

# Fc galactosylation of anti-platelet human IgG1 alloantibodies enhances complement activation on platelets

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## Abstract

Approximately 20% of patients receiving multiple platelet transfusions develop platelet alloantibodies, which can be directed against human leukocyte antigens (HLA) and, to a lesser extent, against human platelet antigens (HPA). These antibodies can lead to the rapid clearance of donor platelets, presumably through IgG-Fc receptor (FcγR)-mediated phagocytosis or via complement activation, resulting in platelet refractoriness. Strikingly, not all patients with anti-HLA or -HPA antibodies develop platelet refractoriness upon unmatched platelet transfusions. Previously, we found that IgG Fc glycosylation of anti-HLA antibodies was highly variable between patients with platelet refractoriness, especially with respect to galactosylation and sialylation of the Fc-bound sugar moiety. Here, we produced recombinant glycoengineered anti-HLA and anti-HPA-1a monoclonal antibodies with varying Fc galactosylation and sialylation levels and studied their ability to activate the classical complement pathway. We observed that anti-HLA monoclonal antibodies with different specificities, binding simultaneously to the same HLA-molecules, or anti-HLA in combination with anti-HPA-1a monoclonal antibodies interacted synergistically with C1q, the first component of the classical pathway. Elevated Fc galactosylation and, to a lesser extent, sialylation significantly increased the complement-activating properties of anti-HLA and anti-HPA-1a monoclonal antibodies. We propose that both the breadth of the polyclonal immune response, with recognition of different HLA epitopes and in some cases HPA antigens, and the type of Fc glycosylation can provide an optimal stoichiometry for C1q binding and subsequent complement activation. These factors can shift the effect of a platelet alloimmune response to a clinically relevant response, leading to complement-mediated clearance of donor platelets, as observed in platelet refractoriness.

## Introduction

Prophylactic and supportive platelet transfusions significantly reduce mortality and hemorrhagic complications in

(onco-hematologic) patients with thrombocytopenia. However, in approximately 5-15% of patients receiving chronic platelet support rapid clearance of the transfused platelets is observed, known as platelet refractoriness

(PR). PR can be caused by either non-immune or immune factors.<sup>1–5</sup> Immune PR occurs in approximately 20% of the cases of PR and is mainly attributable to the formation of antibodies against human leukocyte antigens (HLA) and occasionally human platelet antigens (HPA).<sup>6,7</sup> Additionally, anti-ABO and drug-induced antibodies have also been described as potential causes of immune PR.<sup>8–10</sup>

Currently, the transfusion of HLA- and HPA-matched platelets is the only treatment for alloimmunized patients,<sup>11–13</sup> but finding compatible donors can be challenging. Unmatched platelet transfusions can trigger an immune response with a broad spectrum of HLA-epitope or HPA-recognizing antibodies and the binding of these antibodies to donor platelets may result in destruction of the platelets. It was originally assumed that this was mainly the result of IgG-Fc receptor (FcγR)-mediated phagocytosis but there is growing evidence that complement activation can also play a role.<sup>14</sup> If platelet transfusions are followed by an insufficient increment in platelet count, bleeding may not be prevented adequately.<sup>15,16</sup> For still unknown reasons, not all patients with anti-HLA or anti-HPA antibodies develop PR to unmatched platelet transfusions. As of yet, no clear differential FcγR- or complement-activating potential of the antibodies present in these patients has emerged which might explain differences in platelet clearance rates.

In the last decade, it has become clear that the initiation of the classical complement pathway by IgG antibodies, recognizing surface-bound antigens, requires a stepwise process. The first step, antigen-IgG binding, can precipitate the assembly of hexameric complexes of IgG facilitated by lateral movement and Fc-Fc interactions.<sup>17</sup> This depends on the target antigen not being rigidly anchored in the membrane and/or cellular cytoskeleton, and most likely can occur more efficiently if the antibody response is polyclonal. This leads to binding of antibodies to many different epitopes, with the result of higher levels of sensitization and an increased likelihood of Fc-Fc interactions.<sup>18</sup> The ensuing hexameric assembly can then be further stabilized by interactions with C1q, followed by activation of C1 and the classical complement pathway. The need for hexamerization might also explain our previous observation that only a combination of anti-HLA monoclonal antibodies activated complement on the surface of platelets.<sup>14</sup>

It has also become evident that complement activity of antigen-specific IgG can be controlled through IgG Fc glycosylation. All IgG subclasses contain a conserved N-linked glycan at position N297 in the CH2 domain of the Fc-region of IgG antibodies which is highly variable. The glycan consists of a bi-antennary core structure composed of *N*-acetylglucosamines (GlcNAc) and mannose residues but can be further extended by a fucose, bisecting GlcNAc, galactose and sialic acid residues. Pre-

viously, we described the glycosylation profile of anti-HLA antibodies in patients with PR, which was highly variable with respect to galactose and sialic acid levels. Although Fc galactosylation was significantly increased in the majority of the patients, sialylation levels were more varied, with both decreased and increased sialylation being observed for anti-HLA IgG.<sup>19</sup>

The causal relation between the N-glycan structures and their influence on IgG effector functions is known; both at the level of FcγR- and complement-mediated effector functions.<sup>19–21</sup> The different types of changes, primarily those of high levels of galactosylation, enhance complement activity through elevated C1q binding<sup>20,22</sup> and can be found in patients with increased platelet clearance in fetal and neonatal alloimmune thrombocytopenia.<sup>19,23</sup> High levels of galactosylation seem to have no effect on the intrinsic affinity of C1q to monomeric IgG, but stimulate hexamerization of IgG on IgG-opsonized surfaces, and enhance downstream complement activation of IgG.<sup>24,25</sup>

Here, we investigated the role of Fc glycosylation in anti-platelet alloantibodies and its effect on complement activation, as well as the potential synergy in inducing complement activation if anti-HLA and anti-HPA-1a antibodies are bound simultaneously.

## Methods

Additional information on materials and methods used can be found in the *Online Supplementary Data*.

### Donor blood

Peripheral blood was obtained from anonymous, healthy volunteers with informed written consent, after approval from the Sanquin Ethical Advisory Board, in accordance with the Declaration of Helsinki.

