSpan Biosciences: murine anti-hIgG1 (clone HP6001), murine anti-hIgG2 (clone 31-7-4), murine anti-hIgG3 (clone HP6050) and murine anti-hIgG4 (clone HP6025).

Supplemental Methods

Clinical study

This monocentric study was called the ACERTP trial ("Anticorps associés aux états réfractaires aux transfusions plaquettaires") and was performed in the hematology department of the "Institut de Cancérologie Strasbourg Europe" (ICANS), a comprehensive cancer center situated in Strasbourg, France. To be eligible for the trial, the patients had to be adults and had to sign the informed consent form before starting the trial. Biological and clinical data were prospectively collected from March 2022 to September 2023. All patients received chemotherapy-based treatment for allogeneic or autologous hematopoietic stem cell transplantation (HSCT) or for induction and consolidation of acute leukemia treatment (Table 1).

Platelet concentrates production and issuing

Platelet concentrates (PC) were produced by the Etablissement Français du Sang Grand Est, and were either apheresis platelets collected on Trima (Terumo BCT), or buffycoat platelets made from 8 whole blood donations, and later divided in 2 twin subunits. All PC were stored in 60-65% additive solution (Intersol, Terumo BCT) and pathogen reduced with amotosalen + UVA (Intercept Blood System, Cerus, USA). Each PC underwent a platelet count, i.e. the platelet count was known for each PC (routine procedure in France). Shelf life was 7 days at +20-24°C, with agitation. Patients' weight and height were specified by the haematologist on the prescription form, and registered in the laboratory information system (LIS: Edgeblood, InLog, France). French guidelines recommend a posology interval between 0,5 and 0,7.10¹¹ platelets/10Kg body weight, and are respected as often as possible, depending of the inventory at issuing.

Anti-HLA-I IgG subclass assay

A high-definition LABScreen single-antigen Class I (One Lambda, Canoga Park, CA) was performed on the LABScan200 flow cytometer (Luminex® Corporation, Austin, TX) to determine the specificity of anti-HLA-I IgG antibodies. A positive result was defined as a mean fluorescence intensity (MFI) greater than 1,000 for total IgG Luminex®, a positivity threshold determined by the HLA-I laboratory based on negative controls. IgG subclasses detection was performed using the same protocol, except for the use of a secondary antibody specific for each subclass. The subclass of an anti-HLA-I antibody, already detected in total IgG Luminex®, was determined to be positive when the MFI was twice that of the corresponding negative control in subclass detection.

Aggregation test

Blood was drawn into a hirudinated tube. PRP or washed platelets were obtained by centrifugation as previously described². Platelet aggregation induced by anti-HLA-I antibodies was measured turbidimetrically as described previously².

Supplemental legends

Supplemental Table 1: Pre-and post-transfusion platelet counts and CCI value in immunized patients

Platelet count pre- and 24h post-transfusion (TS) (G/L) and the CCI value at 24h was reported for each allo-immunized patient without non-immune factors.

Supplemental Figure 1: Anti-HLA-I IgG analysis in transfused patients enrolled in a clinical study

A. Anti-HLA-I total IgG were analyzed by Luminex® and represented on a heat map. The MFI values of the anti-HLA-I total IgG are plotted against their specificities in the group of transfusions with a CCI value < 7 (left part) or ≥ 7 (right part). **B.** Anti-HLA-I IgG subclasses were analyzed by Luminex® and represented on a heat map per IgG subclass (IgG1, IgG2 and IgG3). The MFI values of HLA-I IgG subclasses are plotted against their specificities in the group of transfusions with a CCI value < 7 (left part) or ≥ 7 (right part).

Supplemental Figure 2 : Effect on IgG4-W632 on platelet activation

Hirudinated PRP was incubated with buffer, CRP (5 $\mu\text{g/mL}$) or human chimeric W632-IgG4 (10 $\mu\text{g/mL}$). P-selectin exposure was measured by flow cytometry.

