ARTICLE

Anti-HLA antibodies with complementary and synergistic interaction geometries promote classical complement activation on platelets

Maaike Rijkers,[1] David Schmidt,[2] Nina Lu,[1] Cynthia S.M. Kramer,[3] Sebastiaan Heidt,[3] Arend Mulder,[3] Leendert Porcelijn,[4] Frans H.J. Claas,[3] Frank W.G. Leebeek,[5] A.J. Gerard Jansen,[1,5] Ilse Jongerius,[6] Sacha S. Zeerleder,[6] Gestur Vidarsson,[2] Jan Voorberg[1,7] and Masja de Haas[3,4,8]

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

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ABSTRACT

High titers of HLA antibodies are associated with platelet refractoriness, causing poor platelet increments after transfusions in a subset of patients with HLA antibodies. Currently, we do not know the biological mechanisms that explain the variability in clinical responses in HLA alloimmunized patients receiving platelet transfusions. Previously we showed that a subset of anti-HLA IgG-antibodies induces FcγRIIa-dependent platelet activation and enhanced phagocytosis. Here, we investigated whether anti-HLA IgG can induce complement activation on platelets. We found that a subset of anti-HLA IgG induced complement activation via the classical pathway, causing C4b and C3b deposition and formation of the membrane-attack complex. This resulted in permeabilization of platelet membranes and increased calcium influx. Complement activation also caused enhanced α-granule release, as measured by CD62P surface exposure. Blocking studies revealed that platelet activation was caused by FcγRIIa-dependent signaling as well as HLA antibody induced complement activation. Synergistic complement activation employing combinations of monoclonal IgGs suggested that assembly of oligomeric IgG complexes strongly promoted complement activation through binding of IgGs to different antigenic determinants on HLA. In agreement with this, we observed that preventing anti-HLA-IgG hexamer formation using an IgG-Fc:Fc blocking peptide, completely inhibited C3b and C4b deposition. Our results show that HLA antibodies can induce complement activation on platelets including membrane attack complex formation, pore formation and calcium influx. We propose that these events can contribute to fast platelet clearance *in vivo* in patients refractory to platelet transfusions with HLA alloantibodies, who may benefit from functional-platelet matching and treatment with complement inhibitors.

Introduction

HLA alloantibodies can develop upon transfusion,[1] transplantation[2] and during pregnancy.[3,4] Leukoreduction of platelet transfusion products reduced HLA immunization by more than 50 percent,[5] however, 20-30% of patients receiving multiple platelet transfusions still develop HLA alloantibodies.[1,3,6] It is known that high titers of HLA antibodies are associated with platelet refractoriness.[7] About 12-15% of patients, in need of chronic platelet transfusion support, become refractory to

Correspondence:
m.dehaas@sanquin.nl

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platelet transfusions and repeatedly show poor increments of platelet counts caused by rapid clearance of the transfused platelets.[3,6] HLA-matched platelet transfusions are commonly used for treatment of HLA alloimmunized patients. However, treatment with HLA-matched platelet concentrates is challenging due to the fact that it is often difficult to find a sufficiently high number of compatible donors for refractory patients. Current transfusion approaches for HLA alloimmunized patients are exclusively based on binding specificity of HLA antibodies but do not take into account functional properties of circulating HLA antibodies. Here, we have further characterized the pathogenic properties of different types of HLA-antibodies.

Previously, we showed that a subset of human monoclonal HLA antibodies and patient sera containing HLA antibodies induce FcγRIIa-dependent platelet activation and enhanced phagocytosis by macrophages.[8] However, it remains unclear to which extent this HLA antibody-mediated activation of platelets contributes to platelet clearance and which other mechanisms contribute to platelet clearance in refractory patients. In the current study we have focused on the role of complement activation by HLA antibodies.

Platelets have been shown to promote complement activation via several mechanisms. It has been reported that activation of platelets, which leads to α-granule release and subsequent CD62P surface exposure, triggers deposition of complement C3b. C3b can bind directly to CD62P exposed on platelet surfaces, suggesting that platelet activation promotes complement deposition on platelets.[9,10] In this case, the alternative pathway of the complement cascade is initiated, where binding of IgG and subsequent C1q deposition is bypassed. Subsequent binding of C3b facilitates further complement activation, finally leading to the formation of a membrane attack complex (MAC), also called the C5b-9 complex.[9] Peerschke *et al.* also showed that C1q can bind to agonist-activated platelets, indicating a possible role for platelets in complement activation *via* the classical complement pathway.[11] Platelet activation can also induce complement activation in the fluid phase, where the release of chondroitin sulfate by activated platelets is the trigger.[12] Also, binding of C3 to activated platelets has been suggested to stimulate formation of platelet-leukocyte interactions.[13] In addition, IgG-complexes can induce platelet aggregation, which is strongly enhanced by addition of C1q.[14] Mouse monoclonal antibodies (mAbs) directed to beta-2 microglobulin (β2M) and a pan HLA mAb have been shown to induce C3b binding and complement dependent cytotoxicity (CDC) on platelets when added at high concentrations.[15,16]

Platelet transfusion-related adverse events might be (partly) explained by complement activation in platelet products as standard storage conditions have been shown to induce complement activation with increasing C3a and C4d levels found in platelet concentrates upon prolonged storage.[17]

Here, we studied complement activation on platelets induced by HLA antibodies. Human HLA mAbs and sera from patients with refractory thrombocytopenia containing HLA antibodies were used to study the effect of complement deposition, formation of a MAC, platelet activation and permeabilization. Our results show that a subset of anti-HLA antibodies can induce complement activation on platelets. We also showed that blocking pathways leading to complement deposition on platelets, prevented complement activation induced pathogenicity of HLA antibodies. Based on our findings, we propose that functional matching of platelet concentrates may be used to further improve treatment of refractory patients with HLA antibodies. Our results also suggest that complement-directed therapeutic interventions may be utilized to increase donor platelet survival in HLA-immunized refractory patients.

Methods

Materials

Detailed information on materials used can be found in the *Online Supplementary Data.*

Patient sera

Blood samples of patients refractory to platelet transfusion were used following informed consent according to the Dutch established codes of conduct for responsible use of patient material and as approved by our institute.[18] HLA antibody specificities in patient sera were determined by single antigen bead assay on Luminex platform (Labscreen SA, One Lambda, Inc.). Twelve sera positive for HLA antibodies and negative for other platelet specific antibodies were used.

Platelet isolation

Platelets were isolated from citrated whole blood from healthy volunteers with known HLA type. All donors gave written informed consent and blood was drawn in accordance with Dutch regulations and after approval from the Sanquin Ethical Advisory Board in accordance with the Declaration of Helsinki. Platelets were isolated and washed as described before.[19] Platelets were resuspended in platelet assay buffer (10 mM HEPES, 140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2$, 10 mM glucose and 0.5 mM $NaHCO_3$, pH 7.4).

Complement deposition and platelet activation

Platelets were used at a final concentration of $0.08*10^8$ platelets/ml and mixed with indicated inhibitors, antibodies/sera (heat inactivated for 30 min at 56°C, 25% of total sample volume) and complement source (normal human serum) (25% of total volume) in platelet assay buffer. Mixtures were incubated for 30 min at 37°C while shaking (300 rpm), and then fixed by adding formaldehyde (final concentration of 1%). Platelets were washed with platelet assay buffer and stained for flow cytometry. Anti-CD42a-FITC or CD41-APC-Cy7 antibodies were used to gate for platelets. Platelets were stained with mouse anti-human CD62P-PE and mouse anti-human C3b-APC or mouse anti-human C4b-APC. For measuring the formation of a MAC, platelets were stained with rabbit anti-human C5b-9 antibody followed by the secondary antibody chicken anti-rabbit Alexa 647. Mean fluorescent intensities and/or percentage positive platelets were measured using flow cytometer FACSCanto II (Becton Dickinson, Franklin Lakes, NJ, USA).