### Surface plasmon resonance

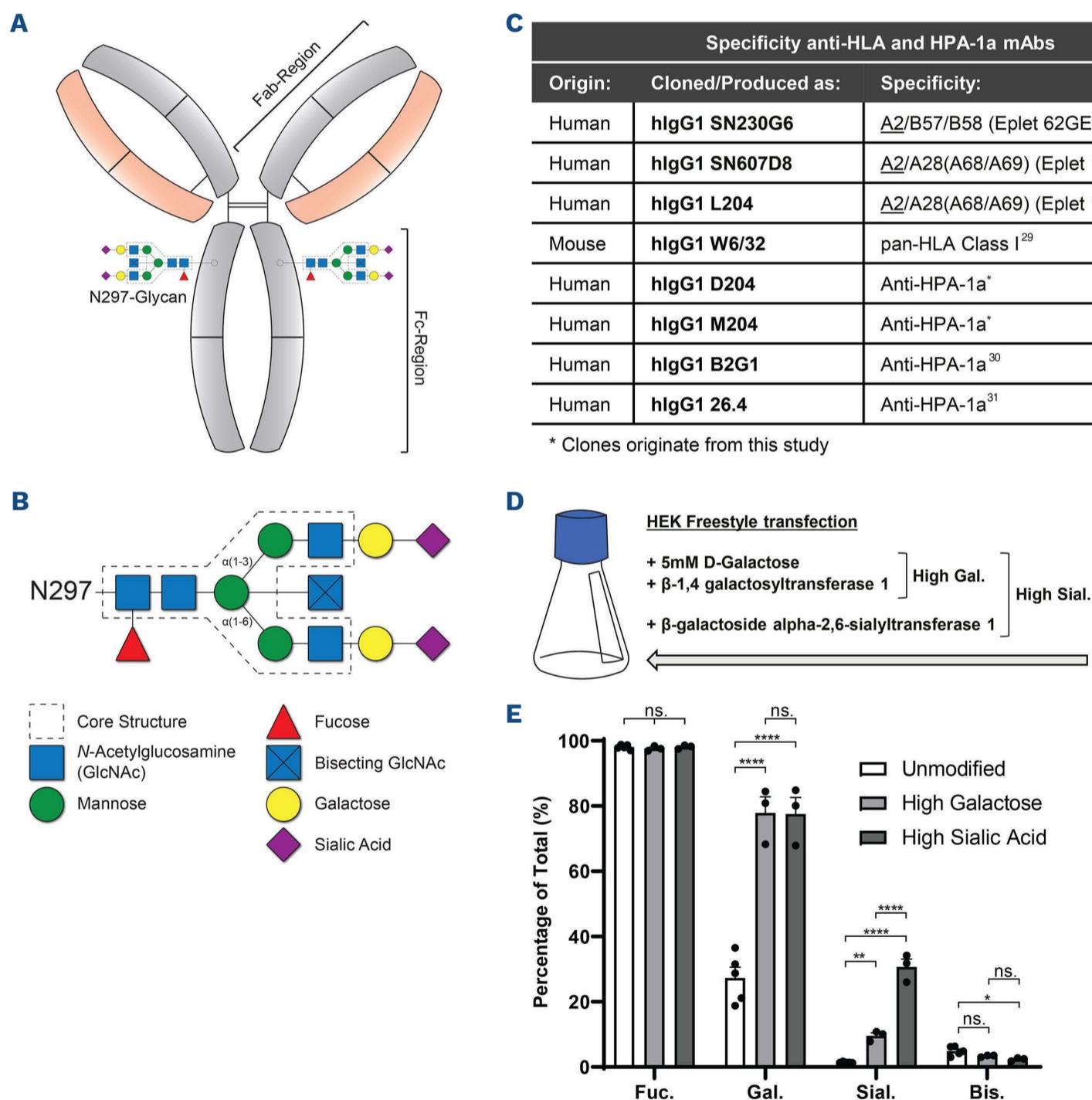
Anti-HLA antibodies (SN230G6, SN607D8 and W6/32) were spotted, via random coupling, using a Continuous Flow Microspotter (Wasatch Microfluidics) onto a SensEye Easy2Spot G-type sensor (Senss). Spotting was done in 10 mM acetate buffer phosphate-buffered saline (PBS) + 0.075%-Tween 80 (Amresco), pH 4.5 in duplicate at a two-fold dilution ranging from 60 nM to 7.5 nM for 15 min. The sensor was deactivated with 100 mM ethanolamine (Merck), pH 8.8 for 7 min. Binding measurements were carried out in an IBIS MX96 (IBIS Technologies). First, 100 nM HLA-A\*02:01 was flowed over the sensor for 5 min, followed by a 5-min flow of 100 nM of each anti-HLA antibody separately. Regeneration was then performed with 10 mM Gly-HCl, pH 2.4. This was repeated for every possible antibody combination.

### Platelet isolation

Citrated whole blood from healthy volunteers with known HLA and HPA-type, corresponding to the specificity of the anti-HLA antibodies (*Online Supplementary Table S1*), was centrifuged at 125 g for 20 min. The platelet-rich plasma was harvested and 10 vol% ACD (acid citrate dextrose, 85 mM Na<sub>3</sub>-citrate·2H<sub>2</sub>O, 71 mM citric acid·H<sub>2</sub>O and 111 mM D-glucose) was added. The platelet-rich plasma was centrifuged (850 g for 8 min) and washed twice with wash buffer (36 mM citric acid·H<sub>2</sub>O, 103 mM NaCl, 5 mM KCl, 5 mM EDTA, 5.6 mM D-glucose, pH 6.5). The platelets were fixed with 1% paraformaldehyde and washed and stored in PBS + 0.5% bovine serum albumin (BSA) at room temperature.

### Flow cytometric complement assay

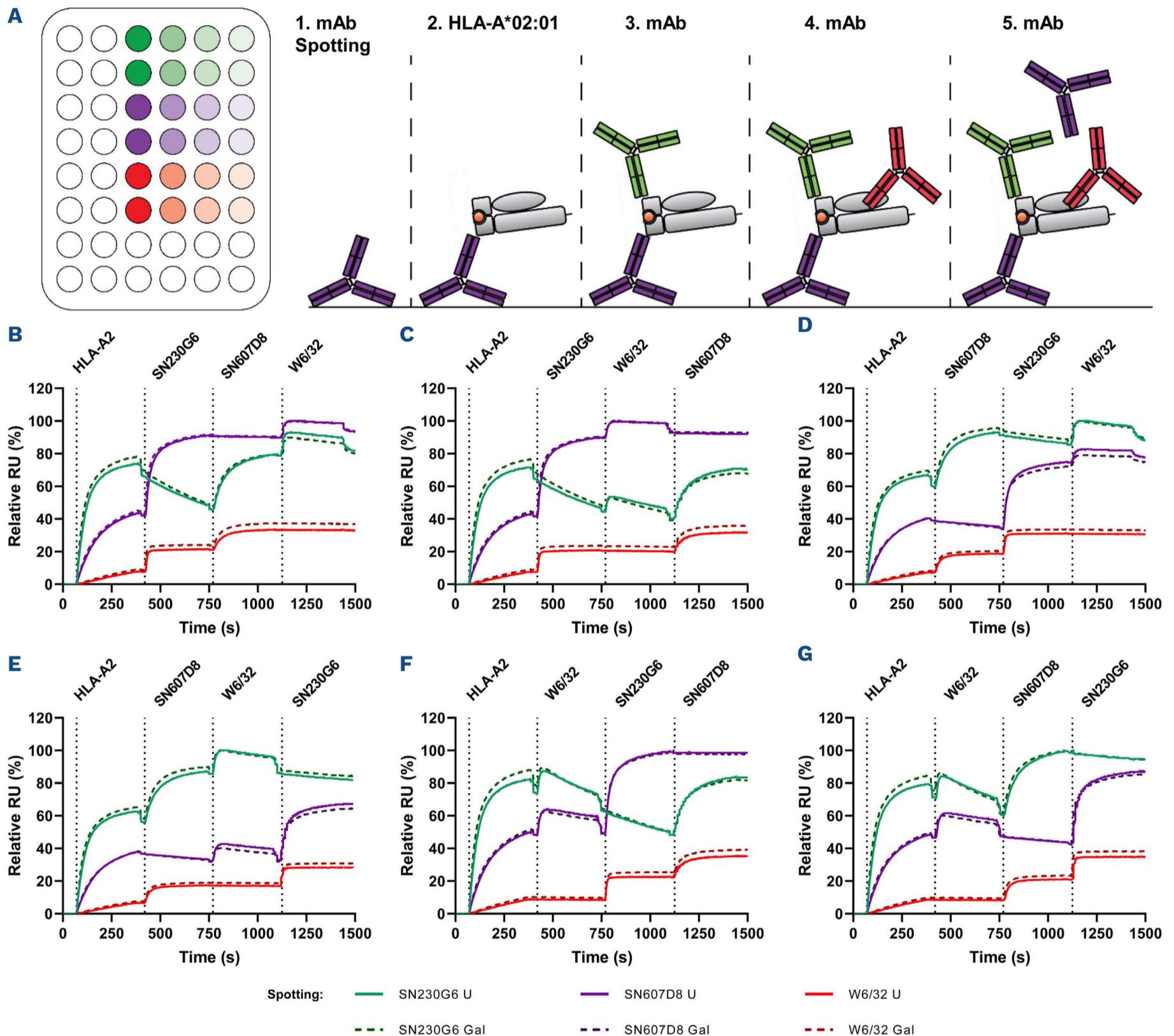
Platelets (5x10<sup>6</sup>) were incubated with equal volumes of anti-platelet antibody (0.001-20 µg/mL) and pooled complement-rich human serum, for 30 min. at 37°C while shaking. For the antibody combinations, the dilution series started with 10 µg/mL of both antibodies, resulting in a total antibody concentration of 20 µg/mL. The platelets were washed four times with PBS + 0.5% BSA + 5 mM EDTA and stained for 20 min. for flow cytometry with Goat F(ab')<sub>2</sub> Anti-Human IgG-PE (1/250, Southern Biotech), anti-complement C3b/iC3b-APC Antibody Clone: 3E7/C3b (1/250, Biolegend) and C1q Polyclonal Antibody-FITC (1/25, Thermo Fisher Scientific). The samples were split and anti-C1q-FITC was measured separately due to the high