Supplemental Figure 3: Effect on IV.3 on platelet activation mediated by anti-CD9 antibodies

Hirudinated PRP was incubated with anti-CD9 antibodies +/- IV.3, a CD32a inhibitor at 20µg/ml. Effect of IV.3 was evaluated by flow cytometry using activation markers such as P-selectin exposure (Psel) and Annexin V binding (A) or by aggregation test (B).

Supplemental Figure 4 : Platelet aggregation tests induced by hIgG1, hIgG2 or hIgG3-W632

(A) Aggregation test with hirudinated PRP incubated with hIgG1 (left panel), hIgG2 (middle panel) or hIgG3 (right panel) with IV.3 (+IV.3) or without (Ctrl). Representative trace of n=3 experiments is shown. (B) Aggregation test with hirudinated PRP incubated with hIgG1 (left panel), hIgG2 (middle panel) or hIgG3 (right panel) with Eculizumab (+Ecu) or without (Ctrl). Representative trace of n=3 experiments is shown. (C) Time-lags (time between the agonist addition and the beginning of aggregation are presented as the mean \pm SEM (n=3); Time =600 sec was considered as the maximal time lag (i.e. no aggregation observed in this time frame).

References

1. 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. *Journal of immunological methods*. 2013;390(1-2):41-51.
2. Cazenave JP, Ohlmann P, Cassel D, Eckly A, Hechler B, Gachet C. Preparation of washed platelet suspensions from human and rodent blood. *Methods in molecular biology*. 2004;272(13-28).

A

CCI ≥ 7

Platelet count pre-TS (G/L)	Platelet count post-TS 24h (G/L)	CCI value 24h
16	36	9.4
59	81	12.4
4	20	7.6
11	27	8.9