Pore formation

Complement assay was initiated as described above. After 30 min incubation at 37°C, platelets were washed once and resuspended in assay buffer containing live/dead marker for 30 min at room temperature (RT). Platelets were washed and stained with anti-CD42a (for gating purposes), anti-C3b and CD62P and analyzed using flow cytometry.

Calcium influx

Platelets were loaded with calcein violet and fluo-4 for 30 min at RT, washed and resuspended in platelet assay buffer. Complement activation assay was initiated as described above. After 20 min incubation at 37°C, platelets were diluted in assay buffer and calcium influx was measured immediately using flow cytometry by measuring fluo-4 signal. Platelets were gated upon their calcein violet fluorescence.

Data and statistical analysis

Flow cytometry data was analyzed using FlowJo version 10 (Ashland, OR, USA). Data are represented as either mean ± standard deviation (SD) or all data points are shown. Statistical analyses were performed using GraphPad Prism 7 Version 7.02 (La Jolla, CA, USA); the analyses used are specified in the respective figure legends.

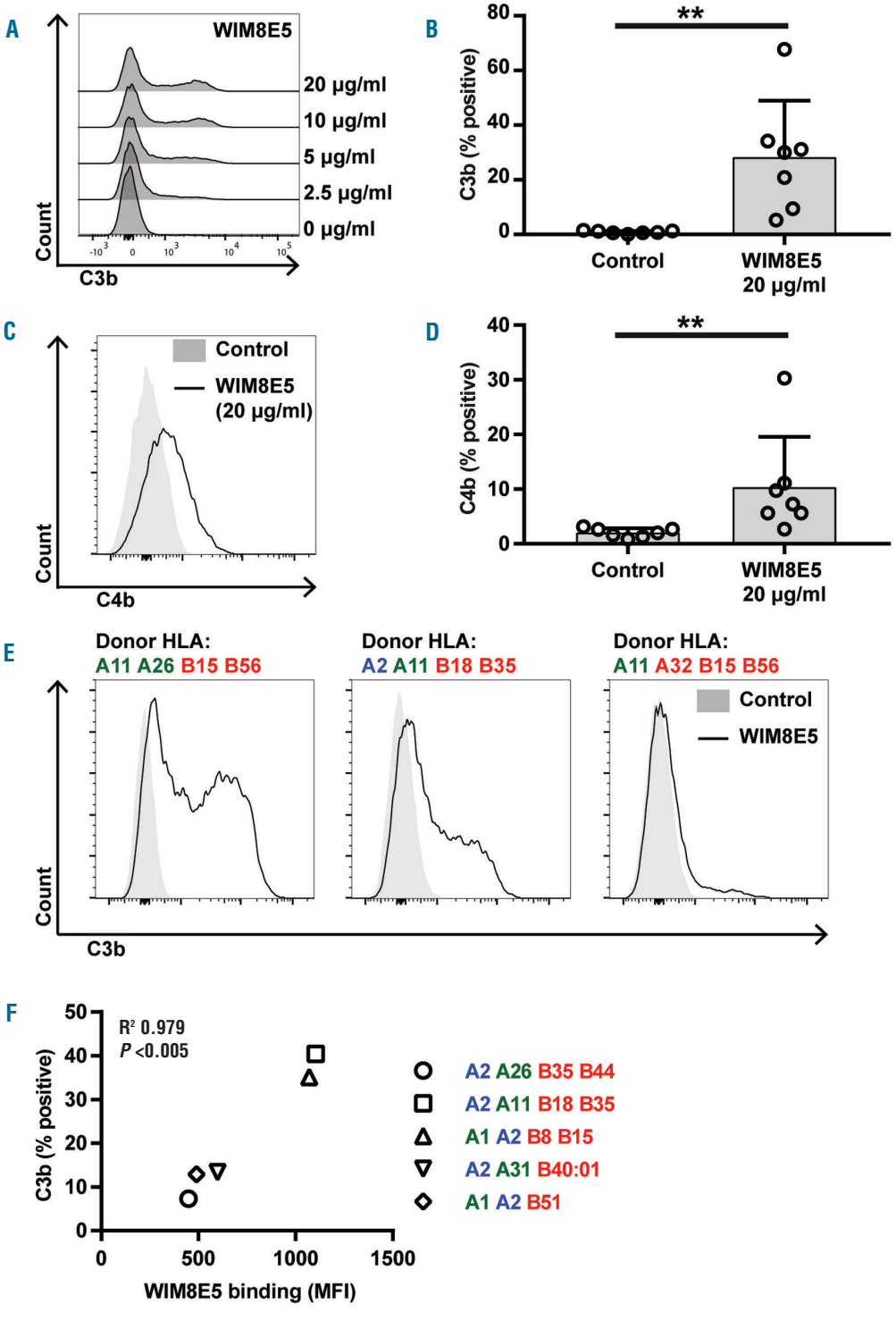


Figure 1. HLA mAb WIM8E5 induces C3b and C4b deposition on platelets. (A) Platelets were incubated with increasing concentrations of WIM8E5 and C3b deposition was measured employing flow cytometry. Representative data from 1 donor. (B) C3b deposition on platelets incubated with 20 µg/ml WIM8E5 were compared to control platelets (no HLA antibody added). (n=7 different donors). (C) Representative flow cytometry graph of C4b deposition on platelets incubated with WIM8E5 (20 µg/ml). (D) C4b deposition on platelets from 7 different donors upon incubation with 20 µg/ml WIM8E5. (E) Comparison of C3b deposition on platelets from 3 donors with different amounts of WIM8E5 reactive antigens. Donor HLA types are indicated in the figure, strong WIM8E5 binding antigens in green, weak binding antigens in blue and non-binding antigens in red. (F) Five donors heterozygous for HLA-A2 (and no A3 or A32) with different HLA expression levels (as measured with anti-human IgG in the absence of serum). Level of WIM8E5 binding correlates with percentage C3b positive platelets. Strong binding antigens are indicated in green, weak binding antigens in blue and non-binding antigens in red. MFI: mean fluorescence intensity. **P<0.01.

Results

HLA antibody WIM8E5 induces complement C3b and C4b deposition on platelets

Human HLA mAbs recognizing different epitopes encoded by different HLA class I subtypes (*Online Supplementary Table S1*) were incubated with platelets from donors with an HLA type matching the specificity of the mAbs, in the presence of normal human serum. Complement deposition of C3b on platelet surfaces increased upon incubation with increasing concentrations of HLA mAb WIM8E5 (specific for an epitope present on all HLA-A antigens except for HLA-A3 and-A32 and with reduced binding to HLA-A2) (Figure 1A). Statistically significant increased C3b deposition was observed with 20 μg/ml WIM8E5 (Figure 1B). Also, C4b deposition on platelet surfaces was significantly increased when platelets were incubated with WIM8E5 (Figure 1C-D). A