**Figure 1. Production of glycoengineered anti-HLA and anti-HPA-1a human IgG1 monoclonal antibodies.** (A) Schematic representation of the immunoglobulin G (IgG) Y-shaped structure; the Fc-N297 glycan and Fab- and Fc-regions are indicated. (B) Composition of the N297-glycan bi-antennary structure with the distinct sugar groups and their respective locations. (C) Antibody specificity of the anti-HLA and anti-HPA-1a monoclonal antibodies (mAbs). (D) Glycoengineering techniques to produce mAbs with increased Fc galactosylation and sialylation, via the addition of relevant substrates and constructs coding for enzymes prior/during transfection. (E) Fc-glycosylation profiles of the mAbs produced using different glycoengineering techniques to increase Fc galactosylation and sialylation, analyzed by mass spectrometry.

fluorescence intensity of the anti-IgG-PE. The flow cytometry data were analyzed using FlowJo vX for Windows (BD Biosciences). The platelets were gated based on the

FSC-A/SSC-A and single cells were selected (FSC-H/FSC-A). The geometric-mean fluorescence intensities of all parameters were calculated.



**Figure 2. Surface plasmon resonance visualizes the co-binding and stabilization of multiple anti-HLA monoclonal antibodies to HLA-A\*02:01.** (A) Spotting of the sensor was performed with an array of unmodified or high galactosylated anti-HLA monoclonal antibodies with three different specificities. The color indicates the different spotted anti-HLA monoclonal antibodies (SN230G6 = green, SN607D8 = purple and W6/32 = red). HLA-A\*02:01 monomers were flowed over the whole array (consisting of all antibodies and controls), followed by each anti-HLA antibody in succession and in different orders, as indicated. An example is given of one of the spots under one specific flow condition using our surface plasmon resonance (SPR) set-up. The same glycovariants were used for all antibodies in the same run; when spotted with unmodified antibodies, all consecutive antibodies were also unmodified and when spotted with high galactosylated antibodies, all following antibodies were high galactosylated as well. (B-G) Sensograms showing the co-binding and stabilization of multiple anti-HLA monoclonal antibodies to HLA-A\*02:01. Runs with unmodified antibodies are indicated by solid lines and runs with high galactosylated antibodies are indicated by dotted lines. Each panel starts with the binding of HLA-A\*02:01 to the spotted antibodies, but differs in the flow order of the three following anti-HLA monoclonal antibodies. Data represent two independent experiments, one for each set of glycovariants, SPR response units (RU) were normalized per experiment, in which the maximum response was set at 100%; the y-axis shows the relative RU in %. mAb: monoclonal antibody.

## Statistics

Statistical analyses were conducted within Graphpad Prism 8.02 (263) for Windows. Curve fitting was performed using nonlinear regression dose-response curves with log(agonist) *versus* response – variable slope (four parameters). Bar graphs were analyzed using ordinary one-way analysis of variance with the Tukey multi-comparison test. *P* values  $\leq 0.05$  were defined as statistically significant, with the levels of significance being indicated by asterisks (\* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$  and \*\*\*\* $P \leq 0.0001$ ).

## Results

### IgG glycoengineering of anti-platelet monoclonal antibodies

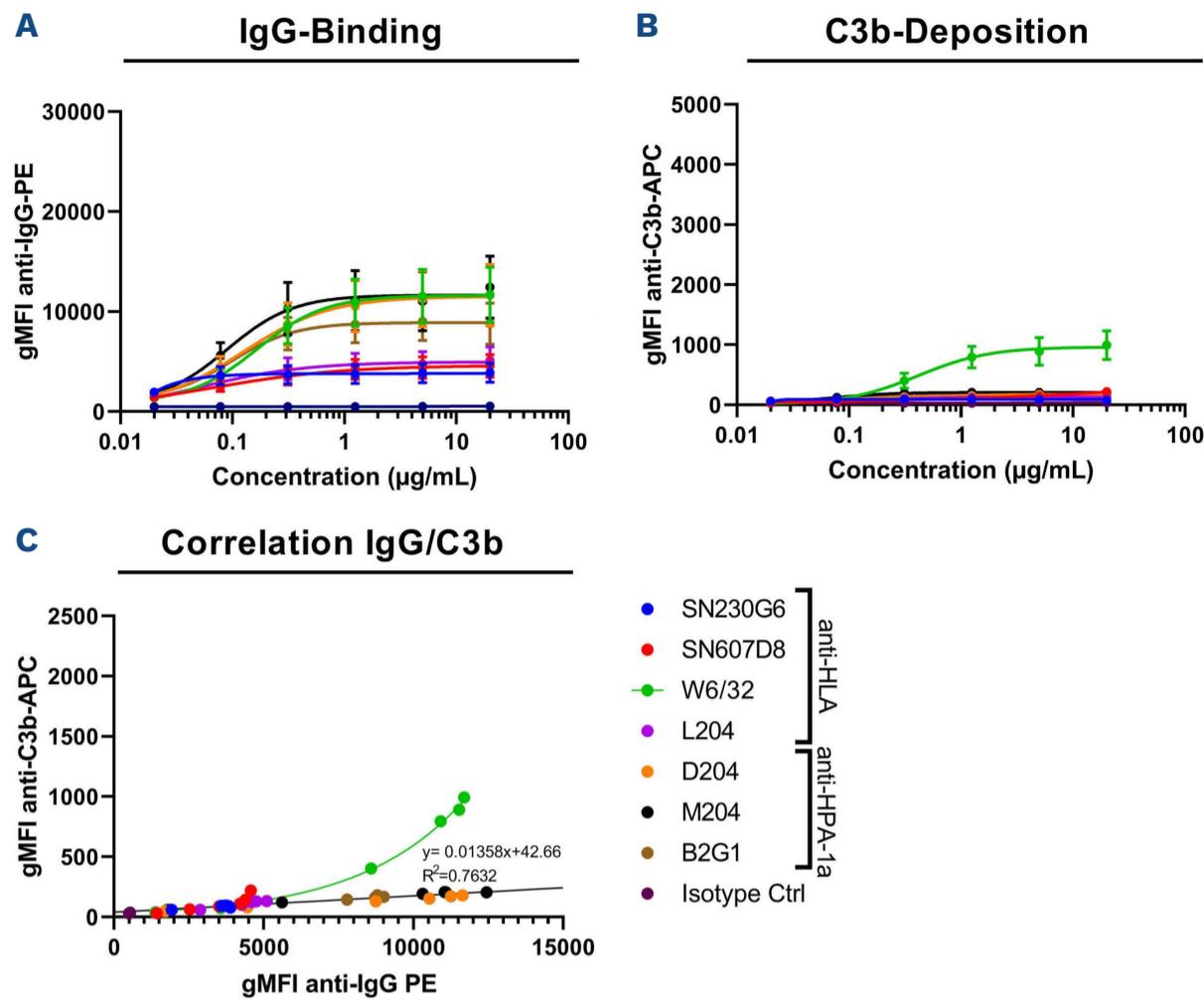
Investigating the effect of Fc glycosylation of anti-platelet alloantibodies on complement activation is a difficult process. Various factors are known to affect complement activation, such as the binding epitope, affinity, iso/allo-type, titer and glycosylation profile of an antibody. In order to circumvent the large variety among PR patients and serum samples, caused by previous pregnancies, transfusions and transplants, a controlled experimental set-up is required. In this study, we produced unmodified and glycoengineered anti-HLA and anti-HPA-1a human IgG1 monoclonal antibodies with different specificities, and enhanced levels of galactosylation and sialylation (Figure 1A-C), as observed in patients with PR. Several specificities and epitopes have been described previously (SN230G6,<sup>26–28</sup> SN607D8,<sup>26–28</sup> W6/32,<sup>29</sup> B2G1<sup>30</sup> and 26.4<sup>31</sup>). Two novel human monoclonal antibodies with HPA-1a specificity (D204 and M204) and one with HLA specificity (L204) were cloned from an alloimmunized individual by manipulation of the RNA of B cells that had secreted anti-platelet or anti-HLA antibodies upon single-cell culture. All monoclonal antibodies were produced as human IgG1\*03 kappa antibodies using a HEK-freestyle based production system.<sup>20,24</sup> After production (Figure 1D), the different glycoengineered antibodies were subjected to glycoanalysis by liquid chromatography mass spectrometry (Figure 1E, *Online Supplementary Figure S1A, B*). The glycosylation profiles of the unmodified antibodies showed nearly 100% fucosylation, ~25% galactosylation, 1% sialylation and 5% bisection. By overexpressing B4GALT1 with elevated substrate availability, Fc galactosylation of IgG1 was increased to 80%. As observed previously, this was accompanied by a minor increase in sialylation and a decrease in bisection.<sup>20,24</sup> Increasing sialylation by overexpressing ST6GALT increased sialylation to approximately 30%. Fucosylation was not affected by any of these glycoengineering techniques.

