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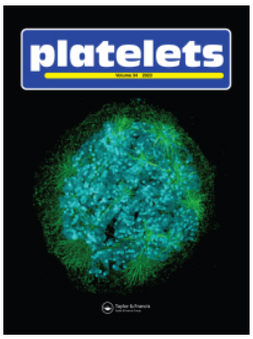

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Phagocytosis of platelets opsonized with differently glycosylated anti-HLA hIgG1 by monocyte-derived macrophages

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Abstract

Immune-mediated platelet refractoriness (PR) remains a significant problem in the setting of platelet transfusion and is predominantly caused by the presence of alloantibodies directed against class I human leukocyte antigens (HLA). Opsonization of donor platelets with these alloantibodies can result in rapid clearance after transfusion via multiple mechanisms, including antibody dependent cellular phagocytosis (ADCP). Interestingly, not all alloimmunized patients develop PR to unmatched platelet transfusions, suggesting variation in HLA-specific IgG responses between patients. Previously, we observed that the glycosylation profile of anti-HLA antibodies was highly variable between PR patients, especially with respect to Fc galactosylation, sialylation and fucosylation. In the current study, we investigated the effect of different Fc glycosylation patterns, with known effects on complement deposition and FcγR binding, on phagocytosis of opsonized platelets by monocyte-derived human macrophages. We found that the phagocytosis of antibody- and complement-opsonized platelets, by monocyte derived M1 macrophages, was unaffected by these qualitative IgG-glycan differences.

Keywords

Alloimmunization, anti-HLA, Fc glycosylation, phagocytosis, platelet transfusion

History

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Introduction

Platelet transfusions are a frequently administered therapy to reduce mortality and hemorrhagic complications in patients with thrombocytopenia. A major problem in the setting of platelet transfusions is platelet refractoriness (PR), which refers to a recurring inadequate platelet count increment after multiple platelet transfusions. The incidence of PR ranges from 5% to 15% and varies depending on both patient characteristics and platelet product preparation [1–4]. In approximately 20% of PR cases platelet clearance is immune mediated, predominantly caused by the presence of alloantibodies directed against class I human leukocyte antigens (HLA) and occasionally human platelet antigens (HPA) [5–9]. Opsonization of donor platelets with these alloantibodies can result in clearance shortly after transfusion, via antibody dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and/or antibody dependent cellular phagocytosis (ADCP) [10–16]. The major management strategy currently available for heavily transfused alloimmunized patients is extensive matching of platelet transfusion products to prevent PR and subsequent worse clinical outcomes [7–9]. Interestingly, not all alloimmunized patients develop PR to unmatched platelet transfusions [17,18], suggesting variation in qualitative traits of the HLA specific IgG responses between patients.

All IgG molecules contain a conserved *N*-linked glycan at position 297, located in the Fc-region, affecting the antibody's structure and function. The glycan is composed of a biantennary core structure of *N*-acetylglucosamines (GlcNAc) and mannose residues, which can be elongated by a fucose, bisecting GlcNAc and up to two galactose residues, which both can be capped by a sialic acid residue. Antibody Fc glycosylation is highly variable, and altered patterns have previously been described in settings of infection and alloimmunization, which are known to affect antibody effector functions and thereby the progression of associated immune responses [19–27]. For example, the absence of the fucose residue leads to an increased binding affinity of the antibody to FcγRIIIa/b, which can result in increased associated effector functions, such as ADCC and ADCP [19–22,25,28,29]. In addition, galactosylation is especially relevant for complement activation [21,24,30,31]. We and others have recently shown that galactosylation increases the antibody's capacity to activate the classical complement pathway, through enhanced hexamerization, which in

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turn increases complement deposition and CDC activity [23,30]. Sialylation slightly enhances complement activation even further [21,23,32], while the bisecting GlcNAc has no effect on either complement activation nor FcγR binding [21].

Previously, we characterized the glycosylation profile of anti-HLA Class I antibodies, detected in hemato-oncological patients receiving platelet transfusions [33] and in patients diagnosed with PR [20]. The anti-HLA IgG-specific glycosylation profile was highly variable between patients. For the majority of patients, we observed an increase in Fc galactosylation and sialylation of HLA-specific IgG in comparison to total IgG. Additionally, a few patients, 2 out of 35, also developed anti-HLA antibodies with extremely low fucosylation levels [33].