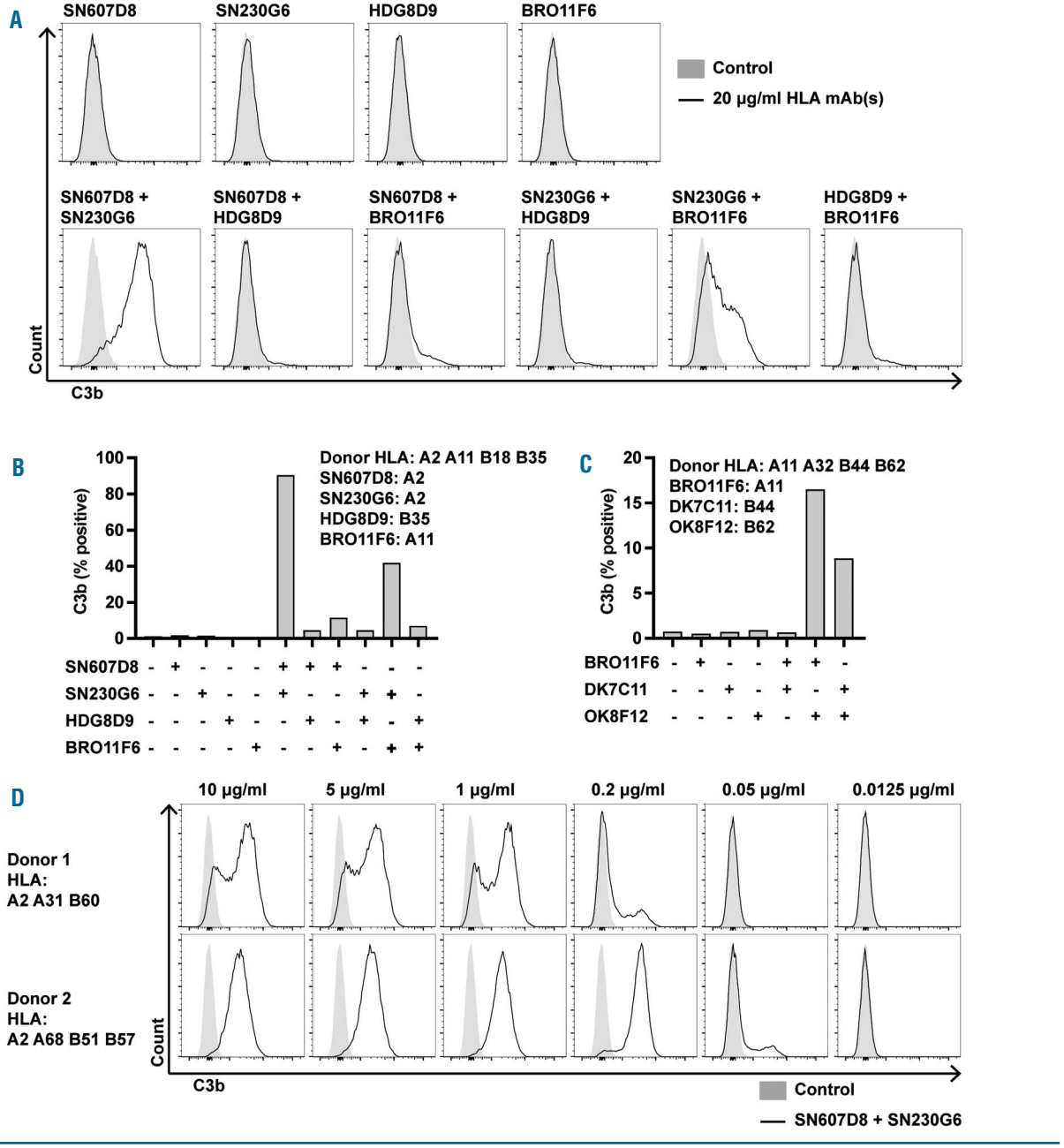


Figure 2. Combinations of HLA mAbs induce enhanced complement activation. (A) Four different HLA mAbs were incubated with HLA matched platelets both separately and in combination (20 μg/ml per antibody) and C3b deposition was measured. (B-C) Examples of C3b deposition induced by HLA mAbs separately and in combination (20 μg/ml per HLA mAb). (D) C3b deposition on platelets of 2 donors by a combination of anti-HLA-A2 antibodies SN607D8 and SN230G6 at 0.0125 - 10 μg/ml. HLA donor 1: A2 A31 B60 HLA donor 2: A2 A68 B51 B57.

strong variation in the level of complement deposition of C3b and C4b was observed between donors, whose various HLA type matched in all cases with the specificity of WIM8E5 (Figure 1B and D). A gene dosage effect was observed; donors with two HLA-A antigen specificities compatible with WIM8E5 binding induced more C3b deposition compared to donors expressing one compatible HLA-A antigen and HLA-A2 (with reduced binding or affinity) (Figure 1E). Only minor C3b deposition was observed when platelets from donors expressing only one compatible HLA-A antigen (e.g., A11 and A32) were used (Figure 1E right panel). Variable levels of C3b deposition on platelets of 5 donors expressing HLA-A2 in combination with another WIM8E5 HLA-A-compatible antigen were observed that correlated with WIM8E5 binding (Figure 1F) This suggests that the level of IgG opsoniza-

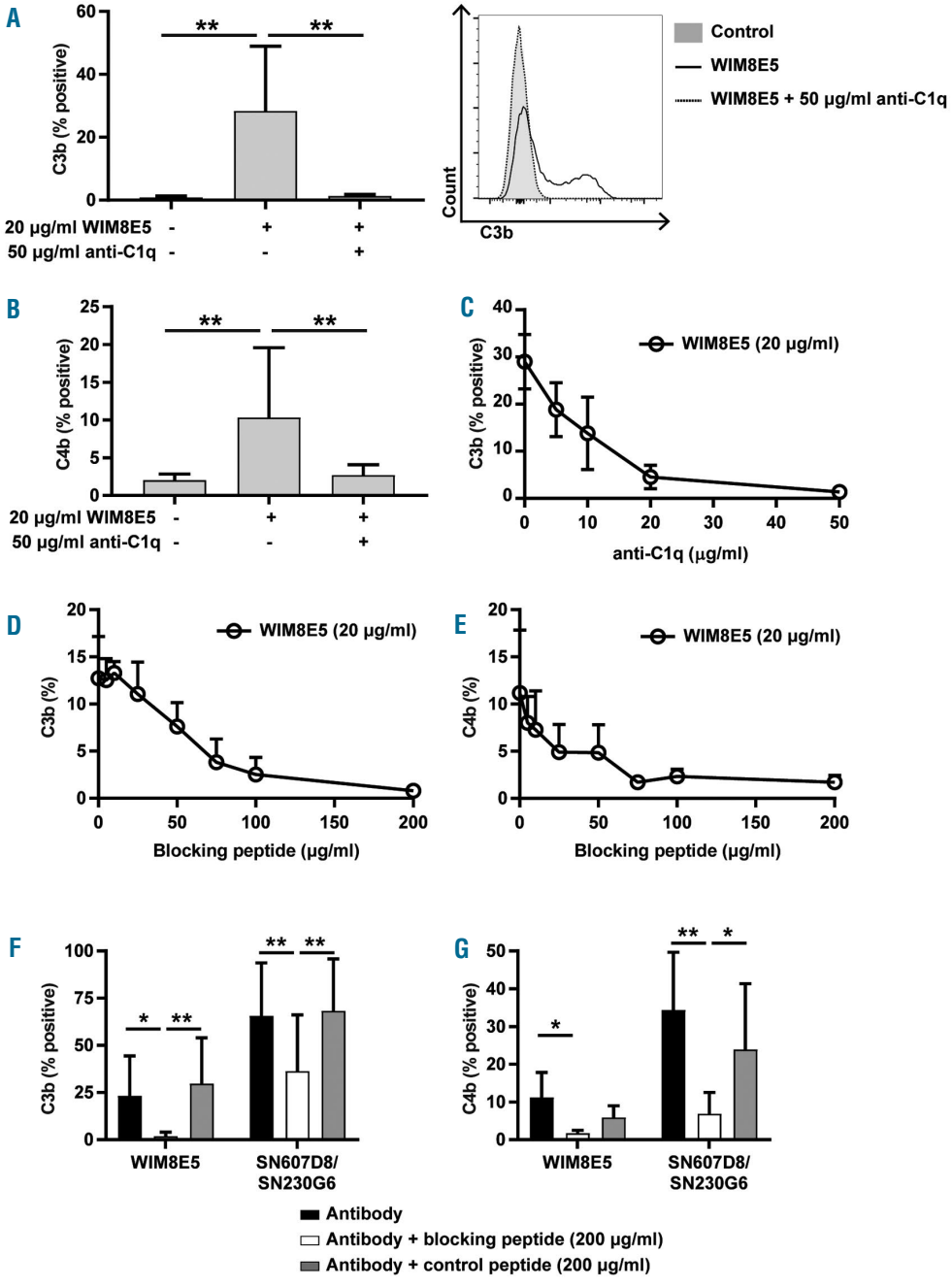


Figure 3. Complement deposition on platelets by HLA mAbs occurs via the classical complement pathway. (A) Pre-incubation of platelets with 50 µg/ml anti-C1q inhibits C3b deposition (n=7), (B) and C4b deposition (n=7). (C) Dose response of increasing concentrations anti-C1q to inhibit C3b deposition induced by 20 µg/ml WIM8E5 (n=4) (D) Effect of increasing concentrations of Fc:Fc blocking peptide DCAWHLGELVWCT on complement deposition induced by WIM8E5 (20 µg/ml) was measured by C3b deposition (n=3), (E) and C4b deposition (n=4). (F) Effect of 200 µg/ml Fc:Fc blocking peptide on C3b deposition by WIM8E5 and SN607D8/SN230G6 was compared to the irrelevant control peptide GWTVFQKRLDGSV (n=4). *P<0.05, **P<0.01.

tion, probably largely affected by the number of available epitopes for WIM8E5, is correlated with the level of platelet complement activation.