### Multiple anti-HLA monoclonal antibodies bind and stabilize each other on a single HLA-monomer

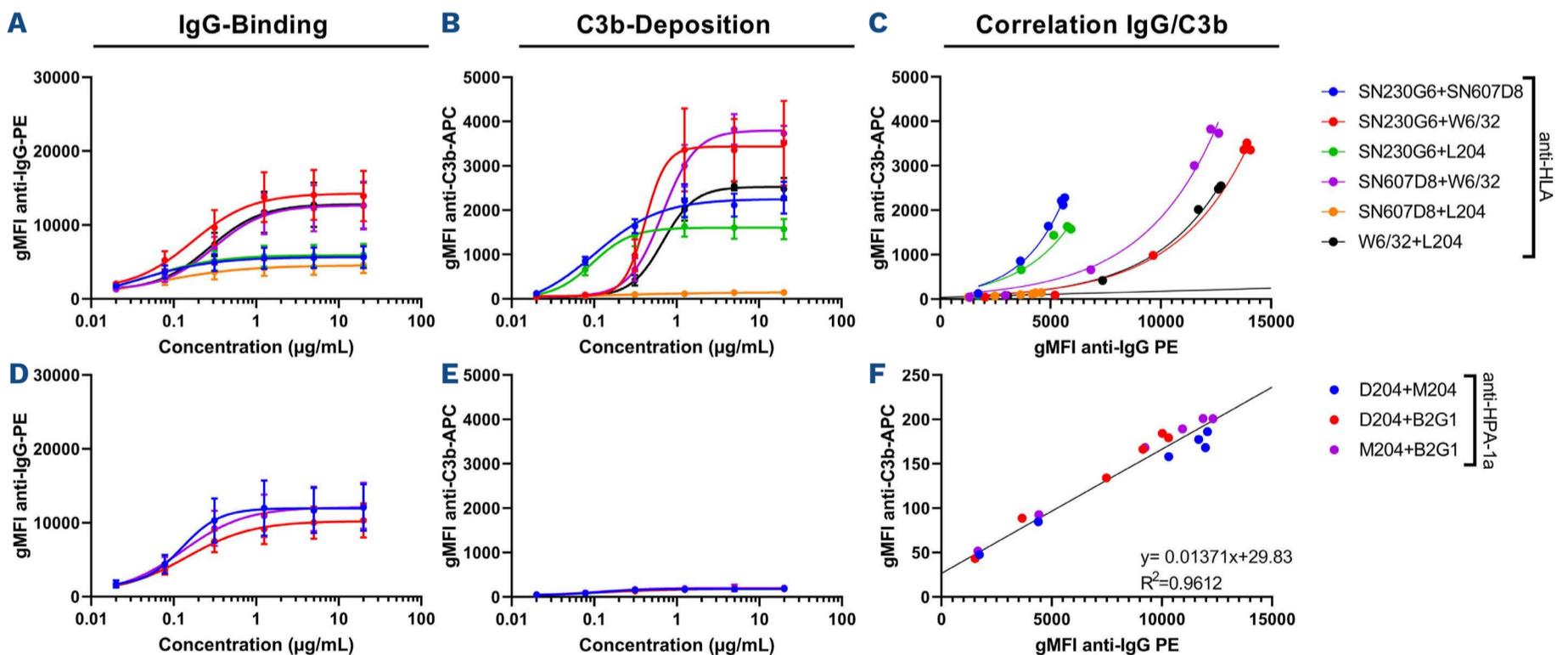
We then analyzed the recognition of HLA-A\*02:01 monomers by the anti-HLA antibodies using surface plasmon resonance. We spotted an array of anti-HLA antibodies with different specificities (SN230G6, SN607D8 and W6/32) and their corresponding glycovariants in parallel and then probed their binding to HLA, followed by each of the antibodies in succession over time (Figure 2A). In our set-up, HLA-A\*02:01 monomers were first flowed over the spotted antibodies on the sensor to visualize the initial binding of HLA-molecules by the spotted antibody. Thereafter, the same anti-HLA monoclonal antibodies were flowed in different orders over the sensor to visualize the subsequent binding of anti-HLA monoclonal antibodies to the same HLA molecules captured by the spotted antibody (Figure 2B-G). This allowed us to see potential overlap (blocking) in binding epitopes between the antibodies. SN230G6 showed the strongest capture of HLA from solution, followed by SN607D8 and then W6/32. After the initial binding of the HLA monomer to the spotted antibody, the remaining antibodies also bound successfully to the same molecule (e.g., purple line, Figure 2C). Although all antibodies were bound successfully, the signal strengths varied depending on the order in which the complex was built, with an apparent dissociation of the antibody/HLA complex over time, recognizable as a decreasing slope. This dissociation was especially notable for the SN230G6 antibody where the antibody in solution showed strong competition with surface-immobilized SN230G6 (e.g., green line, Figure 2B, C). Remarkably, this dissociation did not occur after the sequential binding of soluble SN230G6 to spotted SN607D8 HLA-A2 complexes (e.g., purple line, Figure 2B) and soluble SN607D8 to spotted SN230G6 HLA-A2 complexes (e.g., green line, Figure 2D), indicating the stabilizing interaction between the antibodies in the overall complex. This stabilizing interaction was not observed for combinations with the W6/32 antibody, probably because of low binding and rapid dissociation of the antibody. Furthermore, no differences were observed between the unmodified and high galactose variants.