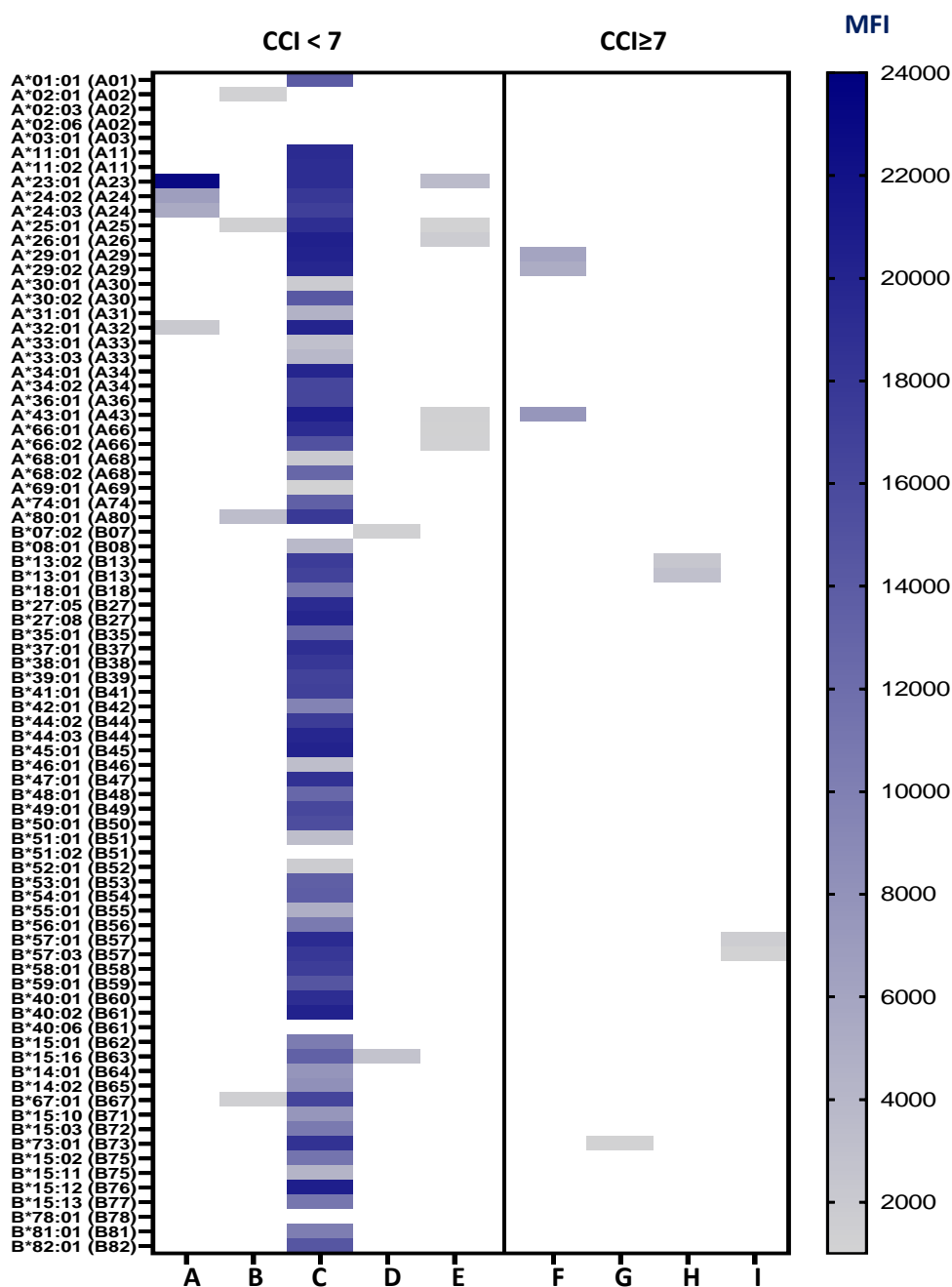
B

CCI < 7

Platelet count pre-TS (G/L)	Platelet count post-TS 24h (G/L)	CCI value 24h
42	50	5.2
4	10	2.9
10	9	0
22	33	5.2
10	21	6.9