Combinations of anti-HLA mAbs induce complement deposition on platelets

Next, less broadly reacting anti-HLA human IgG1 mAbs were tested for their ability to induce C3b deposition on platelet surfaces. No C3b deposition was observed when SN607D8, SN230G6, HDG8D9 and BRO11F6 were incubated separately (Figure 2A; upper panel). However, by combining these HLA mAbs, thereby mimicking the polyclonal nature of HLA alloimmunized patient sera, several combinations promoted C3b deposition on platelets (Figure 2A; lower panel and Figure 2B). This effect was very strong when a combination of anti- HLA-A2 mAbs, SN607D8 and SN230G6, were used (Figure 2B). Also, combinations of monoclonal antibodies anti-HLA-A2

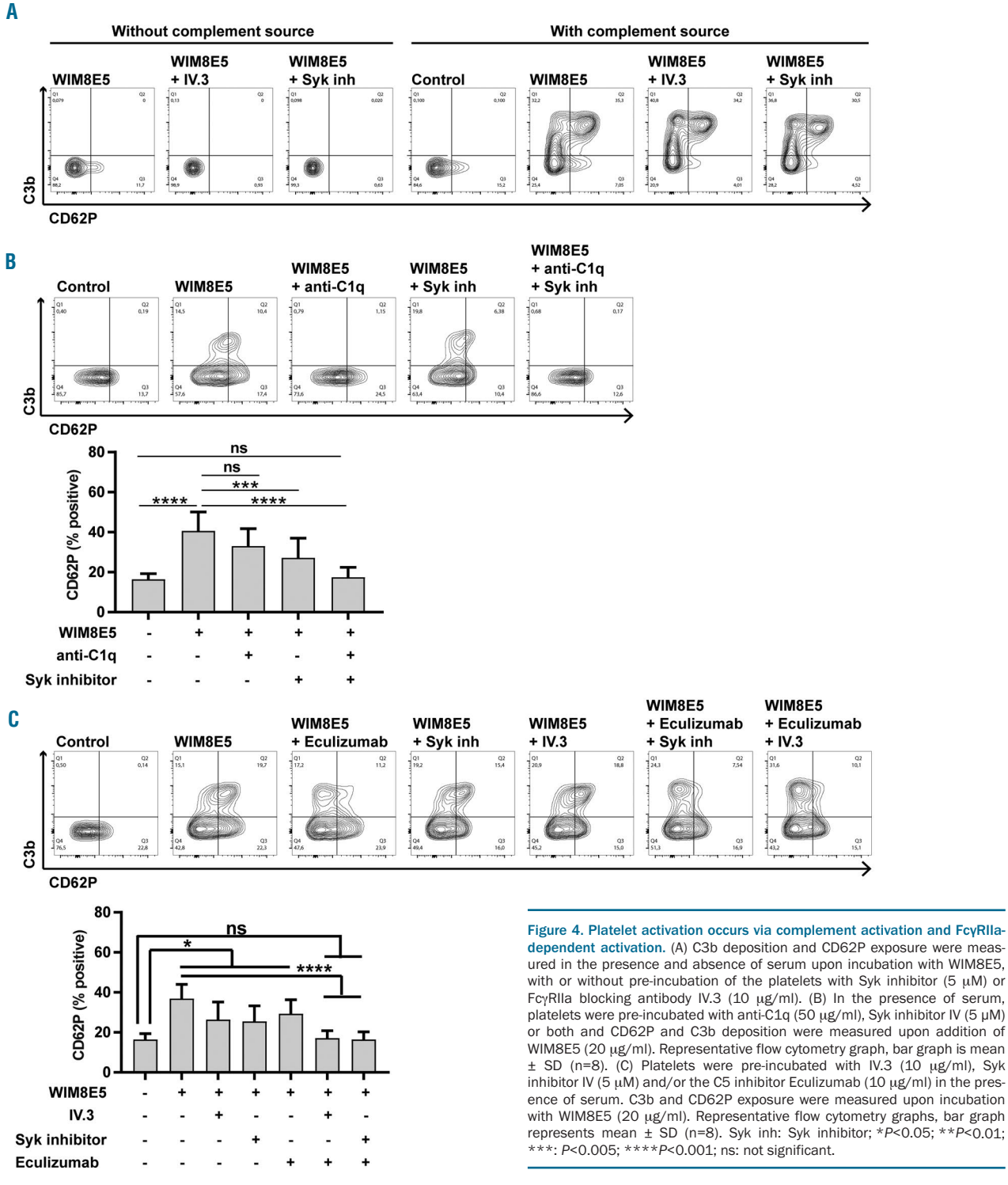


Figure 4. Platelet activation occurs via complement activation and FcγRIIa-dependent activation. (A) C3b deposition and CD62P exposure were measured in the presence and absence of serum upon incubation with WIM8E5, with or without pre-incubation of the platelets with Syk inhibitor (5 μM) or FcγRIIa blocking antibody IV.3 (10 μg/ml). (B) In the presence of serum, platelets were pre-incubated with anti-C1q (50 μg/ml), Syk inhibitor IV (5 μM) or both and CD62P and C3b deposition were measured upon addition of WIM8E5 (20 μg/ml). Representative flow cytometry graph, bar graph is mean ± SD (n=8). (C) Platelets were pre-incubated with IV.3 (10 μg/ml), Syk inhibitor IV (5 μM) and/or the C5 inhibitor Eculizumab (10 μg/ml) in the presence of serum. C3b and CD62P exposure were measured upon incubation with WIM8E5 (20 μg/ml). Representative flow cytometry graphs, bar graph represents mean ± SD (n=8). Syk inh: Syk inhibitor; *P<0.05; **P<0.01; ***: P<0.005; ****P<0.001; ns: not significant.

(SN230G6) and anti-HLA-A11 (BRO11F6) (Figure 2B), anti-HLA-A11 (BRO11F6) and anti-HLA-B52 (OK8F11), or anti-HLA-B44 (DK7C11) and anti-HLA-B62 (OK8F11) induced C3b deposition on platelets. Only when incubated together, the two anti-HLA-A2 antibodies SN607D8 and SN230G6, which bind to opposite sides of the peptide binding groove in HLA-A2,[20,21] were very effective in inducing complement activation. Even at concentrations as low as 0.2 μg/ml, these antibodies induced C3b deposition (Figure 2D). The levels of complement activation are however dependent on the donor. Together, these results suggest that the geometry of binding of anti-HLA antibodies determines whether or not complement deposition can occur.