### Anti-platelet monoclonal antibodies only activate the complement system when incubated simultaneously

Next, we studied the possible synergistic effects of the anti-HLA and -HPA-1a monoclonal antibodies, for both antigen-binding and complement-activating properties on the surface of human platelets employing flow cytometry. The gating strategy is shown in *Online Supplementary Figure S2*. All individual antibodies were capable of binding to the surface of platelets, to either HLA molecules or glycoprotein IIIa (GPIIIa), but differed in their maximum binding (Figure 3A) due to differences in bind-



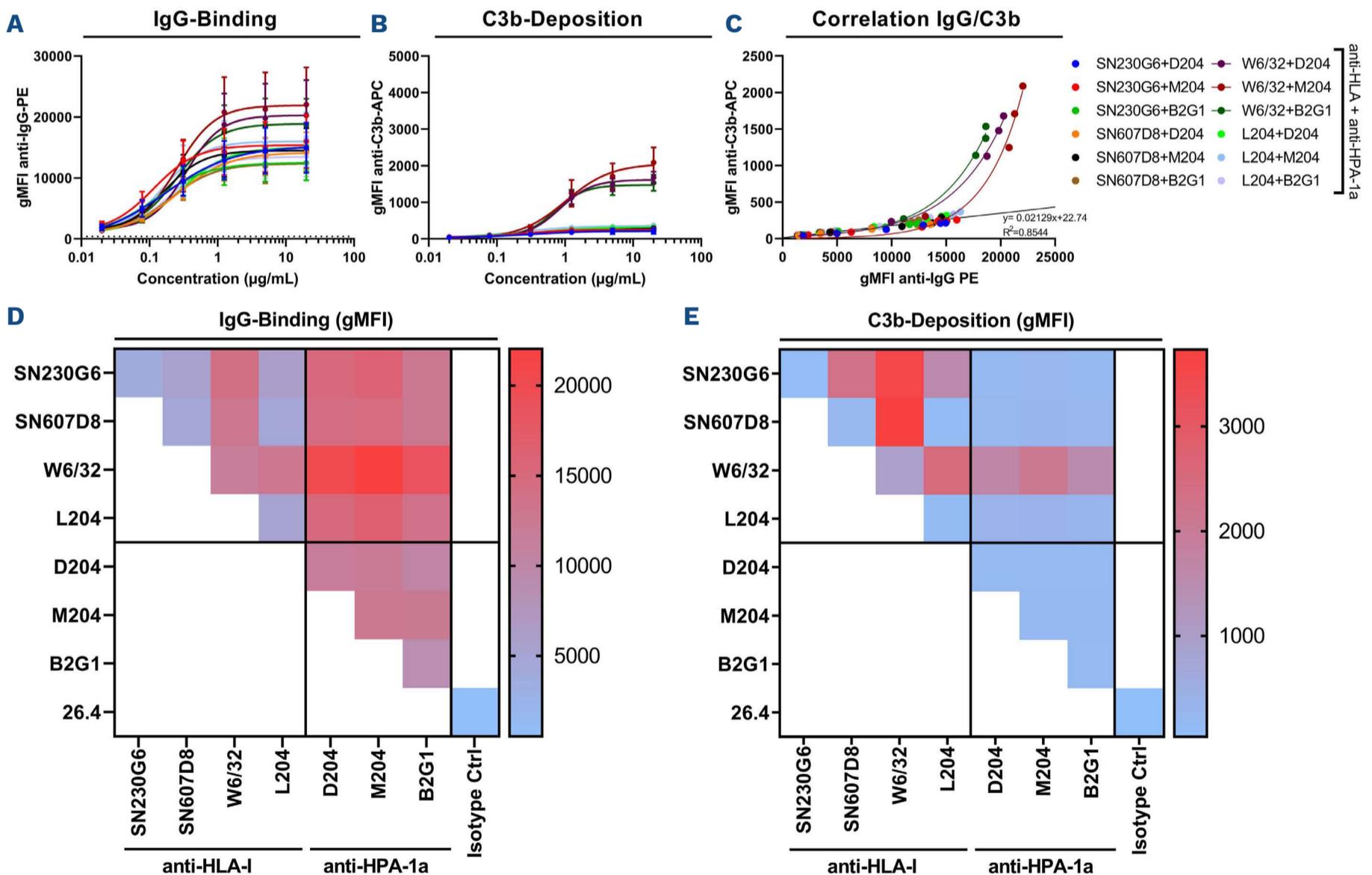
**Figure 3. Binding and complement-activating properties of single unmodified anti-HLA or anti-HPA-1a monoclonal antibodies in the presence of complement-rich serum on the surface of matched platelets analyzed by flow cytometry.** (A) IgG binding is shown, (B) C3b deposition, and (C) the correlation between IgG binding and C3b deposition. Data represent the mean and standard error of mean of three independent experiments using platelet donors with the following HLA-typing: HLA-A01/A02/B07/B08 and HLA-A02/A31/B40/B44. Curve fitting was performed using linear regression and non-linear regression dose-response curves with log(agonist) versus response - variable slope (four parameters) in GraphPad Prism 8.0.2. gMFI: geometric-mean fluorescence intensity.



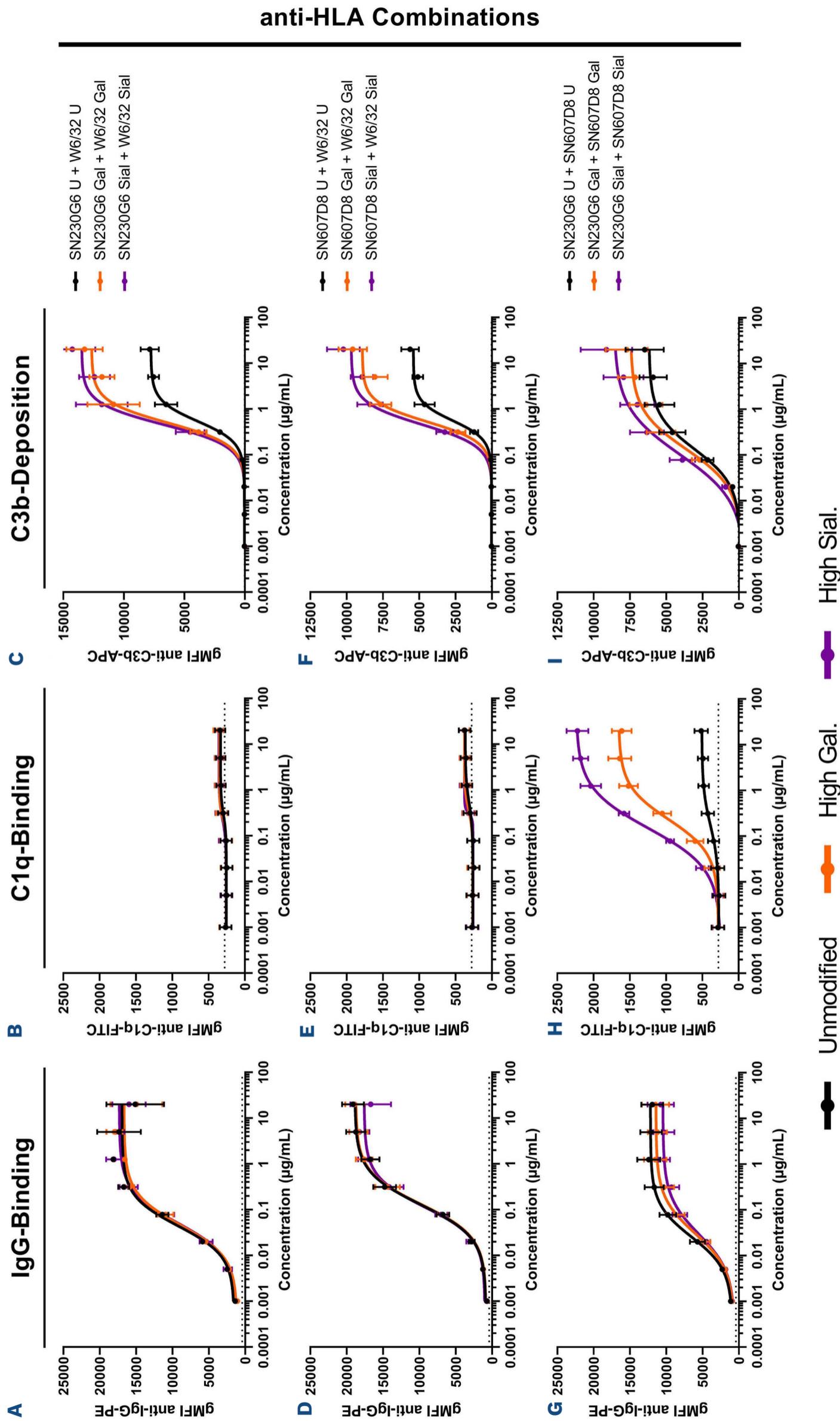
**Figure 4. Binding and complement-activating properties of combinations of unmodified anti-HLA or anti-HPA-1a monoclonal antibodies in the presence of complement-rich serum on the surface of matched platelets analyzed by flow cytometry.** (A-C) Simultaneous incubation of different anti-HLA monoclonal antibody combinations. (D-F) Different combinations of anti-HPA-1a monoclonal antibodies. IgG binding is shown in (A, D), C3b deposition in (B, E), and the correlation between IgG binding and C3b deposition in (C, F). Data represent the mean and standard error of mean of three independent experiments using platelet donors with the following HLA-typing: HLA-A01/A02/B07/B08 and HLA-A02/A31/B40/B44. Curve fitting was performed using linear regression and non-linear regression dose-response curves with log(agonist) versus response - variable slope (four parameters) in GraphPad Prism 8.0.2. gMFI: geometric-mean fluorescence intensity.

