SUPPLEMENTARY APPENDIX

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

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Supplementary data

Supplemental Materials

Human IgG1 mAbs directed to HLA (Supplementary Table S1) were produced by hybridoma technology as previously described,[1] and purified using protein A. Antibodies directed to CD62P (clone AK-4), C3b/iC3b (clone 3E7/C3b), CD41 (clone HIP8) and human IgG Fc (clone HP60717) were obtained from BioLegend (San Diego, CA, USA). Anti-C5b-9 (A227) was from Complement Technology (Tyler, TX, USA). Anti-CD42a (clone ALMA.16) was from BD Biosciences (San Jose, CA, USA). Blocking antibody anti-CD32a (clone IV.3) was from Bio X Cell (West Lebanon, NH, USA). Syk inhibitor IV (BAY 61-3606) was obtained from Merck (Kenilworth, NJ, USA). Monoclonal anti-C5 antibody Eculizumab was from Alexion Pharmaceuticals (New Haven, CT, USA). Monoclonal blocking antibody anti-C1q-85 has been described before,[2,3] Anti-C4 antibody clone C4-10[4] was from Sanquin Research (Amsterdam, The Netherlands). Human plasma derived purified C1-inhibitor (Cetor®) was from Sanquin Blood Supply Foundation (Amsterdam, The Netherlands), gel filtration was used as extra purification step. Live/dead marker near IR fluorescent reactive dye and fluo-4 were from Thermo Fisher Scientific (Waltham, MA, USA). Calcein violet was from BioLegend. Fc:Fc interaction-blocking peptide DCAWHLGELVWCT[5] and control peptide GWTVFQKRLDGSV[6] were synthesized by JPT Peptide Technologies (Berlin, Germany).