Complement deposition induced by anti-HLA antibodies occurs via the classical pathway

Both classical- and alternative complement pathway activation have been described to occur on platelets.[9,11,22] To discriminate between these pathways, platelets were

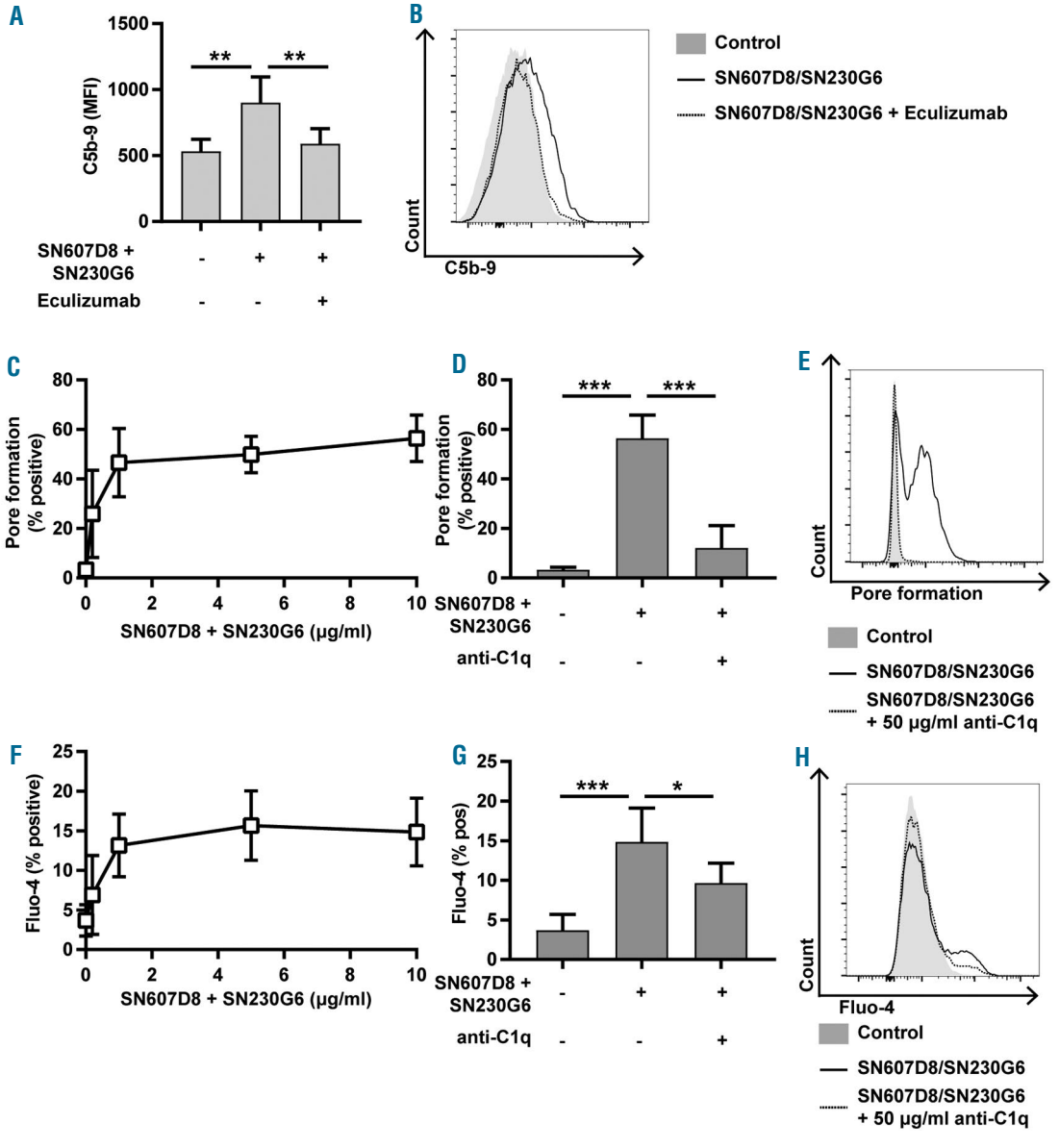


Figure 5. HLA antibodies induce the formation of a MAC, pore formation and calcium influx. (A) Complement activation was induced by SN607D8/SN230G6 and was inhibited by Eculizumab. Formation of a MAC was measured employing an anti-C5b-9 antibody. (n=4) (B) Representative flow cytometry plot showing SN607D8/SN230G6 induced formation of the C5b-9 complex (solid line). In the presence of Eculizumab the formation of the C5b-9 complex is inhibited (dashed line). (C) Pore formation upon incubation with increasing concentrations of SN607D8 and SN230G6 was measured using an impermeable dye, binding to free amines: referred to as live/dead marker (L/D). (n=4) (D) Pore formation was blocked with anti-C1q. (n=4) (E) Representative flow cytometry plot of pore formation measured by L/D marker upon complement activation by 10 μg/ml SN607D8/SN230G6 in presence and absence of 50 μg/ml anti-C1q. (F) Platelets were loaded with fluo-4 to measure a calcium influx upon incubation with increasing concentrations of SN607D8 and SN230G6. On the y-axis, the percentage of Fluo-4 positive platelets is depicted (n=4). (G) Pre-incubation of platelets with 50 μg/ml anti-C1q inhibited calcium influx induced by 10 μg/ml SN607D8/SN230G6. (n=4) (H) Representative flow cytometry plot of fluo-4 signal induced by 10 μg/ml SN607D8/SN230G6 in the presence and absence of 50 μg/ml anti-C1q. Data are represented as mean ± SD. MFI: mean fluoresence intensity, *P<0.05; **P<0.01; ***P<0.005.

pre-incubated with a blocking antibody directed to the tail of C1q (anti-C1q), preventing binding of C1q to Fc-tails of immunoglobulins. Complete blockage of WIM8E5 induced C3b and C4b deposition on platelet surfaces was achieved by anti-C1q at a concentration of 50 μg/ml (Figure 3A-B). The blocking effect of anti-C1q was dose dependent (Figure 3C). These results indicate a crucial role for C1q binding in WIM8E5 induced C3b and C4b deposition, suggesting that complement activation occurs via the classical pathway.

It has been described that IgG molecules can form hexamers to which C1q can efficiently bind.[23] Employing a synthetic peptide shown to interfere with Fc-dependent hexamer formation and subsequent complement deposition,[23,24] we studied whether anti-HLA antibody-induced complement deposition was blocked by this peptide. IgG-Fc:Fc blocking peptide inhibited C3b and C4b deposition by WIM8E5 or by the combination of SN607D8 and SN230G6 in a dose dependent manner (Figure 3D-E). No effects were observed with an irrelevant control peptide (Figure 3F-G). Together, these results suggest that anti-HLA antibody-induced complement deposition on platelets involves Fc tail-mediated assembly of IgG hexamers, creating a suitable binding platform for C1q.

Anti-HLA antibodies induce complement- and FcγRIIa-dependent platelet activation

We have recently shown that a subset of anti-HLA mAbs (including WIM8E5) can activate platelets directly through FcγRIIa in the absence of a complement source.[8] Under these conditions, WIM8E5-induced CD62P exposure was completely blocked with either anti FcγRIIa-blocking antibody IV.3 or through Syk inhibitor IV (which blocks downstream signaling of FcγRIIa[25]) (Figure 4A). Incubation of platelets with WIM8E5 in the presence of serum as a complement source resulted in a marked increase in CD62P exposure on platelets (Figure 4A). Under these conditions, CD62P exposure was only slightly inhibited by blocking FcγRIIa or with Syk inhibitor IV (Figure 4A). These results suggest that in the presence of complement activation, platelet activation is only partially FcγRIIa-dependent. To study the effect of complement activation on α-granule release, complement activation was blocked with anti-C1q. This significantly inhibited C3b deposition, but also only partly blocked α-granule release as measured by CD62P exposure (Figure 4B). However, a combination of Syk inhibitor and anti-C1q blocked WIM8E5-induced CD62P exposure as well as C3b deposition completely (Figure 4B).