ing affinity<sup>27,32</sup> and the surface expression of their targets. The monoclonal antibodies SN230G6, SN607D8 and L204 were only able to bind HLA-A2 molecules and therefore showed similar maximum binding. W6/32 binds all HLA class I molecules<sup>33</sup> and showed a maximum binding capacity similar to that of the anti-HPA-1a antibodies. Despite significant binding of all antibodies, neither anti-HPA-1a nor anti-HLA antibodies provoked, by themselves, high levels of complement deposition on platelets, as determined by the amount of C3b deposition (Figure 3B), with the exception of W6/32. Nevertheless, the small amount of complement deposition correlated directly with IgG binding (Figure 3C). Again, W6/32 formed an exception, in line with its polymorphic HLA recognition. Complement activation was strongly enhanced when antibodies with different specificities were combined. IgG binding was increased when multiple anti-HLA antibodies were incubated simultaneously (Figure 4A), to a similar

degree as seen in the surface plasmon resonance data (Figure 2). The combinations of anti-HLA antibodies generally increased C3b deposition (Figure 4B), suggesting enhanced classical complement activation. No synergy was observed for the combination of SN607D8 and L204 as these compete for the same binding epitope. Significant synergistic effects were, however, seen for all other antibody combinations (Figure 4B, *Online Supplementary Figure S3A*), with C3b deposition surpassing a linear correlation (Figure 4C). As expected, when using different combinations of anti-HPA-1a antibodies (all with identical single amino-acid epitopes), no synergistic effects were observed (Figure 4D-F, *Online Supplementary Figure S3B*). The strongest IgG binding was observed when combining anti-HLA and anti-HPA-1a IgG (Figure 5A). Interestingly, however, no synergistic complement activation was observed between anti-HLA and anti-HPA-1a antibodies, except for the combinations including W6/32 (Figure 5B, C



**Figure 5. Binding and complement-activating properties of combinations of unmodified anti-HLA and anti-HPA-1a monoclonal antibodies in the presence of complement-rich serum on the surface of matched platelets analyzed by flow cytometry.** (A-C) Different combinations of anti-HLA and anti-HPA-1a monoclonal antibodies. IgG binding is shown in (A), C3b deposition in (B), and the correlation between IgG binding and C3b deposition in (C). (D, E) Heatmaps of the amount of IgG binding and C3b deposition when incubating with individual or combinations of anti-HLA and anti-HPA-1a monoclonal antibodies at 20 μg/mL, calculated as the average geometric mean fluorescence intensity. Data represent the mean and standard error of mean of three independent experiments using platelet donors with the following HLA-typing: HLA-A01/A02/B07/B08 and HLA-A02/A31/B40/B44. Curve fitting was performed using linear regression and non-linear regression dose-response curves with log(agonist) versus response - variable slope (four parameters) in GraphPad Prism 8.0.2. gMFI: geometric-mean fluorescence intensity.



**Figure 6. Binding and complement-activating properties of combinations of glycoengineered anti-HLA monoclonal antibodies in the presence of complement-rich serum.** IgG binding, C1q binding and C3b deposition were measured using flow cytometry. Data are arranged showing IgG binding in the first column (A, D, G), C1q binding in the second column (B, E, H), and C3b deposition in the last column (C, F, I). The data represent three independent experiments using a platelet donor with the following HLA-typing: HLA-A02/A26/B35/B44. Curve fitting was performed using nonlinear regression dose-response curves with log(agonist) versus response – variable slope (four parameters) in Graphpad Prism 8.0.2. gMFI: geometric-mean fluorescence intensity; U: unmodified; Gal: high galactose; Sial: high sialic acid.

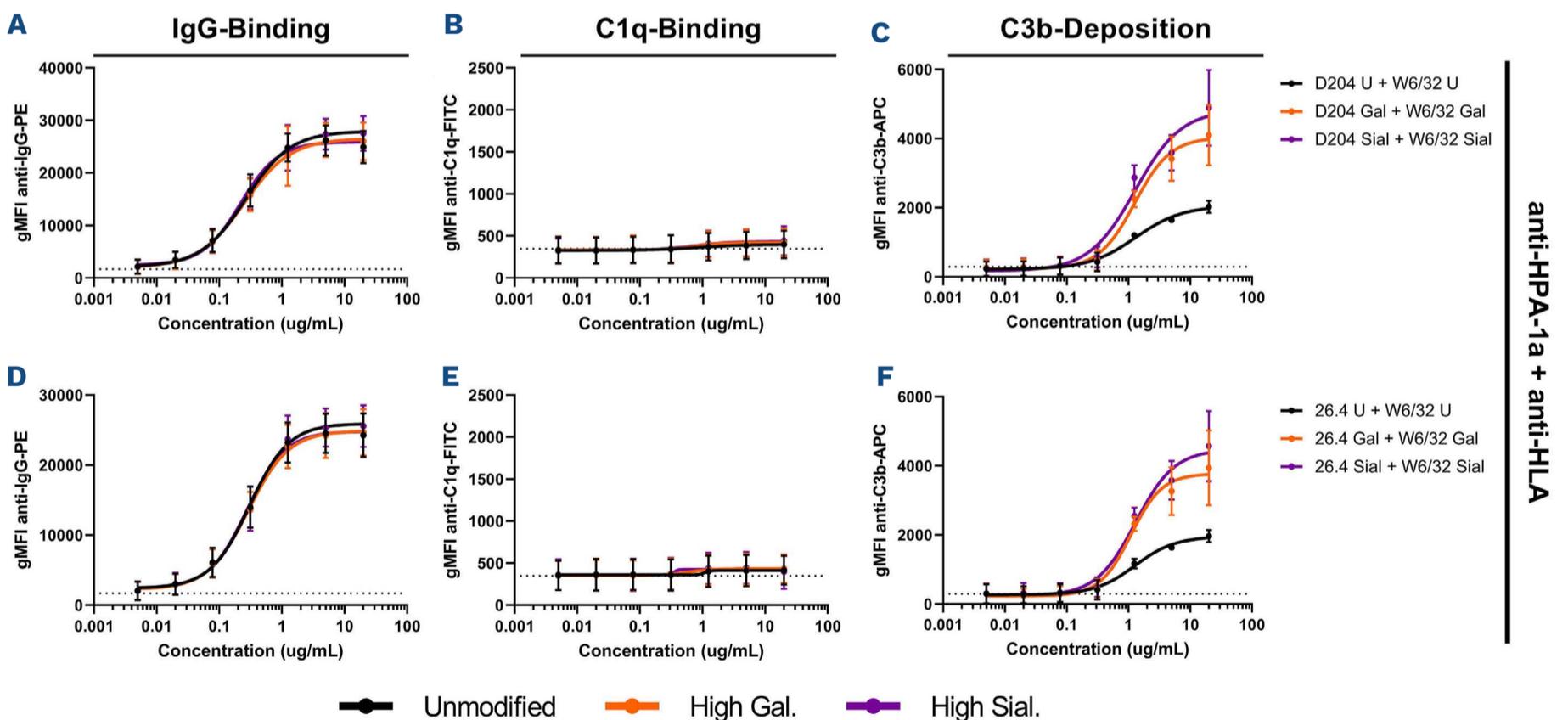
and *Online Supplementary Figure S3C-F*). An overview of the relative binding and complement deposition of all antibody combinations is depicted as heatmaps (Figure 5D, E), clearly illustrating the superior complement-activating potential of the anti-HLA antibodies, especially in combinations, and then particularly with the promiscuous W6/32, recognizing all HLA-I alleles.