In the context of alloimmunization and PR, transfused platelets are mainly thought to be cleared by mononuclear macrophages in the spleen after opsonization with anti-HLA or -HPA alloantibodies [8,10,11,13,34,35]. Several pathways exist in which phagocytes can detect their targets for phagocytosis, via non-opsonic and opsonic receptors. The non-opsonic receptors are essential for the recognition of pathogen-associated molecular patterns (PAMPs) and apoptotic cells, whereas the opsonic receptors recognize cells targeted for clearance with opsonins, such as IgG and complement components. The most important and efficient phagocytic receptors for recognizing IgG are FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) and Complement Receptor 3 (CR3 or CD11b/CD18) for the recognition of iC3b and C3d [36,37]. Splenic macrophages have a high expression of CR3, FcγRI, FcγRII, and FcγRIII [14,38–41]. Upon opsonization of platelets by IgG or complement components, platelets become susceptible to bind phagocytic receptors expressed by splenic macrophages, which leads to subsequent phagocytosis and destruction. This process is further enhanced by the slow passage of platelets through splenic sinusoids [7,11,14]. However, involvement of the complement system as well as platelet-intrinsic factors, such as platelet apoptosis and activation upon opsonization, have also recently been suggested to be involved in reduced platelet survival in PR [12,15,35,42–45].

Interestingly, very little is known about the effect of antibody Fc glycosylation on platelet clearance by macrophages upon opsonization with differently glycosylated anti-HLA alloantibodies. Especially in light of recent findings regarding effects of antibody Fc glycosylation on both FcγR binding and complement deposition, it is important to gain more insight into effects of antibody Fc glycosylation on clearance mechanisms involved in PR upon alloimmunization. In the current study, we investigated the influence of different Fc glycosylation patterns that are known to affect complement deposition and FcγR affinity on opsonized platelet phagocytosis by monocyte-derived human macrophages. Monocyte-derived M1 macrophages were used due to their high expression levels of FcγRs as well as CR3, which are also highly expressed by human splenic macrophages. We found that an increase in complement deposition and/or FcγRIIIa/b affinity via altered Fc glycosylation profiles did not affect phagocytosis of IgG opsonized platelets by human monocyte-derived macrophages in this *in vitro* model.

Material and methods

Human blood samples

Monocytes were isolated from buffy coats obtained from anonymized Sanquin blood donors after written informed consent. Platelets were isolated from citrated whole blood from anonymous, healthy volunteers with informed written consent. Monocytes and platelets were not obtained from the same individuals. All procedures were approved by the Sanquin Ethical Advisory Board and are in accordance with the declaration of Helsinki and Dutch regulations.

Production of recombinant glycoengineered anti-HLA monoclonal antibodies

The production and glycoengineering techniques of the anti-HLA mAbs used in this study have been described in detail [46–52]. In brief, the protein sequences of the variable regions of all anti-HLA mAbs were used to assemble pcDNA3.1 expression vectors encoding for full human IgG1 and PG LA LA Fc mutants (P329 G, L234A, and L235A), which are incapable of binding complement and FcγRs [53]. The expression vectors were used for the production of recombinant antibodies in our in-house HEK Freestyle system. For obtaining antibodies with certain desired glycan-profiles, the chemical inhibitor 2-deoxy-2-fluoro-L-fucose (2FF, Carbosynth) was used to decrease fc fucosylation and 5 mM D-galactose (Sigma Aldrich) and the constructs coding for the enzymes β-1,4 galactosyltransferase 1 (B4GALT1) and β-galactoside alpha-2,6-sialyltransferase 1 (ST6GALT1) were used prior/during transfection to increase galactosylation and sialylation. Monoclonal antibodies were purified 6 days post-transfection and subjected to liquid chromatography–mass spectrometry-based IgG Fc glycosylation analysis [46,47,54].