Previously, Wiedmer *et al.* described a mechanism where the formation of a MAC on platelet surfaces induces platelet activation and α-granule release caused by calcium influx through the MAC.[26] We used a C5 blocking antibody (Eculizumab) to prevent cleavage of C5 and thereby the formation of a MAC. Like anti-C1q, Eculizumab partly inhibited CD62P surface exposure (Figure 4C). WIM8E5-induced CD62P exposure was completely inhibited when Eculizumab was combined with either Syk inhibitor IV or FcγRIIa blocking antibody IV.3 (Figure 4C). Similar results were obtained employing combinations of HLA mAbs (*Online Supplementary Figure S1*). Together, these results suggest that in presence of a complement source, platelet activation by anti-HLA antibodies is dependent on formation of a MAC as well as FcγRIIa-dependent signaling.

MAC formation leads to calcium influx

To confirm MAC formation, platelets activated with a combination of SN607D8 and SN230G6 were stained with an anti-C5b-9 antibody. As expected,

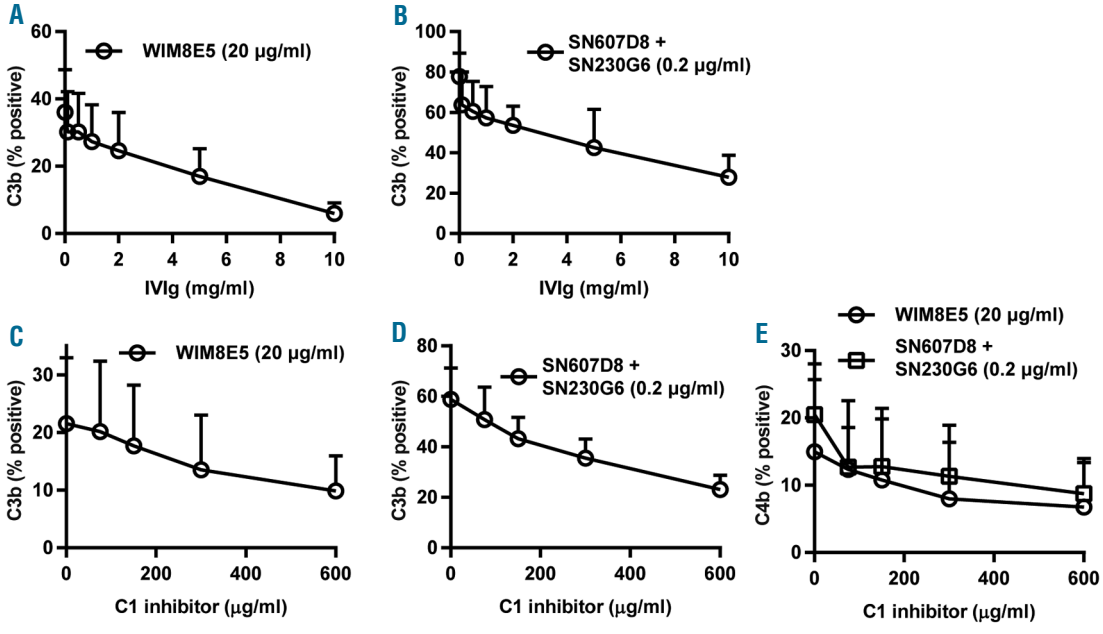
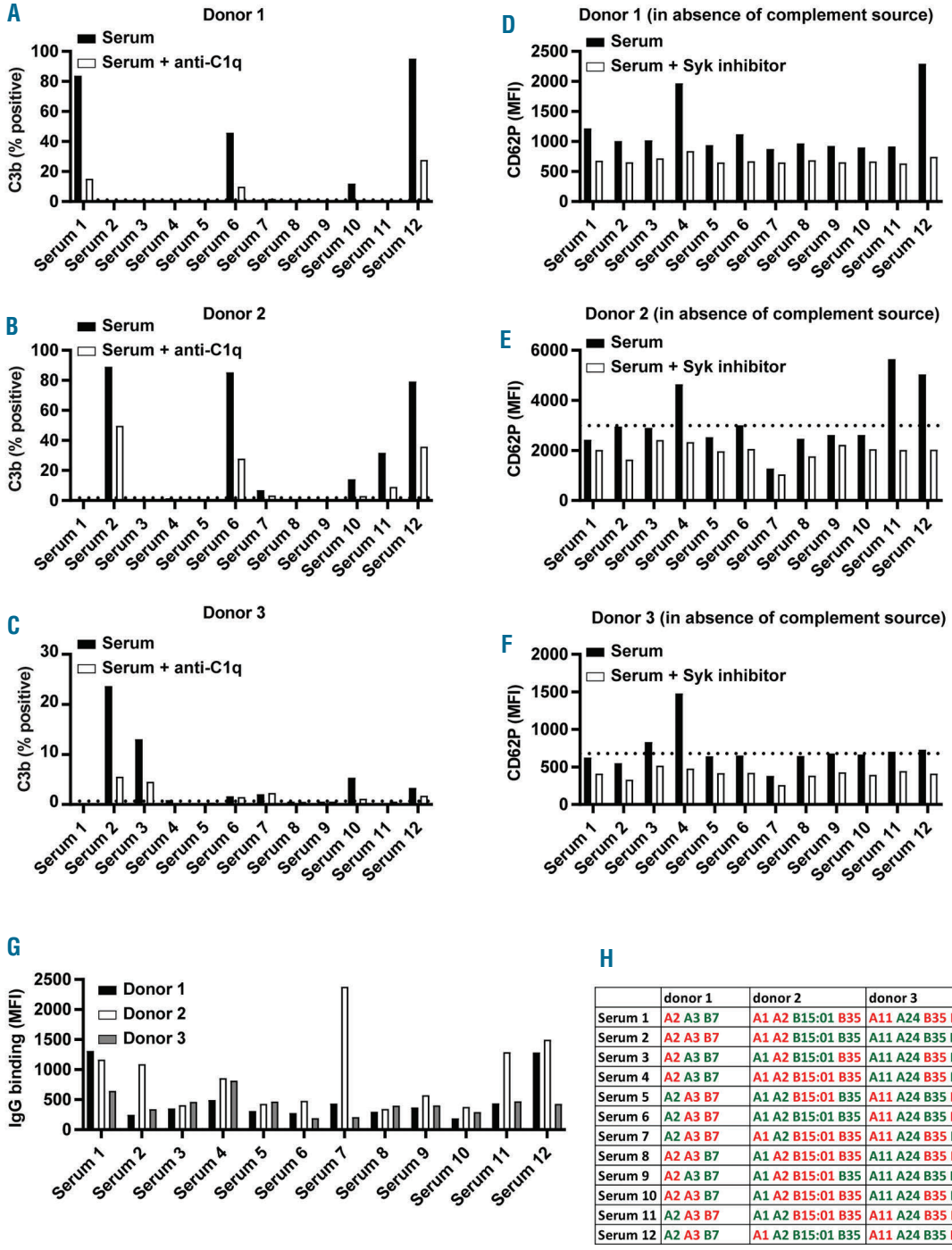


Figure 6. IVIg and C1 esterase inhibitor inhibit complement deposition on platelets. (A-B) Platelets were incubated with increasing concentrations of IVIg (0-10 mg/ml), after which WIM8E5 (20 μg/ml) (A) or SN607D8 and SN230G6 (0.2 μg/ml) (B) were added. C3b deposition was measured on the platelet surfaces. (n=3). (C-E) Platelets were pre-incubated with 0-600 μg/ml C1 esterase inhibitor. C3b deposition upon incubation with (C) 20 μg/ml WIM8E5 or (D) 0.2 μg/ml SN607D8 and SN230G6 was measured. (E) Inhibitory effect of C1 esterase inhibitor on C4b deposition was measured upon incubation with WIM8E5 (20 mg/ml) or SN607D8 and SN230G6 (0.2 μg/ml) (n=3).

SN607D8/SN230G6 induced enhanced C5b-9 on the platelet surface, which was blocked with anti-C1q (Figure 5A-B and *Online Supplementary Figure S2*).