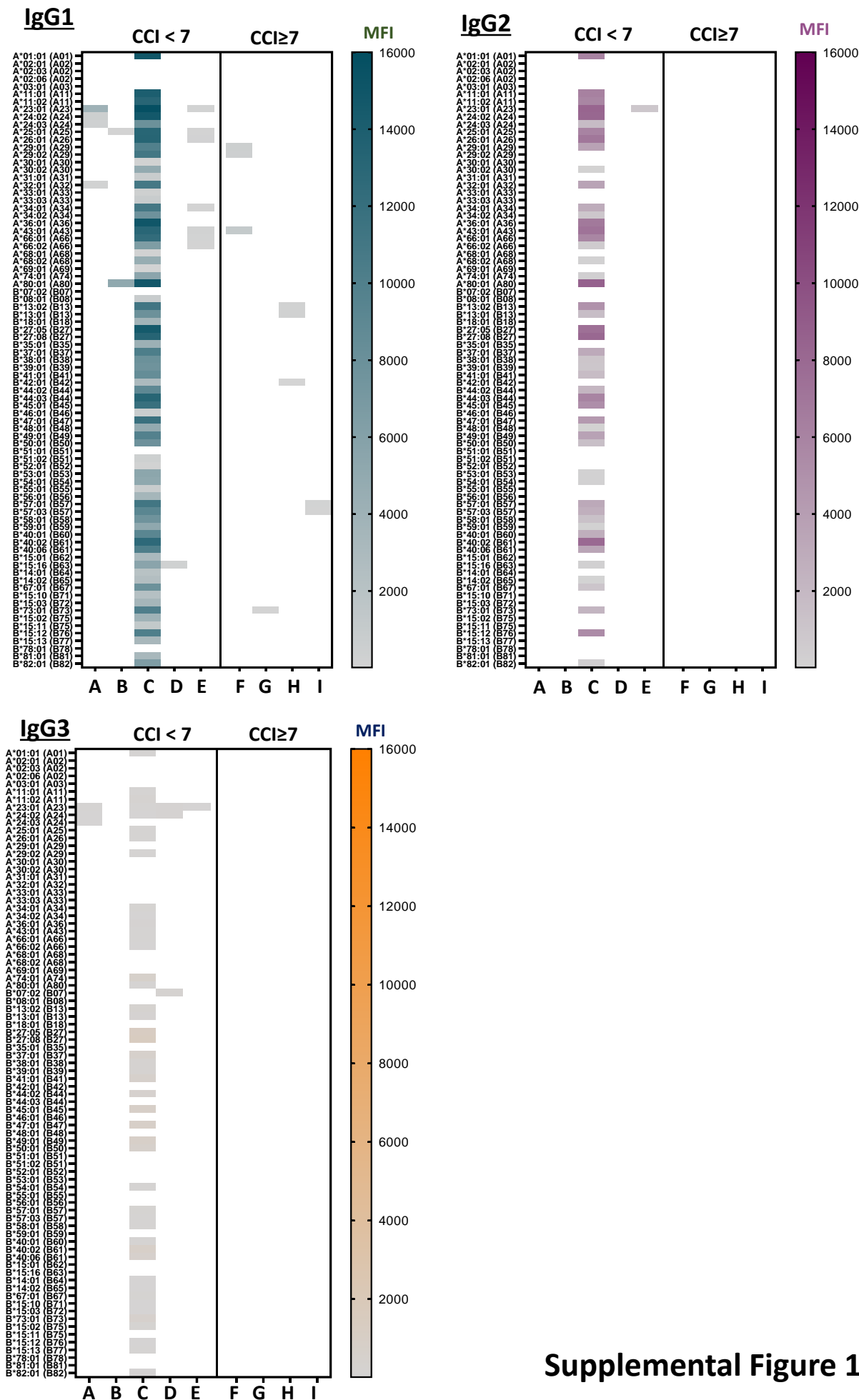
A

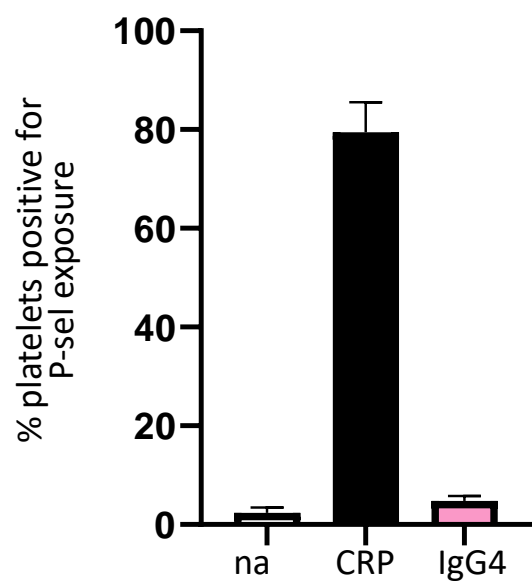
Total anti-HLA-I IgG