Surface Plasmon Resonance (SPR)

The antibody binding to human FcγR classes was assessed by surface plasmon resonance (SPR) on the IBIS M×96 (IBIS technologies) as previously described [55,56]. All C-terminally biotinylated hFcγRs were spotted using a Continuous Flow Microspotter (Wasatch Microfluidics) onto a single SensEye G-streptavidin sensor (Ssens), which allows binding affinity measurements for each antibody to all hFcγR simultaneously. The biotinylated hFcγRs were spotted in threefold dilutions, ranging from 30 nM to 1 nM for hFcγRIIa-H131, hFcγRIIIa-F158, hFcγRIIIb-NA1 and hFcγRIIIb-NA2, from 10 nM to 0.3 nM for hFcγRIIa-R131 and hFcγRIIb, and ranging from 100 nM to 3 nM for hFcγRIIIa-V158 in PBS supplemented with 0.075% Tween-80 (VWR, M126–100 ml), pH 7.4. Regeneration was performed after each sample with 10 nM Gly-HCl, pH 2.0. The dissociation constant (KD) was calculated using equilibrium fitting to Rmax = 500. Analysis and calculation of all binding data was done with Scrubber software version 2 (Biologic Software) and Excel.

Monocyte isolation and differentiation to monocyte-derived macrophage

Monocytes were isolated from buffy-coat derived PBMCs using CD14+ magnetic microbead separation (Miltenyi Biotec) and subsequently frozen until further use as previously described [44,57]. Flow cytometry was used to determine monocyte purity, which was >90%. Monocytes were differentiated to monocyte-derived macrophages as described [58]. Briefly, monocytes were thawed on day 0 and differentiated in a 24-well culture plate ($0.25x10^6$ monocytes per well) in the presence of 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF, CellGenix) in IMDM 1640 (Lonza) containing 10% fetal calf serum (Bodinco) 100 U/mL penicillin and 100 U/mL streptomycin (both Gibco) at 5% CO_2 37°C. Cells were cultured for a total of 9 days and fresh medium and GM-CSF were added on day 3 of culture.

Platelet isolation, labeling and opsonization

Platelets from healthy volunteers with known HLA typing were isolated from Platelet Rich Plasma (PRP), by centrifuging citrated

whole blood at 125 *g* for 20 minutes, optimized with methods described previously to avoid platelet activation [12,16]. Hereafter, 10 vol% ACD (acid citrate dextrose, 85 mM Na_3-citrate·2 H_2O, 71 mM citric acid·H_2O and 111 mM D-glucose) was added. The PRP was centrifuged (850 *g* for 8 min) and washed 2 times with wash buffer (WB; 36 mM citric acid·H_2O, 103 mM NaCl, 5 mM KCl, 5 mM EDTA, 5.6 mM D-glucose, pH 6.5). The platelets were set to a concentration of 6×10^8 cells/mL in PBS and incubated for 20 min at RT with 3.75 µM PKH26 (Sigma Aldrich) on a roller bank. 10 vol% FCS was added to terminate the labeling process and the labeled platelets were washed with WB and resuspended in PBS +0.5% BSA. 5×10^6 platelets were incubated with equal volumes of recombinant anti-HLA antibodies and pooled complement sufficient human serum, for 30 min at RT. For some conditions, the serum was pre-incubated for 30 min at 56°C to inactivate complement. The platelets were washed 3 times with PBS +0.5% BSA +5 mM EDTA and resuspended in macrophage culture medium. Complement deposition (C3b) was assessed by staining a small fraction of the platelets with anti-complement C3b/iC3b-APC Antibody Clone: 3E7/C3b (1/250, Biolegend)