Figure S1

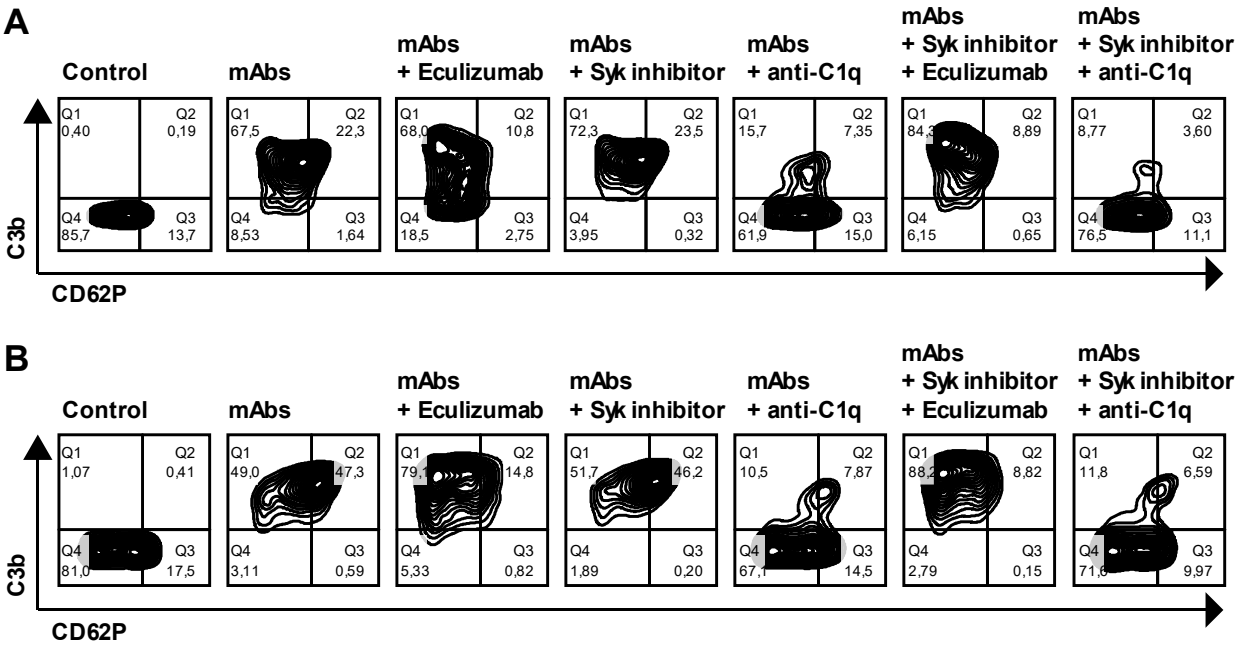

Figure S1. Platelet activation occurs via complement activation and Syk-dependent activation. A mix of anti-HLA mAbs was used to induce platelet complement activation and platelet activation (CD62P exposure). Complement and/or Syk-dependent activation was inhibited by pre-incubation with Syk inhibitor IV (5 µM), anti-C1q (50µg/ml), Eculizumab (10 µg/ml) or a combination of these inhibitors. (A) 20 µg/ml of SN607D8, SN230G6, GV5D1 and GV2D5 was used to induce complement activation. (B) 20 µg/ml of SN607D8 and SN230G6 was used to induce complement activation. These data confirm that platelet activation induced by anti-HLA antibodies occurs via both complement dependent activation (reduced CD62P exposure upon pre-incubation with either anti-C1q or Eculizumab) and Syk-dependent activation (reduced CD62P exposure upon pre-incubation with Syk inhibitor IV). A combination of a complement inhibitor and Syk inhibitor IV completely blocks CD62P exposure.