### Enhanced Fc galactosylation and sialylation of anti-platelet monoclonal antibodies enhance complement activation on platelets

We then investigated the IgG binding, C1q binding and C3b deposition of the same anti-HLA and anti-HPA-1a antibody combinations after glycoengineering, resulting in enhanced galactosylation and sialylation (Figures 6 and 7). As expected, no differences were observed for antigen-binding between IgG-glycovariants (Figure 6A, D, G and Figure 7A, D). C1q binding was particularly prominent for the combination of SN230G6+SN607D8 and not for the combinations with W6/32 (Figure 6B, E, H and Figure 7B, E). For the combination of SN230G6+SN607D8 a striking effect of elevated Fc galactosylation, and additionally of elevated sialylation, was observed, with a 4-fold increase in maximum C1q binding (Figure 6H, *Online Supplementary Figure S4*). Substantial effects of glycoengineering were also evident on C3b deposition for all antibody combinations (Fig-

ure 6C, F, I and Figure 7C, F). Elevated Fc galactosylation increased C3b deposition 1.3- to 2-fold, compared to the unmodified antibody combinations (*Online Supplementary Figure S4F-J*), and the deposition was slightly further increased, up to 2.5-fold, by elevated sialylation. These higher levels of complement deposition most likely translate into more complement-dependent lysis as more cell death was observed for these glycovariants, when performing the experiments with non-fixed platelets (*Online Supplementary Figure S5*). When comparing the maximum responses of C1q binding and C3b deposition of all antibody combinations, the differences between glycovariants were highly significant (*Online Supplementary Figure S4*).

These results led to the conclusion that multiple anti-HLA antibodies, with different epitopes, are capable of binding to a single HLA-Class I molecule on the surface of platelets in an allo-immune response (Figure 8A). The glycan composition of these antibodies, especially Fc galactosylation, affects Fc:Fc interactions (Figure 8B)<sup>24,25</sup> and thereby promotes the formation of hetero-hexameric IgG complexes between different anti-HLA antibodies but also between anti-HLA and anti-HPA-1a antibodies. These hexamers are the optimal platform for C1q to bind (Figure 8C), which subsequently activates the classical complement pathway, leading to the formation of the MAC-complex and platelet lysis (Figure 8D).

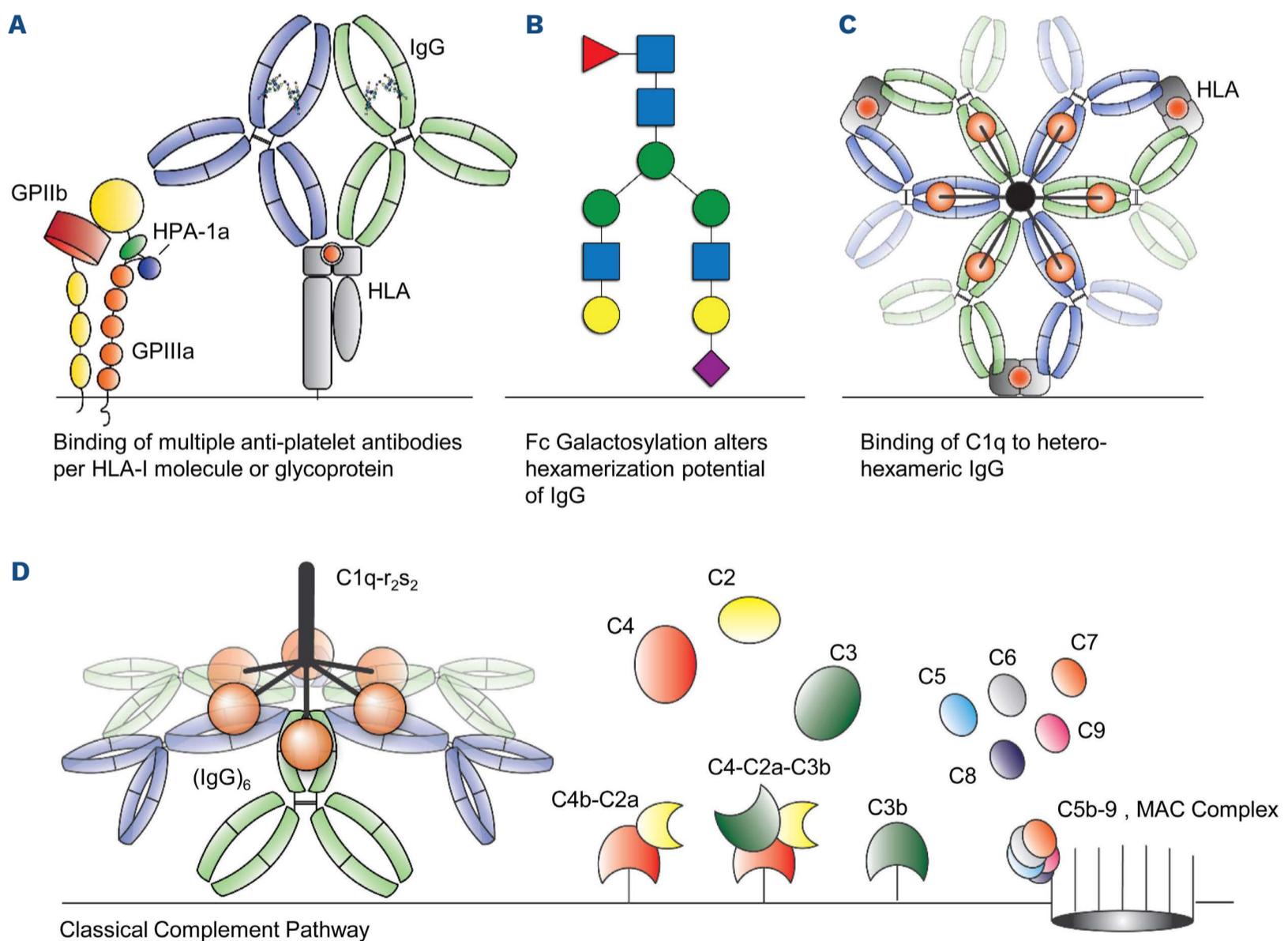


**Figure 7. Binding and complement-activating properties of combinations of glycoengineered anti-HPA-1a and anti-HLA monoclonal antibodies in the presence of complement-rich serum.** IgG binding, C1q binding and C3b deposition were measured using flow cytometry. Data are arranged showing IgG binding in the first column (A, D), C1q binding in the second column (B, E), and C3b deposition in the last column (C, F). The data represent three independent experiments using different platelet donors with the following HLA-typing: HLA-A02/B35/B40 and HLA-A02/A24/B07/B35. Curve fitting was performed using nonlinear regression dose-response curves with log(agonist) versus response – variable slope (four parameters) in Graphpad Prism 8.0.2. gMFI: geometric-mean fluorescence intensity; U: unmodified; Gal: high galactose; Sial: high sialic acid.