Phagocytosis of opsonized platelets by macrophages

For the phagocytosis assay, opsonized platelets were incubated for 30 min at 37°C with allogeneic macrophages at a 1:40 macrophage:platelet ratio. For some conditions, the macrophages were pre-incubated for 30 min at RT with 10 µg/mL FcγR blocking antibodies (anti-CD16, anti-CD32, and/or anti-CD64) and isotype control. Anti-CD64 (clone 10.1, blocking FcγRI) was ordered as f (ab')2 fragments from Ancell Corporation, whereas the anti-CD32 (clone AT10, blocking FcγRIIa/b/c), anti-CD16 (clone 3G8, blocking FcγRIIIa/b) and isotype (anti-biotin) were cloned and produced as hIgG1 N297A P329 G, L234A, and L235A, making them incapable of binding C1q and FcγRs [53]. Hereafter, cells were washed with PBS and harvested using 130 mM lidocaine (Sigma Aldrich) with 10 mM EDTA (Merck). After harvesting, macrophages were kept on ice and washed with ice-cold PBS +0.5% bovine serum albumin (BSA, Sigma Aldrich) + 2 mM EDTA (Merck) and subsequently with ice-cold PBS. After harvesting, cells were fixed with 3.7% paraformaldehyde (Sigma Aldrich) in PBS for 15 min at RT. Next, cells were washed with PBS +0.5% BSA and stained using APC-labeled anti-HLA-DR (clone L243, BD Biosciences) and BV421-labeled anti-CD42a (clone ALMA.16, BD Biosciences) for 20 min at RT in PBS +0.5% BSA. Cells were washed with PBS +0.5% BSA and analyzed using BD LSR II flow cytometer and imaging flow cytometry (ImageStreamX Mark II Imaging Flow Cytometry, Merck Millipore). Flow cytometry data was analyzed using Flowjo v 10.8.1; cells were gated based on FSC/SSC, single cells, and HLA-DR+, after which cells were gated on positivity for PKH26-labeled platelets and anti-CD42a-BV421 (PKH26+ CD42a-: phagocytosed; PKH+ CD42a+: bound platelets; Supplementary Figure S3A). Imaging flow cytometry data was analyzed using IDEAS v6 software and involved gating on Aspect Ratio Intensity/Area Ch01 to gate cells, and subsequently cells in focus (Gradient RMS Ch01) and HLA-DR+ cells to gate macrophages, after which cells were gated on positivity for PKH26-labeled platelets and anti-CD42a-BV421 (Supplementary Figure S3B).

Statistics

Statistical analyses were executed within GraphPad Prism 8.02 (263) for Windows. Bar graphs were analyzed using ordinary one-way ANOVAs with Dunnett multi-comparison test. The level of significance was set at $p \leq .05$. *, **, *** and **** denote a statistical significance of $p < .05$, ≤.01, ≤.001 and ≤.0001, respectively.

Results

In order to determine the effect of Fc glycosylation of anti-HLA alloantibodies on complement and/or FcγR-mediated phagocytosis of platelets, a system was set up to monitor platelet phagocytosis by human monocyte-derived macrophages. Anti-HLA monoclonal antibodies (mAbs) with altered glycosylation profiles (Supplemental Figure S1A-C), were produced as described [46,47] and subjected to liquid chromatography–mass spectrometry based IgG Fc glycosylation analysis to confirm the intended glycosylation profiles (Supplemental Figure S1D).

Platelets were incubated with unmodified and glycoengineered anti-HLA hIgG1 mAbs (SN230G6, SN607D8 and W6/32) in presence of complement sufficient serum, enabling antibody, and complement opsonization. Only combinations of SN230G6 and SN607D8 antibodies resulted in strong C3b deposition, although the pan-HLA class I-recognizing W6/32 (Supplemental Figure S1B) caused complement deposition on its own (Supplemental Figure S1E), in line with our previous observations [47]. Antibodies with elevated galactosylation and sialylation significantly enhanced complement deposition. Afucosylation of the mAbs had no effect on complement deposition (Supplemental Figure S1E). Neither the PG LALA Fc mutant, incapable of binding C1q and FcγRs [53], nor heat inactivated serum (HI serum) resulted in C3b deposition (Supplemental Figure S1F). Binding of these glycoengineered antibodies to human FcγRs was evaluated by SPR array, which confirmed the increase in affinity of the afucosylated variants for FcγRIIIa/b, whereas no affinity differences were observed for FcγRIIa/b (Supplemental Figure S2).

Opsonized PKH26-labeled platelets were co-incubated with monocyte-derived M1-like macrophages (Figure 1A). These differentiated macrophages express all classes of FcγR (FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16)) as well as CR3 (CD11b/CD18) (Figure 1B), all crucial receptors involved in phagocytosis [8,11,36,37,59]. Internalization of platelets was determined employing imaging and conventional flow cytometry (gating strategies are depicted in Supplemental Figure S3A and 3B, respectively). The platelet-specific marker CD42a was used to detect platelets on the exterior of the macrophages (Figure 1C). The majority of platelet positive (PKH+) macrophages was CD42a-. Moreover, using imaging flow cytometry the majority of PKH+ CD42a+ events was shown to be comprised of macrophages that had both phagocytosed (PKH+ CD42a-) and bound (PKH+ CD42a+) platelets simultaneously, indicating the total PKH+ compartment to be relevant for the quantification of platelet phagocytosis (Figure 1D-E and Supplementary Figure S3A, lower panel). Therefore, the total compartment of PKH+ macrophages was analyzed by conventional flow cytometry to assess platelet phagocytosis as exclusion of PKH+ CD42a+ events would lead to an underestimation of phagocytosis efficiency.