Figure S2

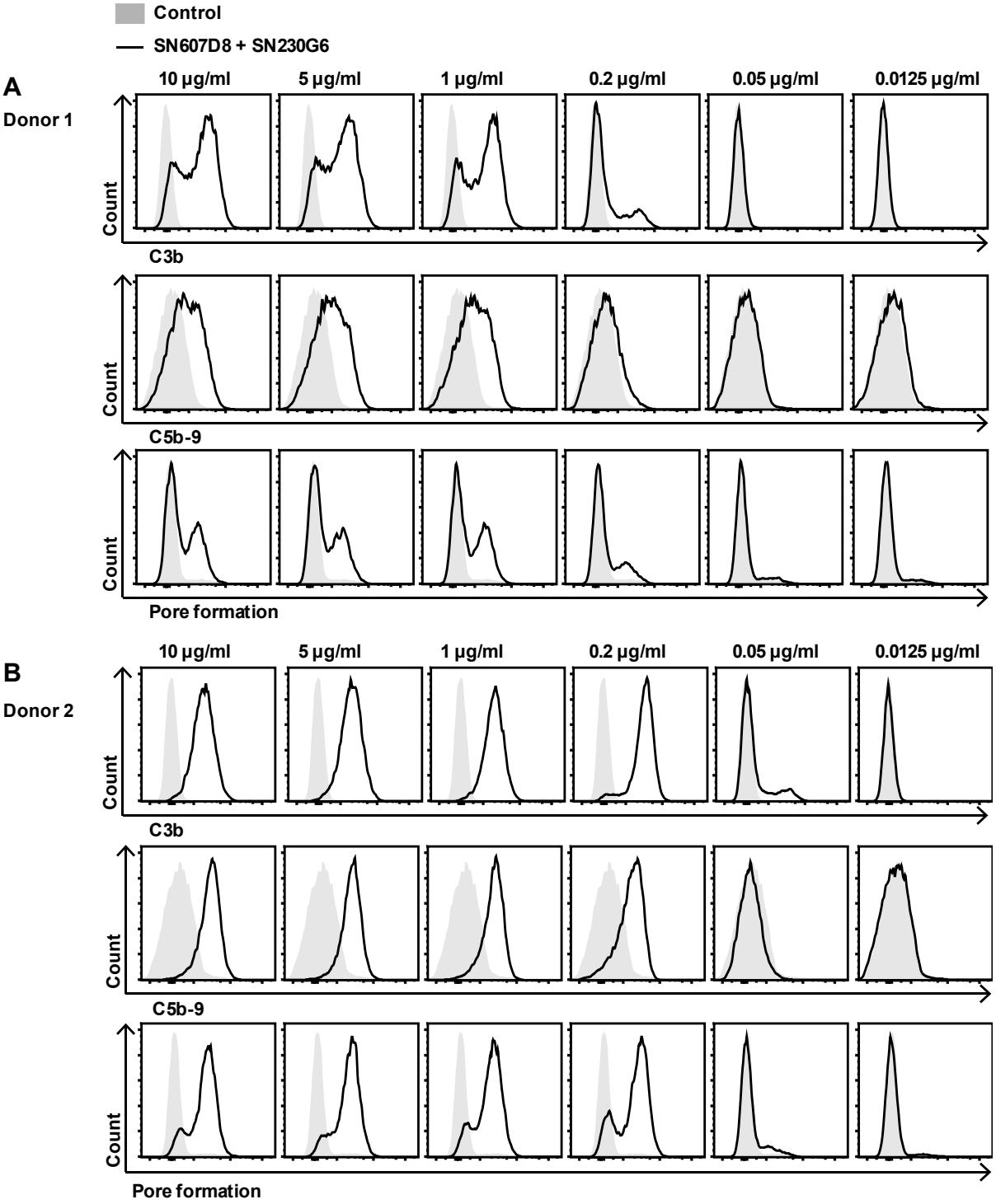

Figure S2. Comparison of anti-HLA antibody induced C3b deposition, C5b-9 deposition and pore formation. Platelets were incubated with decreasing concentrations of SN607D8 and SN230G6 (10 µg/ml till 0.0125 µg/ml) and C3b deposition, C5b-9 deposition and pore formation were measured employing the same samples. (A) Platelets from donor expressing HLA A2 A31 B60. (B) Platelets from a donor expressing HLA A2 A68 B51 B57. Concentration-dependent induction of C3b deposition, C5b-9 deposition and pore formation by anti-HLA antibodies. The signals observed for

both C3b deposition, C5b-9 deposition and pore formation decline at the same concentration of anti-HLA mAbs. Pore formation is not observed for all platelets positive for C3b deposition and C5b-9 complex formation. Dose-response curves for C3b deposition, C5b-9 complex formation and pore formation are platelet donor-dependent.

Figure S3

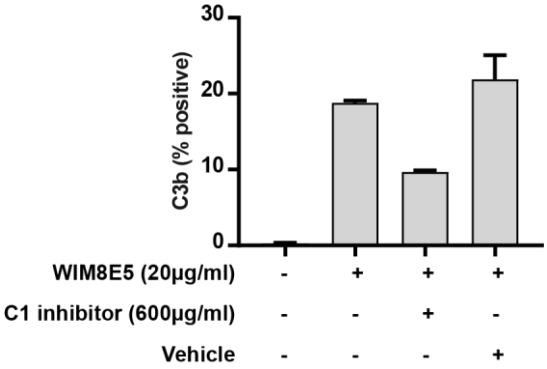

Figure S3. No effect of vehicle control on C3b deposition. Platelets were pre-incubated with either 600 mg/ml C1 inhibitor or an equal volume of vehicle control (PBS). Subsequently 20 µg/ml WIM8E5 was added and complement deposition of C3b was measured employing flow cytometry. No inhibiting effect of the vehicle was observed.