Formation of a MAC is associated with cellular lysis through its end product which is pore formation.[27] To measure the permeability of platelet membranes, a live/dead marker (binding free amines, fluorescent signal increases in case of damaged membranes due to accessibility of intracellular amines) was added to platelets after incubation with SN607D8/SN230G6. Incubation with



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	donor 1	donor 2	donor 3
Serum 1	A2 A3 B7	A1 A2 B15:01 B35	A11 A24 B35 B38
Serum 2	A2 A3 B7	A1 A2 B15:01 B35	A11 A24 B35 B38
Serum 3	A2 A3 B7	A1 A2 B15:01 B35	A11 A24 B35 B38
Serum 4	A2 A3 B7	A1 A2 B15:01 B35	A11 A24 B35 B38
Serum 5	A2 A3 B7	A1 A2 B15:01 B35	A11 A24 B35 B38
Serum 6	A2 A3 B7	A1 A2 B15:01 B35	A11 A24 B35 B38
Serum 7	A2 A3 B7	A1 A2 B15:01 B35	A11 A24 B35 B38
Serum 8	A2 A3 B7	A1 A2 B15:01 B35	A11 A24 B35 B38
Serum 9	A2 A3 B7	A1 A2 B15:01 B35	A11 A24 B35 B38
Serum 10	A2 A3 B7	A1 A2 B15:01 B35	A11 A24 B35 B38
Serum 11	A2 A3 B7	A1 A2 B15:01 B35	A11 A24 B35 B38
Serum 12	A2 A3 B7	A1 A2 B15:01 B35	A11 A24 B35 B38

Figure 7. HLA antibody containing sera can induce complement activation on platelets. Twelve heat inactivated sera containing HLA antibodies were used to test complement activation and Syk-dependent activation on platelets from 3 different donors. (A-C) Complement activation (in presence of serum) was induced in presence and absence of 25 μg/ml anti-C1q. (D-F) Syk-dependent activation (as measured by CD62P exposure) was induced in presence and absence of 5 μM Syk inhibitor IV in the absence of a complement source. HLA typing of donor 1: A2 A3 B7, donor 2: A1 A2 B15:01 B35, donor 3: A11 A24 B35 B38. (G) IgG binding measured in absence of complement source employing an anti-human IgG antibody. (H) In green the antibodies present in sera matching the HLA typing of the platelet donors, and in red the expressed HLA-antigens for which no matching antibodies are present are indicated. Dotted lines in panel E-F correspond to background values of heat inactivated serum controls (no anti-platelet antibodies present). MFI: mean fluorescence intensity.

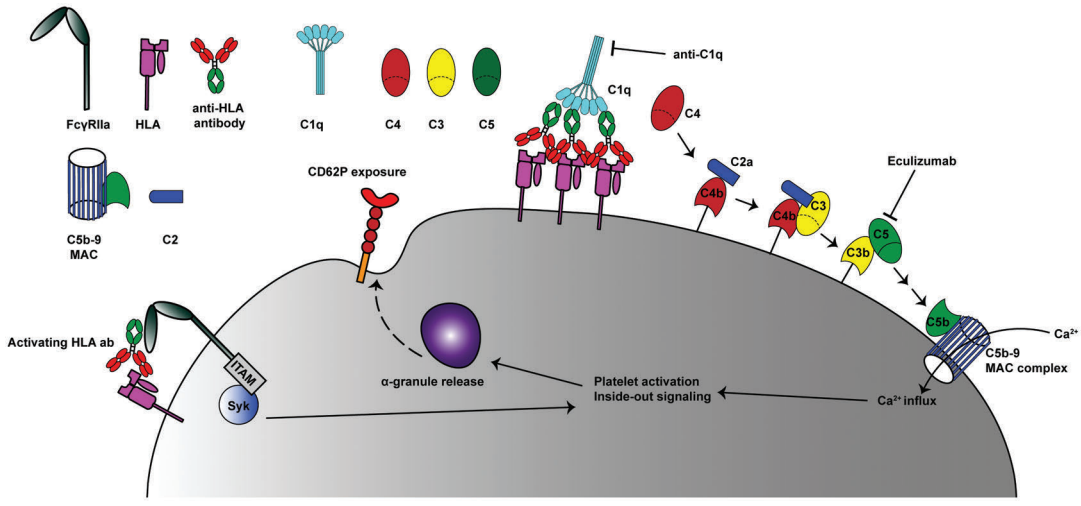

Figure 8. Proposed mechanism of anti-HLA antibody induced complement deposition. Upon binding of anti-HLA antibodies to HLA molecules on platelets, C1q can bind to HLA-bound IgGs. This leads to initiation of the classical complement pathway which results in C4b and C3b deposition on the platelet surface. Eventually this leads to the formation of a MAC which promotes the influx of Ca^{2+}. Elevated intra-platelet Ca^{2+} levels induce platelet activation as measured by CD62P exposure. Anti-HLA antibody-induced complement activation can be inhibited using anti-C1q blocking antibody or Eculizumab at the C1q of C5 level, respectively. Via a separate mechanism binding of HLA antibodies to HLA molecules can cross-link with FcγRIIa and induce platelet activation independent of the complement pathway.

these HLA mAbs induced a large increase in membrane permeability providing evidence for pore formation in platelet membranes (Figure 5C). Anti-HLA antibodies induced pore formation in a concentration-dependent manner (*Online Supplementary Figure S2*) and pore formation could be inhibited by anti-C1q (Figure 5D-E). Comparison of C3b deposition, C5b-9 complex formation and pore formation revealed a clear correlation between these three parameters upon incubation of platelets derived of two different donors with increasing concentrations of SN607D8/SN230G6 (*Online Supplementary Figure S2*). Under the same conditions, an increase in calcium influx in platelets loaded with fluo-4 was measured (Figure 5F-H). Together, these results suggest that complement activation induced by HLA antibodies leads to formation of a MAC, with subsequent pore formation in platelet membranes resulting in Ca^{2+} influx.

Immunoglobulins (IVIg) and C1 esterase inhibitor inhibit HLA antibody-induced complement activation

IVIg can inhibit FcγRIIa-dependent platelet activation by anti-HLA antibodies.[8] Similar effects have been observed in the context of heparin-induced thrombocytopenia.[28,29] Here, we also tested if IVIg affects complement activation and observed a dose-dependent inhibition of C3b deposition by IVIg (Figure 6A-B). Similarly, by inhibiting C1 employing the C1 esterase inhibitor, C3b and C4b deposition induced by HLA mAbs was inhibited (Figure C-E and *Online Supplementary Figure S1*). These results show that complement activation on platelets induced *in vitro* by HLA mAbs can be inhibited by IVIg and C1 esterase inhibitor in a dose dependent manner.

Sera containing HLA antibodies can induce complement activation on platelets, which is not correlated to Syk-mediated activation

In order to confirm our data obtained with human monoclonal HLA antibodies, sera containing HLA antibodies from 12 patients refractory to platelet transfusions were tested for their ability to induce C3b deposition on platelets (*Online Supplementary Table S2*). Depending on the HLA type of the donor platelets, C3b deposition was observed upon incubation with sera containing HLA antibodies (Figure 7A-C). In agreement with the results obtained for human mAbs, incubation with anti-C1q blocked C3b deposition on platelet membranes (Figure 7A-C). In case of strong complement activation, 25 μg/ml anti-C1q only partly inhibited C3b deposition. C3b deposition, however, was completely blocked by anti-C1q when the anti-HLA sera were diluted (*Online Supplementary Figure S4*). This confirms that HLA antibodies, as present in patient sera, induce complement activation via the classical pathway.