## Discussion

Anti-HLA and anti-HPA antibodies formed after pregnancies or incompatible platelet transfusions can play a devastating role in immune PR. They are responsible for the rapid clearance of donor platelets, theoretically via several immunological pathways, such as complement-dependent cytotoxicity, antibody-dependent cellular cytotoxicity and antibody-dependent cellular phagocytosis. The role of FcγR-mediated phagocytosis, in which opsonized platelets are phagocytosed by macrophages in the spleen, has been studied in detail and is an important clearance pathway.<sup>34–38</sup> However, the role of the complement system, capable of both direct complement-dependent cytotoxicity and stimulation of myeloid antibody-dependent cellular phagocytosis through C3b opsonization,<sup>39</sup> in immune PR by anti-HLA or anti-HPA antibodies has been implied but inadequately investigated in clinical studies.<sup>40</sup> In contrast to solid organ

transplantation, in which C1q-binding anti-HLA antibodies correlated with antibody-mediated rejection,<sup>41,42</sup> Jackman *et al.* found no significant correlation between corrected platelet count increments and either C1q binding or low-level anti-HLA IgG antibodies.<sup>43,44</sup> However, C1q-fixing solid-phase screening showed significantly better corrected count increments when compatible platelets were selected for transfusion.<sup>45</sup> Importantly, a pilot trial with the complement inhibitor eculizumab showed that a single injection resolved PR in four out of ten patients.<sup>46</sup> Platelet activation is also known to induce complement activation<sup>47–49</sup> and prolonged storage of platelet concentrates could lead to increased levels of complement components (C4d, C3a, C5a and C5b-9), which can accelerate the complement cascade once they are transfused into patients.<sup>50</sup> Furthermore, *in vitro* work has clearly shown the ability of anti-HLA and anti-HPA antibodies to activate the complement system on the surface of human platelets.<sup>14,51–54</sup> Together, these results



**Figure 8. The alloimmune response leading to platelet lysis following transfusion of incompatible platelets.** (A–D) Incompatible platelet transfusions result in the formation of anti-platelet antibodies, which can be directed against HLA and/or HPA. A polyclonal antibody response will lead to the binding of multiple antibodies, recognizing different epitopes, to a single HLA-class I molecule or glycoprotein on the surface of platelets. Fc-glycosylation affects the potential of antibodies to form hetero-hexamers with neighboring Fc-tails. These hexamers are the optimal platform for C1q, leading to activation of the C1q-r<sub>2</sub>-s<sub>2</sub> complex, starting the complement cascade by depositing C4b and C3b in the process, which ultimately leads to the formation of the membrane attack complex and lysis of platelets. MAC: membrane attack complex.

strongly implicate complement in the pathogenesis leading to platelet clearance by anti-HLA or anti-HPA antibodies in immune PR.

One of the factors that can affect the complement-activating properties of an antibody is its Fc glycosylation composition.<sup>20,22</sup> We previously found that the Fc glycosylation profile of anti-HLA antibodies in patients with PR showed varying levels of galactosylation (~40-80%), bisection (~5-30%) and sialylation (~3-30%).<sup>19</sup> Interestingly, fucosylation levels were comparable to those of the total IgG1 (~85-100%), whereas afucosylated IgG are specifically evoked in alloimmune responses, i.e., anti-HPA-1a alloantibodies in fetal neonatal alloimmune thrombocytopenia,<sup>19,23</sup> and against enveloped viruses. Afucosylated IgG increases the binding affinity to FcγRIIIa and FcγRIIIb by approximately 20- to 40-fold and directly translates into increased associated effector functions.<sup>19-21,23,55-57</sup> Fc galactosylation and sialylation of antigen-specific IgG is often increased after recent or active immunization, as seen after COVID-19 infection,<sup>21</sup> vaccination<sup>58,59</sup> or alloimmunization.<sup>19,23</sup> While bisection has been reported to have no effect on either complement or Fc-receptor binding or function, galactosylation has been found to enhance complement activity through increased C1q binding.<sup>20,22,25</sup> The effect of Fc sialylation on complement activity is less clear. On the one hand, Quast *et al.* showed that Fc sialylation impaired complement-dependent cytotoxicity,<sup>60</sup> whereas Dekkers *et al.* and Wada *et al.* found slightly increased C1q binding by sialylated IgG1.<sup>20,6</sup>

Here, we mimicked the Fc glycosylation profile of anti-HLA monoclonal antibodies as observed in patients with PR, using glycoengineering. We have observed that anti-HLA monoclonal antibodies with different epitopes are able to bind to the same HLA molecules and interact with each other to activate the complement system. This synergy was especially observed for anti-HLA antibodies and only between anti-HLA and anti-HPA-1a antibodies in combination with W6/32. Thus, despite significant IgG binding, not all antibody combinations were able to form hetero-hexamers and thereby initiate the classical complement pathway. Overall, the more antibodies with non-competing epitopes, the more antibodies will bind to the surface of platelets, resulting in more antibodies in close vicinity, enabling Fc:Fc interaction and hetero-hexamer formation, leading to complement activation. When only a single monoclonal antibody is present, either anti-HLA or anti-HPA-1a, the antibody density is too low or the distance between antibodies is too large to form hexamers. In this study, platelet donors were primarily selected on the basis of expression of HLA-A2, which was the only HLA class I (A, B or C) matching the antibody specificity of SN230G6, SN607D8 and L204. W6/32 binds all HLA class I,<sup>33</sup> which possibly explains why it is the

only anti-HLA monoclonal antibody for which synergy with anti-HPA-1a monoclonal antibodies was observed. We hypothesize that either its binding epitope or the overall antibody density is of importance.

We also found that measurement of C1q binding to alloantibody opsonized platelets was a less suitable biomarker for complement activation on platelets compared to measurement of C3b deposition, most likely due to the low affinity of C1q to IgG as opposed to covalent binding of activated C3b to targets.

Furthermore, elevated Fc galactosylation increased the complement-activating properties of anti-HLA and anti-HPA-1a monoclonal antibody combinations and a minor contributing effect was observed for elevated Fc sialylation. These results are in agreement with reports describing increased complement activation by elevated Fc galactosylation,<sup>20,22,25,60</sup> which enhances the Fc:Fc interaction and hexamerization of IgG and thereby improves C1q binding and downstream complement activation.<sup>24,25</sup> We also noted slightly increased complement deposition after opsonization of platelets with antibodies engineered to have elevated levels of sialylation, which is in line with our previous results using antibodies to model antigens (TNP and biotin).<sup>20,24</sup>

In conclusion, a single anti-HLA or anti-HPA-1a monoclonal antibody is an insufficient representation of the *in vivo* opsonization of donor platelets in a patient after a polyclonal alloantibody response, leading to immune PR. It is the interaction between antibodies that leads to efficient activation of the complement system, initiating complement-dependent lysis and/or opsonization for subsequent phagocytosis of platelets. Furthermore, the anti-HLA antibodies observed in PR patients are highly galactosylated and occasionally sialylated, which enhance the complement-activating properties of the antibodies even further. Our work provides more insight into the mechanisms of classical complement activation of anti-platelet antibodies, which can be formed as a consequence of alloimmunization in fetal-neonatal alloimmune thrombocytopenia and immune PR but also in auto-immune diseases, such as immune thrombocytopenia. It especially highlights the importance of pre-transfusion HLA and HPA-matching between donor and recipient or functional matching based on the complement-activating potential of patients' antibodies. Furthermore, our work also underscores the findings of the pilot trial by Vo *et al.*<sup>46</sup> that inhibition of complement activation could improve the effectiveness of platelet transfusions in HLA or HPA immunized patients. However, larger randomized trials are required to investigate this further.

#### Disclosures

*No conflicts of interest to disclose.*

**Contributions**

TLJvO, RK, LP, CEvS, MdH, MW, JV and GV designed and supervised the experimental work. TLJvO, JJO, AEHB, CAMK and JN performed experiments and collected data. DMG, JYM, WJEvE, AM and SH developed vital reagents. TLJvO and GV wrote the manuscript, which was edited by all authors. All authors analyzed and interpreted data and approved the manuscript.

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**Data-sharing statement**

Data can be requested by contacting Dr. Gestur Vidarsson (G.Vidarsson@sanquin.nl).

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