Figure S4

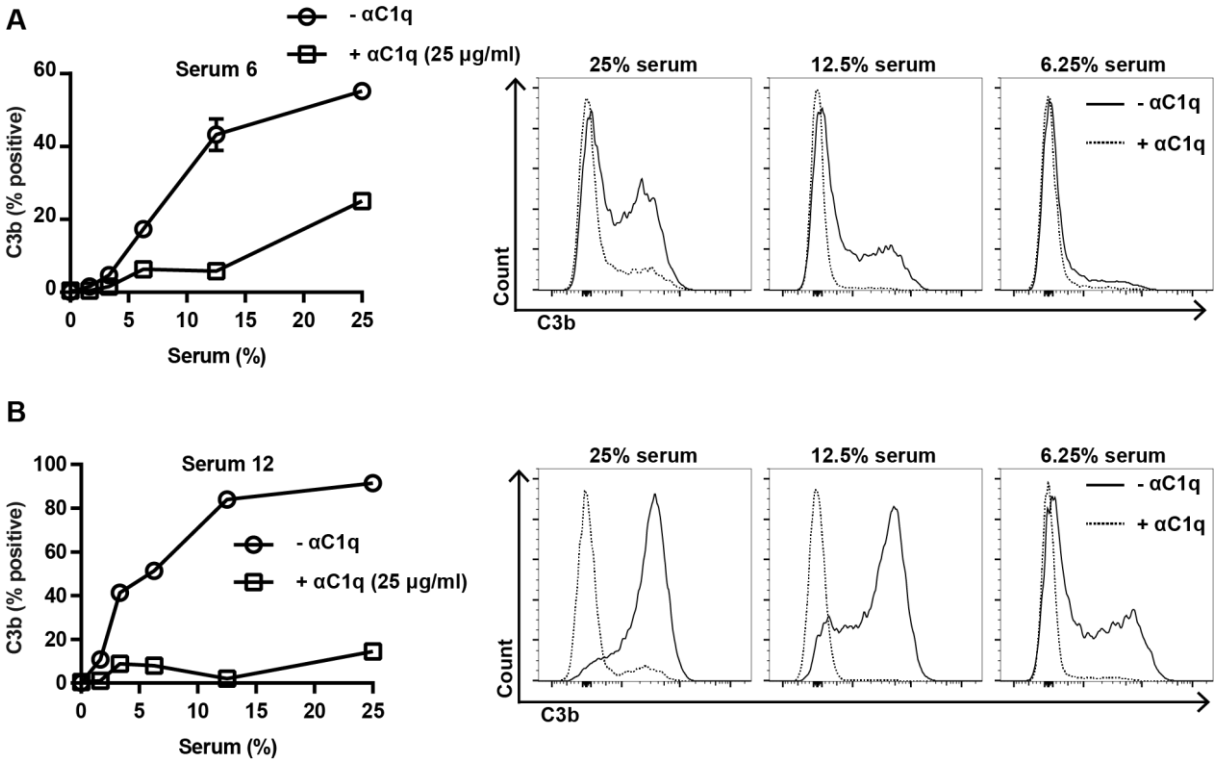

Figure S4. Complement deposition induced by different dilutions of anti-HLA antibodies containing patient sera can be completely inhibited with anti-C1q. Platelet from donor (HLA A2 A31 B40:01) were incubated with different concentrations of HLA antibody containing sera (A: serum 6, matching antibodies A2 B40:01. B: serum 12, matching antibodies A2 B40:01). Complement activation was inhibited with 25 µg/ml αC1q. Using large volumes of patient sera only partial inhibition of C3b deposition by 25 µg/ml αC1q was observed. When smaller volumes of sera were used C3b deposition could be completely blocked with 25 µg/ml αC1q.