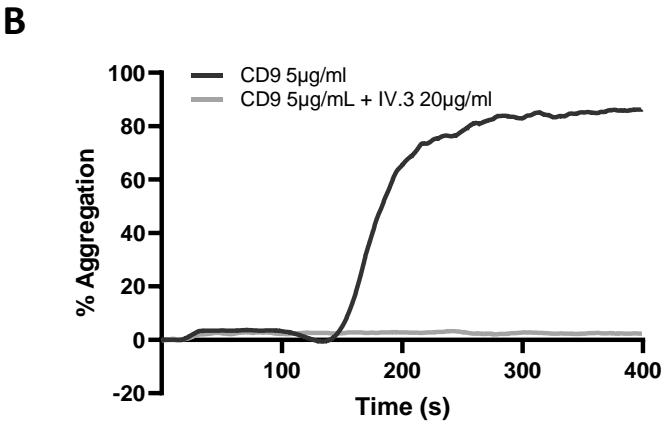
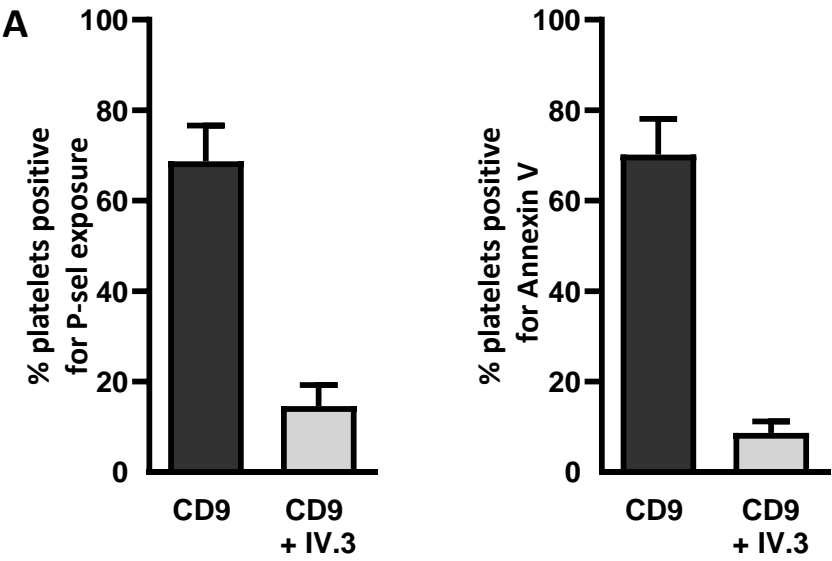
Supplemental Figure 1