Platelet phagocytosis was significantly increased when macrophages were incubated with platelets opsonized with unmodified anti-HLA mAbs SN230G6+SN607D8, compared to unopsonized platelets (Figure 2A,B). Phagocytosis was observed in an antibody concentration-dependent manner with the maximum % of phagocytic MQ at 1 µg/mL. Although the glycoengineered mAbs with elevated galactosylation and sialylation levels significantly enhanced complement deposition on platelets (Supplemental Figure S1E), these did not result in higher levels of subsequent phagocytosis (Figure 2C). Also, opsonization with afucosylated IgG, which increases the affinity to FcγRIII ([21] and

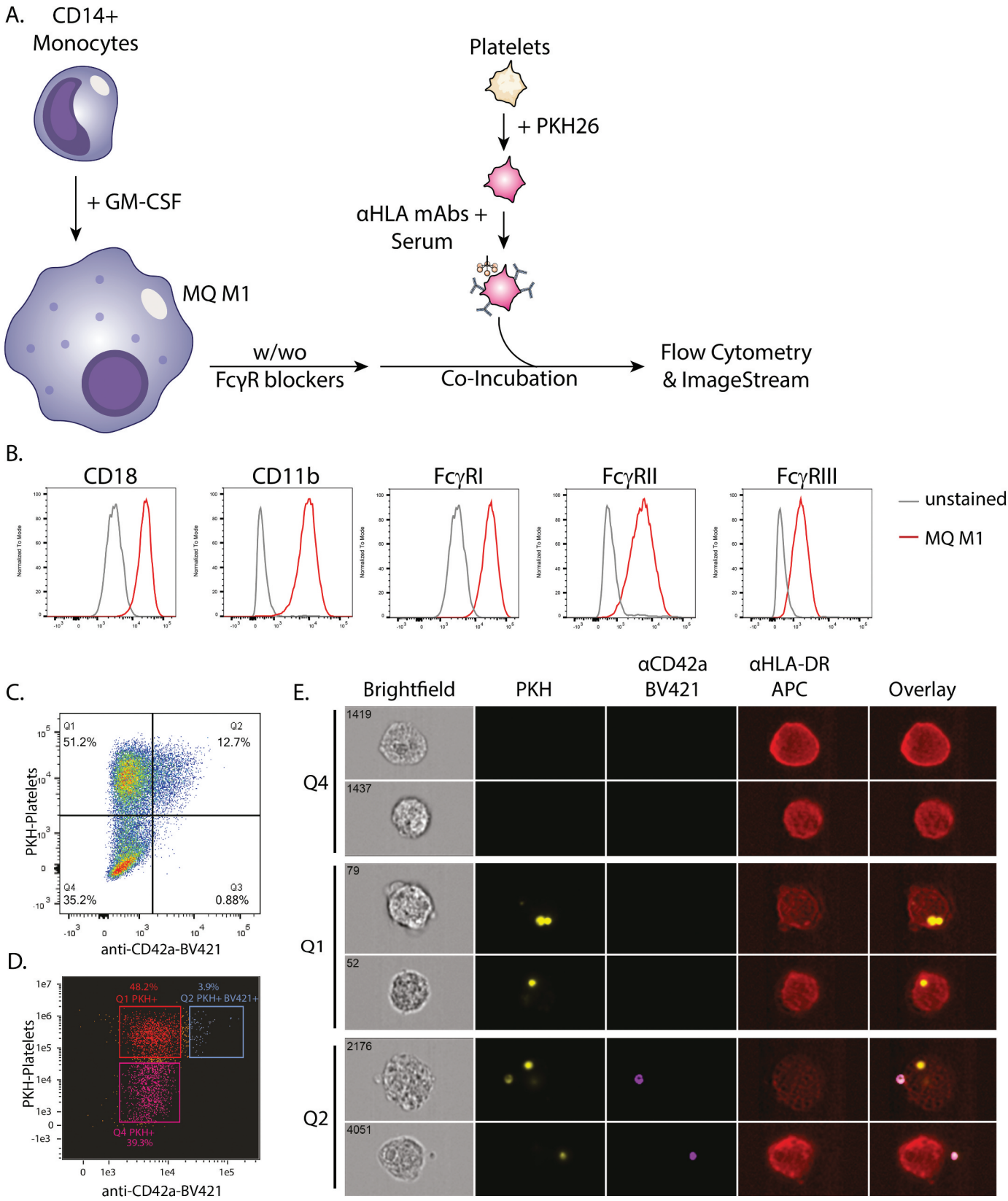

Figure 1. **A)** Schematic overview of the experimental set-up to monitor phagocytosis of opsonized platelets: CD14+ monocytes were cultured for 9 days with GM-CSF to differentiate into monocyte-derived macrophages (MQ M1). Hereafter, the macrophages were pre-incubated with and without FcγR-blockers. Freshly isolated platelets from HLA-A2+ donors were labeled with PKH26 and pre-incubated with unmodified and glycoengineered anti-HLA monoclonal antibodies in the presence of complement sufficient or heat-inactivated (HI) serum, for antibody and complement opsonization. The macrophages and platelets were washed and co-incubated for 30 minutes at 37°C and analyzed by flow cytometry and Imagestream **B)** Expression levels of complement receptor 3 (Cd11b/cd18) and FcγR's (FcγRI, FcγRII, FcγRIII) on the surface of the monocyte-derived macrophages as analyzed by flow cytometry. **C-E)** Internalization of platelets was determined employing **C)** conventional and **D-E)** imaging flow cytometry. Anti-CD42a-BV421 staining, in combination with PKH26, was used to identify platelets attached to the exterior of the cell. Representative images are shown for indicated quadrants obtained by imaging flow cytometry.

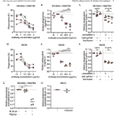