Figure S5

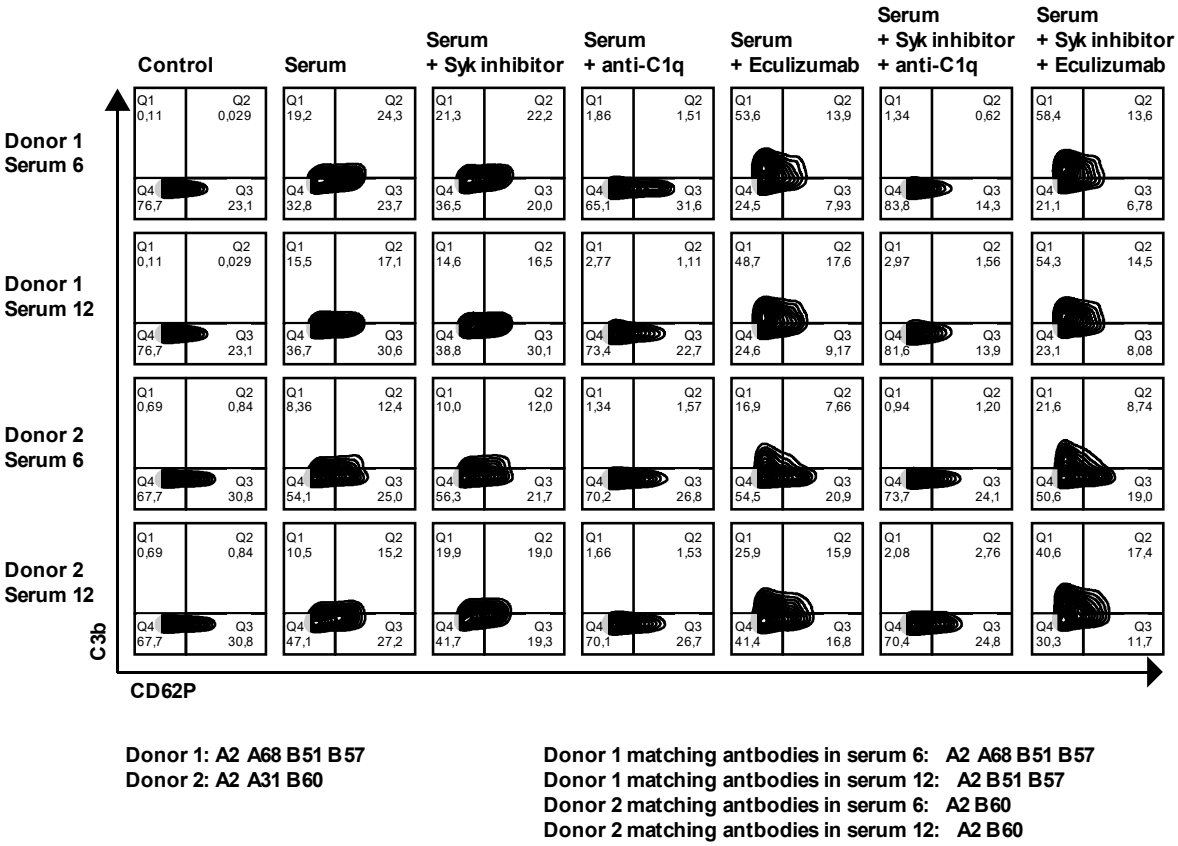

Figure S5. Blocking complement deposition and CD62P exposure induced by anti-HLA antibodies containing patient sera with Syk inhibitor, anti-C1q and Eculizumab. Platelets from 2 donors, with an HLA typing as indicated in the figure, were incubated with anti-HLA antibody containing sera (serum 6 or serum 12) in presence or absence of Syk inhibitor IV (5 µM), anti-C1q (50µg/ml), eculizumab (10 µg/ml) or a combination of these inhibitors. Enhanced C3b deposition and CD62P exposure was observed for all 4 platelet-serum combinations. Limited effect of Syk inhibitor alone on CD62P exposure was observed. Anti-C1q could completely block C3b deposition induced by the sera. Eculizumab inhibited CD62P exposure, but not C3b deposition, and with combinations of Syk inhibitor IV and anti-C1q or Syk inhibitor IV and Eculizumab, CD62P positive events were similar to that of control samples.