B

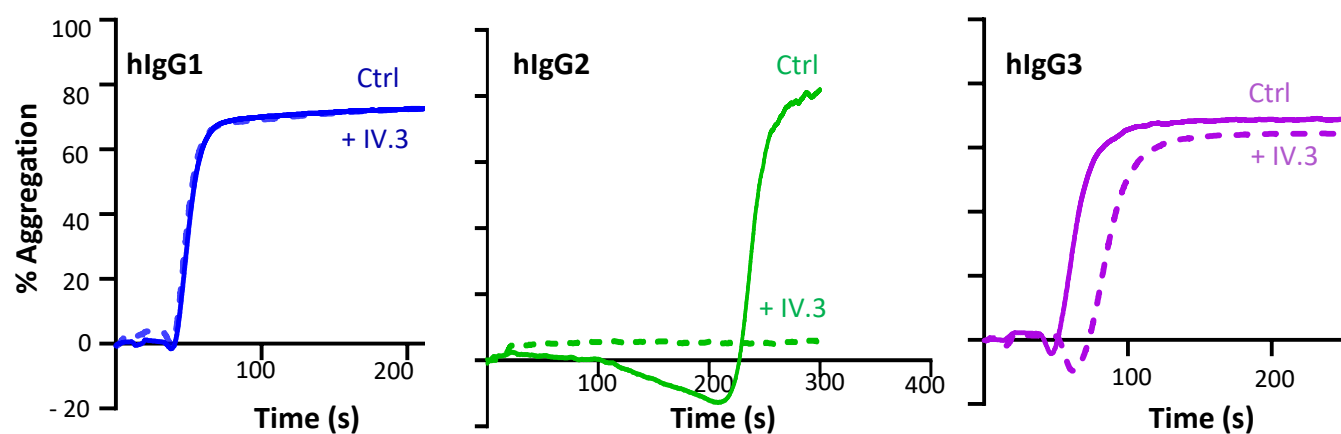
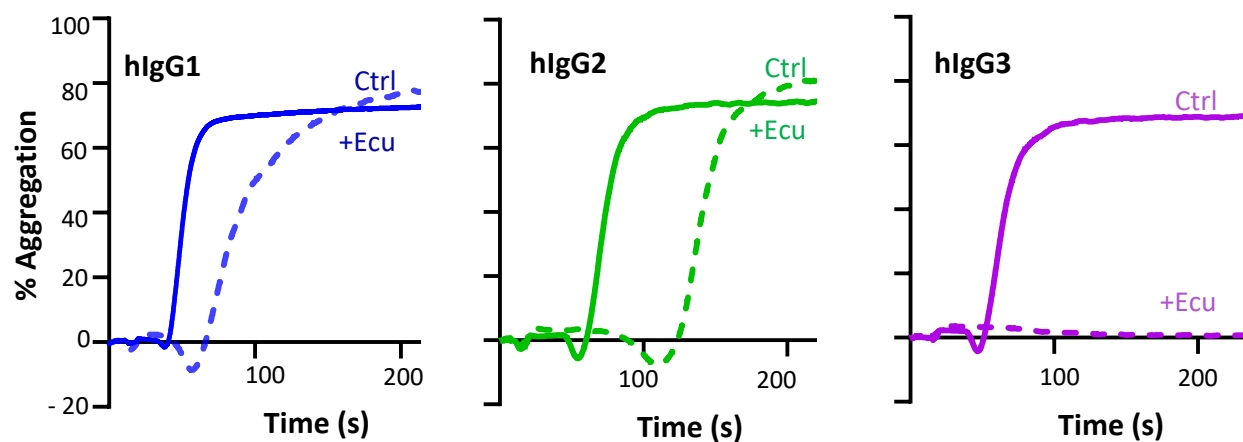
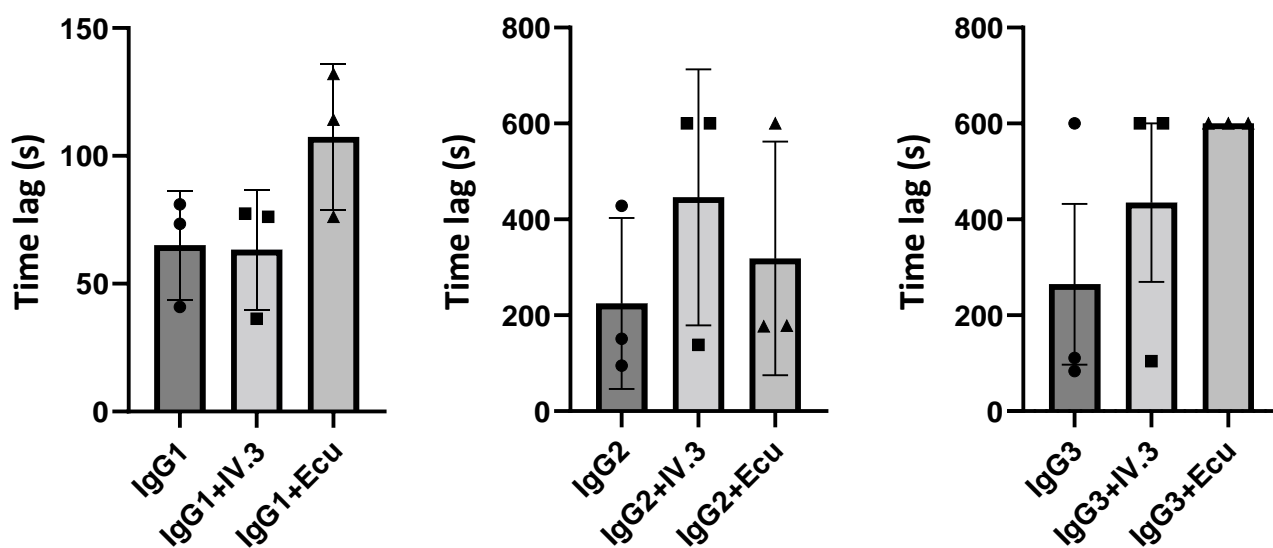




Supplemental Figure 2



Supplemental Figure 3

A**B****C****Supplemental Figure 4**