Figure 2. **Phagocytosis of complement and antibody opsonized platelets by monocyte-derived macrophages.** A) Absolute and B) relative levels of phagocytosis of platelets incubated with various concentrations of unmodified hIgG1 anti-HLA monoclonal antibodies (mAb) SN230G6+SN607D8 in the presence of complement sufficient serum. C) Differences in phagocytosis between platelets pre-incubated with unmodified and glycoengineered hIgG1 SN230G6+SN607D8 mAbs in the presence of complement sufficient serum. D) Absolute and E) relative levels of phagocytosis of platelets pre-incubated with various concentrations of unmodified pan-anti-HLA Class I hIgG1 mAb W6/32 in the presence of complement sufficient serum. F) Differences in phagocytosis between platelets pre-incubated with unmodified and glycoengineered hIgG1 W6/32 mAb in the presence of complement sufficient serum. G,H) Relative phagocytosis levels of platelets pre-incubated with 1 µg/ml unmodified or PG-LALA Fc mutant anti-HLA mAbs (SN230G6+SN607D8 or W6/32) in the presence of complement sufficient or heat-inactivated (HI) serum. A-H) the level of phagocytosis (%) was defined as the percentage of the PKH26+ macrophage fraction (Q1+Q2). The data represents the mean and SD of 2 technical replicates of 2-3 different monocyte donors used in 2-4 independent experiments. For each independent experiment also a different platelet donor was used. The color of the data points indicates the different monocyte donors. For normalization, the level of phagocytosis of platelets incubated with 1 µg/ml unmodified anti-HLA mAbs was set at 1, as indicated. An ordinary one-way ANOVA with Dunnett's multi-comparison test was performed for the statistical analysis. *$p \leq .05$, ****$p \leq .0001$ and ns = non-significant.

Supplemental Figure S2), did not enhance phagocytosis compared to the unmodified antibodies (Figure 2C). Moreover, incubating the platelets with the separate unmodified anti-HLA mAbs SN230G6 or SN607D8 resulted in enhanced phagocytosis (Supplementary Figure S3A,B) and was not affected by antibody Fc glycan modifications (Supplementary Figure S3C,D). Similar to the combination SN230G6 + SN607D8, incubating the platelets with the pan-HLA Class I antibody W6/32 resulted in

a significantly increased phagocytosis compared to unopsonized platelets (Figure 2D,E). Phagocytosis was observed in an antibody concentration-dependent manner and was unaffected by any of the antibody glycan modifications (Figure 2E,F). These results suggested that the observed phagocytosis was not primarily mediated through either complement receptors or FcγRIIIa/b.