FcγRIIa-dependent α-granule release was also investigated with the same donors and platelet donors, in absence of complement source (Figure 7D-F). Some sera induced both C3b deposition and FcγRIIa-dependent platelet activation, however, similar to the results obtained with HLA mAbs, there were also sera which activated only one of these two pathways. These results suggest that HLA antibodies may induce complement activation and FcγRIIa-dependent platelet activation via distinct mechanisms. To confirm that complement activation induced by HLA antibodies present in patients sera correspond to that of HLA mAbs, platelet complement activation and Syk-dependent activation were blocked. Similar to results obtained with HLA mAbs (Figure 4 and *Online Supplementary Figure S1*), both Syk-dependent and complement-dependent platelet activation occurs when platelets are incubated with HLA antibody containing sera (*Online Supplementary Figure S5*). Furthermore, C3b deposition induced by these sera could be inhibited employing the IgG-Fc:Fc blocking peptide (*Online Supplementary Figure S6*).

The level of IgG binding differed among sera and also depended on the donor platelets used (Figure 7G and

Online Supplementary Figure S3). Although some association between IgG binding and complement deposition and FcγRIIa-dependent platelet activation was observed, some sera displaying only limited IgG binding were still capable of inducing complement activation or Syk-dependent activation. This suggests that only a subset of anti-HLA antibodies present in patient sera can induce C3b deposition on platelets.

Discussion

Development of HLA alloantibodies remains a major cause of refractoriness to platelet transfusions. As yet, the pathogenic properties of HLA alloantibodies have not been sufficiently characterized. Here, we have shown that HLA antibodies are capable of inducing complement activation on platelets, leading to C4b and C3b deposition on platelet surfaces and the formation of a functional MAC.

It was previously shown that platelet activation can lead to the binding of C3b,[9] inducing complement activation via the alternative pathway. We have previously shown that HLA antibodies can induce FcγRIIa-dependent platelet activation, leading to CD62P surface exposure.[8] Therefore, we tested whether the complement activation by HLA antibodies occurs via the classical pathway, or whether CD62P surface exposure directly leads to C3b binding followed by activation of the alternative complement pathway. Since a blocking antibody directed to C1q completely blocked C3b and C4b deposition, complement activation by HLA antibodies appears to be fully dependent on the classical pathway. In line with this observation, blocking FcγRIIa-dependent activation had no effect on C3b deposition on platelets. Similar results were obtained in patient sera containing HLA antibodies. Also, the ability of HLA antibody-containing sera to induce complement activation was not directly correlated to FcγRIIa-dependent platelet activation. Some sera were capable of inducing both complement- and FcγRIIa-dependent activation of platelets.

We observed enhanced CD62P exposure in the samples in which complement was activated. In the presence of a complement source, CD62P exposure was only partly blocked upon inhibition of FcγRIIa-signaling. Simultaneous blocking of complement activation and FcγRIIa-signaling resulted in complete inhibition of platelet activation as measured by CD62P exposure. This suggests that HLA antibodies can induce platelet activation by promoting complement deposition as well as FcγRIIa-dependent signaling. We speculate that both mechanisms may contribute to the rapid clearance of platelets in refractory patients.

In our study, we demonstrated that not all HLA mAbs and patient sera induce complement activation to a similar extent. Specific combinations of mAbs showed significantly enhanced C3b deposition; strong, synergistic complement deposition induced by incubation of platelets with low concentrations of two distinct HLA mAbs (SN607D8 and SN230G6) was observed. This suggests that structural organization of platelet bound IgG is crucial for initiation of the complement cascade. It has been shown that C1q binds most efficiently to IgG hexamers, for which Fc:Fc interactions are needed.[23,30,31] In line with these observations, we could completely block complement deposition induced by HLA mAbs by adding an IgG-Fc blocking peptide[24] described to prevent these Fc:Fc interactions. The same blocking peptide inhibited C3b deposition on platelets induced by HLA antibody-containing patient sera. These results suggest that the propensity of polyclonal (patient)-derived HLA antibodies to form IgG hexamers on the platelet surface provides a binding platform for C1q which finally promotes the formation of C5b-9 complexes on platelet membranes. Additional properties of HLA antibodies such as specific subclass or composition of the glycan in the Fc part of IgGs are expected to potentially modulate complement activation of HLA antibodies on platelets.

In this respect, it is interesting to note that glycosylation of IgG1 has been described to affect the binding of IgG1 to C1q which also might affect the ability of individual antibodies to induce complement activation.[32] Altogether, this might explain why only a subset of HLA mAbs and patient sera containing HLA antibodies induce complement activation. Interestingly, sera containing HLA antibodies matching the HLA type of the platelet donor did not always induce complement activation. This may partly be explained by differences in concentration and binding specificity of the different antibodies in these polyclonal sera, and varying levels of HLA expression on the donor platelets used in this study.[7] Also, the presence of specific IgG subclasses might play a role, as different subtypes bind with different affinity to C1q.[33] In this study, we show that low concentrations of specific HLA antibodies can induce complement activation in a synergistic manner. This observation suggests that depending on the HLA profile and HLA density on donor platelets, specific combinations of patient-derived HLA antibodies can potently induce complement activation. Our results indicate that the propensity of HLA antibodies to induce complement activation is not per se related to the titer and binding specificity of HLA antibodies in serum. Rather, our findings suggest that the ability of HLA antibodies to form hexameric IgG complexes on platelets determines their ability to induce complement activation.

Ideally, the ability of HLA antibodies of individual patients to induce platelet activation[8] and /or complement deposition on donor platelets should be assessed before transfusion. Previous studies have shown that platelet cross match does increase platelet recovery in patients, but data on occurrence of hemorrhage and mortality are lacking.[34] Implementation of functional tests focusing on HLA antibody-induced platelet activation and complement deposition may have the potential to functionally stratify platelet concentrates for specific HLA alloimmunized patients. This would further increase the pool of suitable donor platelets for treatment of HLA alloimmunized patients who are currently dependent on treatment with HLA-matched platelet concentrates. This would increase transfusion safety, lower the costs and avoid delays in provision of donor platelets, which is important for transfusion support in chronically transfused patients.

There are several treatment options for refractory thrombocytopenia, which from an immunological perspective could target both FcγR- and complement-dependent clearance of donor platelets. Immunoglobulins (IVIg) could inhibit C3b deposition on platelets induced by HLA mAbs. Inhibition of complement deposition by IVIg has previously been shown in a number of autoimmune disorders.[35,36] A randomized trial in HLA alloimmunized patients treated with IVIg revealed increased platelet-cor-

rected count increments one hour after transfusion compared to placebo; however, no beneficial effects were observed 24 hours after transfusion.[37] Thus, IVIg might be an effective way to prevent rapid platelet clearance induced by HLA antibodies. Similarly, Eculizumab could block formation of a MAC.[38,39] Preliminary data of a clinical trial showed that Eculizumab could overcome platelet transfusion refractoriness in patients with HLA-alloantibodies.[40] The C3 inhibitor Compstatin was not tested here, but is also used in clinical practice already[41] and might have a beneficial effect for patients refractory for platelet transfusion caused by HLA alloantibodies. Also, C1 esterase inhibitor, which blocks complement activation at the beginning of the complement cascade, inhibited C3b and C4b deposition induced by HLA mAbs. Blocking complement activation at this point might be even more beneficial than blocking after formation of C4b and C3b. The potential beneficial effect of complement inhibitors in refractory patients receiving platelet transfusions needs to be confirmed in clinical studies.

In conclusion, in this study we have shown that HLA antibodies can induce complement activation on platelets through the classical complement pathway, as is schematically represented in the visual abstract. We propose that C3b complement deposition induced by HLA alloantibodies in refractory patients might contribute to platelet clearance through their binding to C11b/CD18 on myeloid cells. Ongoing complement activation on HLA-sensitized platelets will lead to formation of a MAC allowing for Ca^{2+}-influx and subsequent platelet activation. Importantly, these effects of HLA antibodies may not only be prevented via HLA-matching between donors and recipient antibodies, but also by functional matching, since not all HLA antibodies are capable of inducing complement activation. Furthermore, inhibition of complement activation, as currently studied in clinical trials, may be of benefit to further optimize platelet support in HLA immunized patients.

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