Figure S6

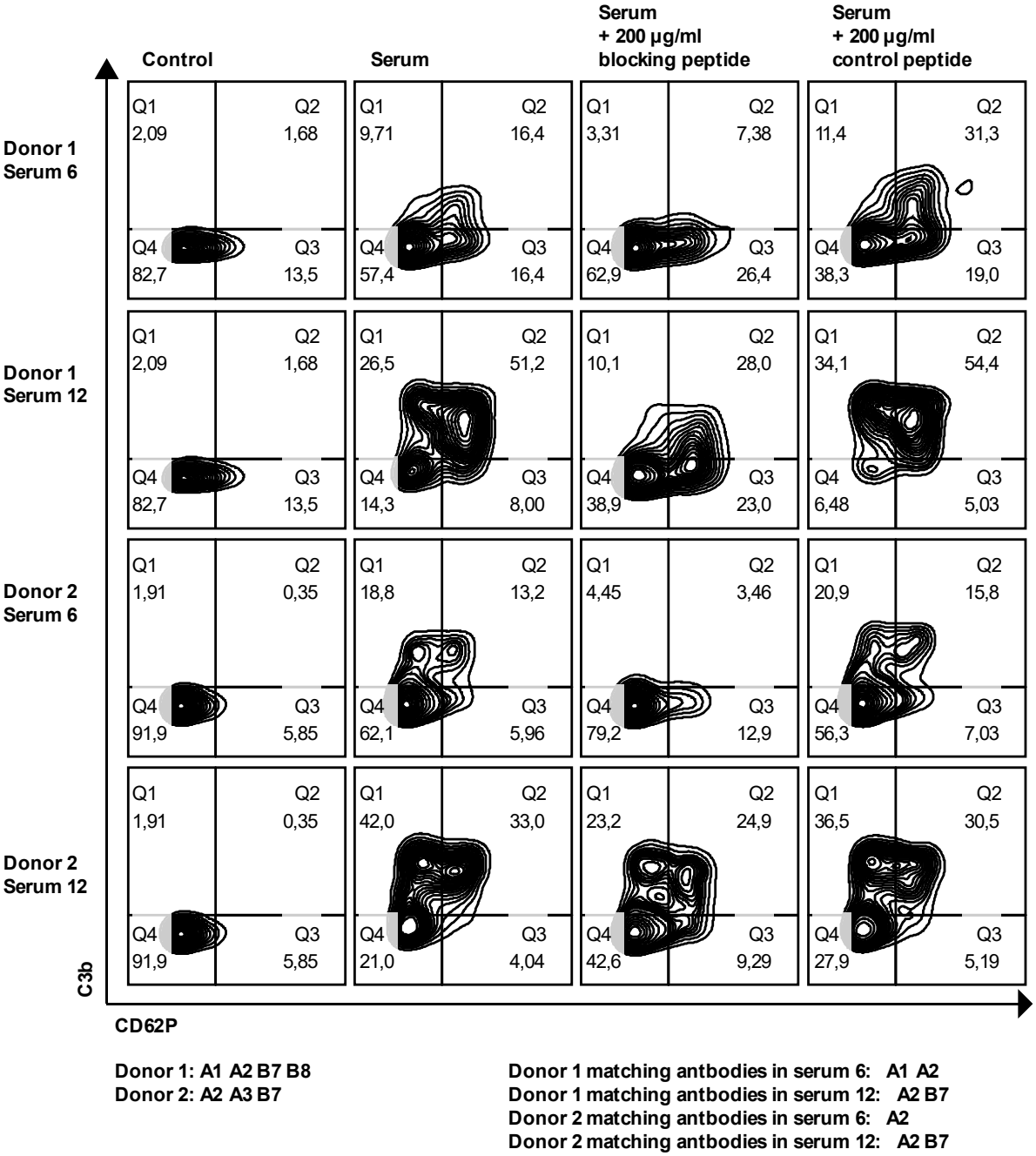

Figure S6. Complement deposition induced by anti-HLA antibodies containing patient sera is inhibited by Fc-IgG:IgG blocking peptide. Platelets from 2 donors, with an HLA typing as indicated in the figure, were pre-incubated with anti-HLA antibody containing sera (serum 6 or serum 12) and washed once in assay buffer. Buffer, 200 µg/ml IgG-Fc:Fc blocking peptide or 200 ug/ml control peptide was added following by the addition of serum (as a complement source). Complement deposition was measured by C3b deposition. The IgG-Fc:Fc blocking peptide inhibits complement deposition on platelets induced by anti-HLA antibody containing patient sera.