In line with this, platelets opsonized with HI sera did not result in diminished phagocytosis by macrophages (Figure 2G). However, platelet opsonization with PG LA LA Fc mutants significantly abrogated phagocytosis (Figure 2G), suggesting phagocytosis to be FcγR dependent but complement independent. The same trend was observed for W6/32, for which phagocytosis appeared to be unaffected by HI serum (Figure 2H). However, it has to be noted that incubating platelets with the unmodified W6/32 antibody in presence of complement sufficient serum only leads to low C3b deposition (Supplemental Figure S1E).

To preliminarily assess which FcγR may be involved in the phagocytosis of the anti-HLA opsonized platelets, FcγR-blocking antibodies were used. While blocking either FcγRII or FcγRIII resulted in a negligible decrease in phagocytosis, blocking FcγRI alone or in combination with FcγRII and FcγRIII, resulted in reduced phagocytosis of opsonized platelets by monocyte-derived macrophages (Supplemental Figure S4E-F). We hypothesized that upon blockade of FcγRI, increased affinity for FcγRIII may become apparent for platelets opsonized with low fucosylated antibodies. Strikingly, the presence of the FcγRI blocking antibody did not seem to affect the phagocytosis of low fucosylated W6/32 antibodies (Supplemental Figure S4G).

Discussion

Alloimmunization to HLA or HPA antigens is associated with a higher incidence of platelet refractoriness (PR). However, not all alloimmunized patients develop PR upon transfusion with unmatched platelet products, which may be explained by variations in the quality of allospecific IgG responses between patients as well as the differential immunogenicity of non-matched transfusion products [1–4,17,18]. Previously, we reported on differences in Fc glycosylation between total IgG and anti-HLA specific IgG detected in patients receiving platelet transfusions [33] and patients diagnosed with PR [20]. We hypothesized that these differences in Fc glycosylation might explain the observed inter-donor variation in immune responses. Observed differences in glycosylation included both increased sialylation/galactosylation of anti-HLA specific IgG, for the majority of the patients, as well as a severe decrease in fucosylation in a few patients [33]. These differences in antibody Fc glycosylation can affect antibody effector functions, including ADCC, ADCP, and/or CDC, and thereby platelet clearance upon opsonization of platelets with differently glycosylated anti-HLA alloantibodies [22,23]. Previously, we have shown that anti-HLA mAbs with different specificities, binding simultaneously to the same HLA-molecules interact synergistically to activate complement on platelets [16,47]. Also, elevated galactosylation and sialylation of anti-HLA mAbs enhanced complement deposition and CDC activity even further [47]. However, the effect of anti-HLA antibody Fc glycosylation on platelet phagocytosis by macrophages has not been studied before.

Here, we used an *in vitro* system to investigate the effect of Fc glycosylation of anti-HLA antibodies on phagocytosis of antibody and complement opsonized platelets by monocyte-derived macrophages. Anti-HLA mAbs were glycoengineered with increased levels of galactosylation and sialylation or decreased fucosylation levels, as observed in patients receiving platelet transfusions and with PR. We found that phagocytosis of opsonized platelets by monocyte-derived macrophages was unaffected by differential Fc glycosylation of anti-HLA antibodies and resulting differences in complement deposition or increased FcγRIII affinity, possibly because phagocytosis appeared primarily mediated through FcγRI.