Figure S7

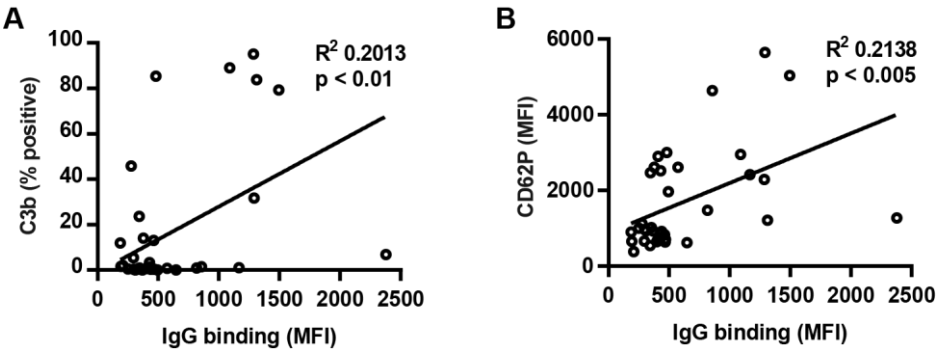

Figure S7 Correlation IgG binding of antibodies in patient sera and C3b deposition and CD62P exposure. Twelve sera (heat inactivated) from refractory patients were incubated with platelets of 3 different donors. Levels of IgG binding, C3b deposition and CD62P exposure were compared. (A) Correlation between IgG binding and C3b deposition. (B) Correlation between IgG binding and CD62P exposure (in absence of human serum (complement source)). Linear regression is indicated in the graphs.

Table S1: HLA monoclonal antibodies

Antibody name	**HLA specificity (determined by CDC on large (n>230) panels of HLA typed peripheral blood lymphocytes)**
WIM8E5	A1/A10 (25/26/34/43/66)/A11/A9 (23+24)/A29/A30/A31/A33/A28(68+69)
SN607D8*	A2/A28(A68/A69)
SN230G6*	A2/B57/B58
HDG8D9	B51/B35
BRO11F6	A3/A11/A24
DK7C11	B12 (B44/B45)
OK8F12	B46/B62/B72
GV5D1[$]	A1/A9
GV2D5[$]	A1

* SN607D8 and SN230G6 originate from the same patient

[$] GV5D1 and GV2D5 originate from the same patient

Table S2: Reactivity of anti-HLA antibodies in patient sera

Serum 1			**Serum 2**			**Serum 3**			**Serum 4**		
Antibodies			**Antibodies**			**Antibodies**			**Antibodies**		
HLA A	**HLA B**	**HLA C**	**HLA A**	**HLA B**	**HLA C**	**HLA A**	**HLA B**	**HLA C**	**HLA A**	**HLA B**	**HLA C**
A3	B7	C1	A11	B8	C03:03(Cw9)	A1	B7		A3	B8	C1
A23	B8	C2	A23	B13	C03:04(Cw10)	A3	B15:01(B62)		A11	B13	C8
A24	B13	C3:03(Cw9)	A24	B14:01(B64)	C8	A11	B15:02(B75)		A23	B14:01(B64)	C12
A25	B15:01(B62)	C3:04 (Cw10)	A25	B15:01(B62)		A23	B15:03(B72)		A24	B14:02(B65)	C14
A30	B15:02(B75)	C7	A26	B15:02(B75)		A24	B15:12(B76)		A25	B15:13(B77)	C15
A31	B15:12(B76)	C8	A29	B15:03(B72)		A30	B15:13(B77)		A32	B15:16(B63)	C16
A32	B15:13(B77)	C12	A32	B15:12(B76)		A31	B15:16(B63)		A33	B18	
A33	B15:16(B63)	C14	A33	B15:13(B77)		A32	B27		A34	B27	
A34	B27	C15	A34	B15:16(B63)		A36	B37		A68	B37	
A66	B40:01(B60)	C16	A43	B15:18(B71)		A80	B38		A69	B38	
A68	B40:02(B61)		A66	B18			B39			B39	
A69	B41		A68	B27			B40:01(B60)			B41	
	B42			B35			B40:02(B61)			B42	
	B44 B45			B37			B41			B44	
	B46			B38			B42			B47	
	B47			B39			B44			B49	
	B48			B41			B45			B51	
	B49			B42			B47			B52	
	B50			B44			B48			B53	
	B51			B45			B49			B54	
	B52			B46			B50			B55	
	B54			B47			B51			B57	
	B55			B48			B52			B58	
	B56			B49			B53			B59	
	B57			B50			B54			B67	
	B58			B51			B55				
	B67			B52			B56				
	B73			B53			B57				
	B81			B54			B58				
	B82			B55			B59				
				B56			B67				
				B57			B73				
				B58			B81				
				B59			B82				
				B67							
				B78							
				B82							