Few studies have looked into the effect of antibody Fc glycosylation on platelet phagocytosis. Previously, we observed an increase of platelet phagocytosis upon anti-TNP opsonization of TNP haptenized platelets with low fucosylated antibodies by neutrophils and FcγRIIIa+ monocytes, whereas no effect of decreased Fc fucosylation was found on phagocytosis by FcγRIIIa- monocytes [20]. In the context of ITP, phagocytosis of anti-GPIIb/IIIa opsonized platelets by splenic macrophages was found to be mediated primarily through FcγRI and FcγRIII [38]. Another study by Bruggeman et al., observed no effect of low Fc fucosylation on anti-RhD opsonized erythrocyte phagocytosis by human monocyte-derived macrophages, whereas NK-cell mediated ADCC was affected [60]. These studies have indicated varying effects of antibody Fc glycan structures on the clearance of platelets and red blood cells by different effector cells, which may largely be explained by the effector cell FcγR expression profile (e.g. expression of high-affinity receptor FcγRI). In line with the literature, we found that monocyte-derived macrophages expressed both CR3 and FcγRI-III [60,61] similarly to *ex vivo* splenic macrophages [14,38]. However, it would be interesting to directly compare the FcγR expression levels and phagocytosis efficiency of both *in vitro* generated and *ex vivo* isolated splenic macrophages to gain more insight on the role of splenic clearance in the setting of PR.

Furthermore, *in vivo* phagocytosis may also differ from our observations due to the presence of aspecific antibodies saturating the high affinity FcγRI, thereby allowing for effects of increased affinity for FcγRIII upon low antibody Fc fucosylation. Interestingly, we did not observe increased platelet phagocytosis upon opsonization by low fucosylated antibodies in conditions in which FcγRI was blocked. More research exploring *in vivo* clearance dynamics are warranted, for example through *in vitro* phagocytosis in the presence of serum or aspecific antibodies or through animal experiments as a proxy for PR in patients.

Next to FcγR-mediated phagocytosis, platelet clearance upon transfusion leading to PR can also be mediated (in part) by complement activation on the platelet surface [8,15]. It can occur directly through the classical complement pathway, leading to the formation of a membrane attack complex (MAC) [35] or indirectly by targeting the platelets with complement deposition for phagocytosis, through binding of iC3b to CR3 on splenic macrophages [36,37,62]. Complement deposition on platelets upon opsonization with anti-HLA alloantibodies has previously been described [16] and shown to be affected by increased levels of galactosylation and sialylation [23,24]. In our current setup, we found no evidence for complement mediated phagocytosis by monocyte-derived macrophages, most likely due to the high affinity of FcγRI. Moreover, platelets were opsonized at room temperature to avoid direct complement mediated lysis, which may play an important role in clearance of transfused platelets *in vivo* in the setting of PR. Furthermore, next to increased complement deposition, platelets may also become activated or damaged due to either opsonization by anti-HLA antibodies or through platelet storage lesion. Apoptosis and subsequent phosphatidylserine exposure have been described to result in increased phagocytosis of platelets by dendritic cells and macrophages [10,12,44]. In addition, platelet activation can occur after binding of some anti-HLA mAbs via crosslinking between HLA and FcγRIIa, through Syk signaling, leading to aggregation and increased phagocytosis by monocyte-derived macrophages [12]. The protocols used in the current study were optimized for minimal platelet activation by means of the addition of calcium chelators during isolation; although baseline activation cannot be completely avoided when handling platelets and can be induced upon opsonization by

anti-HLA antibodies and complement deposition [12,16]. However, the introduced Fc glycosylation changes of the anti-HLA mAbs have no effect on FcγRIIa affinity/binding [21] and thereby are unlikely to affect platelet activation. Therefore, we do not consider it likely that differences in platelet activation due to antibody Fc glycosylation changes influence our observed findings.

In summary, we have shown that phagocytosis of anti-HLA opsonized platelets by monocyte-derived macrophages appeared primarily mediated by FcγRI, and differential antibody Fc glycosylation did not affect phagocytosis efficiency in the current *in vitro* setup. However, future studies are warranted, especially to account for the multiple pathways potentially involved in platelet clearance upon alloimmunization *in vivo* in the setting of PR, which can all be affected by the glycosylation profile of alloantibodies. Detailed understanding of platelet clearance upon alloimmunization is important to gain more insight in the pathophysiology of PR and the potential use of complement inhibitors or other immune suppressors in the treatment of PR upon platelet transfusion.

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Authorship contributions

T.L.J.v.O., J.S., J.V., S.M.v.H., M.W., A.t.B. and G.V. designed and supervised experimental work. T.L.J.v.O., J.S., J.N., C.A.M.K., A.E.H. B. performed experiments and collected data. S.H. and A.M. developed vital reagents. T.L.J.v.O, J.S., A.t.B. and G.V. wrote the manuscript, which was edited by all authors. All authors analyzed and interpreted data and approved the manuscript.

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