Serum 5		
Antibodies		
HLA A	HLA B	HLA C
A1	B13	
A2	B15:02(B75)	
A23	B*15:12(B76)	
A24	B*15:18(B71)	
A25	B27	
A26	B35	
A29	B37	
A30	B39	
A31	B*40:01(B60)	
A32	B*40:02(B61)	
A33	B41	
A34	B44	
A43	B45	
A66	B47	
A74	B49	
	B50	
	B82	

Serum 6		
Antibodies		
HLA A	HLA B	HLA C
A1	B13	C*03:03(Cw9)
A2	B15:01(B62)	C*03:04(Cw10)
A23	B15:02(B75)	
A24	B15:03(B72)	
A29	B*15:12(B76)	
A33	B*15:13(B77)	
A34	B*15:16(B63)	
A68	B*15:18(B71)	
A69	B35	
	B37	
	B*40:01(B60)	
	B*40:02(B61)	
	B41	
	B44	
	B45	
	B46	
	B49	
	B50	
	B51	
	B52	
	B53	
	B55	
	B56	
	B57	
	B58	
	B59	
	B78	
	B82	

Serum 7		
Antibodies		
HLA A	HLA B	HLA C
A2	B13	
A23	B*15:13(B77)	
A24	B*15:03(B72)	
A25	B*15:12(B76)	
A32	B*15:16(B63)	
A36	B27	
A69	B37	
	B38	
	B40:01(B60)	
	B40:02(B61)	
	B41	
	B44	
	B45	
	B47	
	B49	
	B50	
	B51	
	B52	
	B53	
	B57	
	B58	
	B59	
	B82	

Serum 8		
Antibodies		
HLA A	HLA B	HLA C
A1	B7	C7
A11	B8	C16
A23	B13	C17
A24	B*15:12(B76)	
A26	B*15:16(B63)	
A29	B27	
A30	B*40:01(B60)	
A31	B*40:02(B61)	
A32	B41	
A33	B42	
A36	B44	
A43	B45	
A66	B47	
A74	B48	
A80	B49	
	B50	
	B51	
	B52	
	B55	
	B67	
	B73	
	B81	
	B82	

Serum 9		
Antibodies		
HLA A	HLA B	HLA C
A1	B8	
A3	B14:01(B64)	
A11	B14:02(B65)	
A24:03	B15:02(B75)	
A25	B15:12(B76)	
A26	B15:13(B77)	
A29	B15:16(B63)	
A30	B15:18(B71)	
A31	B18	
A32	B35	
A33	B51	
A34	B53	
A36	B59	
A43	B78	
A66		
A68		
A69		
A80		

Serum 10		
Antibodies		
HLA A	HLA B	HLA C
A1	B7	C2
A11	B13	C17
A23	B15:12(B76)	
A24	B15:13(B77)	
A25	B15:16(B63)	
A32	B27	
A36	B37	
A66	B38	
A80	B40:01(B60)	
	B40:02(B61)	
	B44	
	B45	
	B47	
	B48	
	B49	
	B50	
	B51	
	B52	
	B53	
	B57	
	B58	
	B59	
	B73	
	B81	

Serum 11		
Antibodies		
HLA A	HLA B	HLA C
A1	B8	
A2	B44	
A23	B45	
A24	B57	
A26	B58	
A29	B82	
A36		
A80		

Serum 12		
Antibodies		
HLA A	HLA B	HLA C
A2	B7	Cw1(01:02)
A23	B8	Cw3(03:07)
A24	B13	Cw8(08:01-03)
	B18	Cw12
	B27	Cw14
	B35	Cw15
	B39	
	B41	
	B42	
	B45	
	B47	
	B48	
	B50	
	B51	
	B53	
	B54	
	B55	
	B56	
	B57	
	B58	
	B60	
	B62	
	B70	
	B75	
	B76	
	B78	

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