SUPPLEMENTAL MATERIAL

Complement & platelets: Prothrombotic cell activation requires membrane attack complex induced release of danger signals

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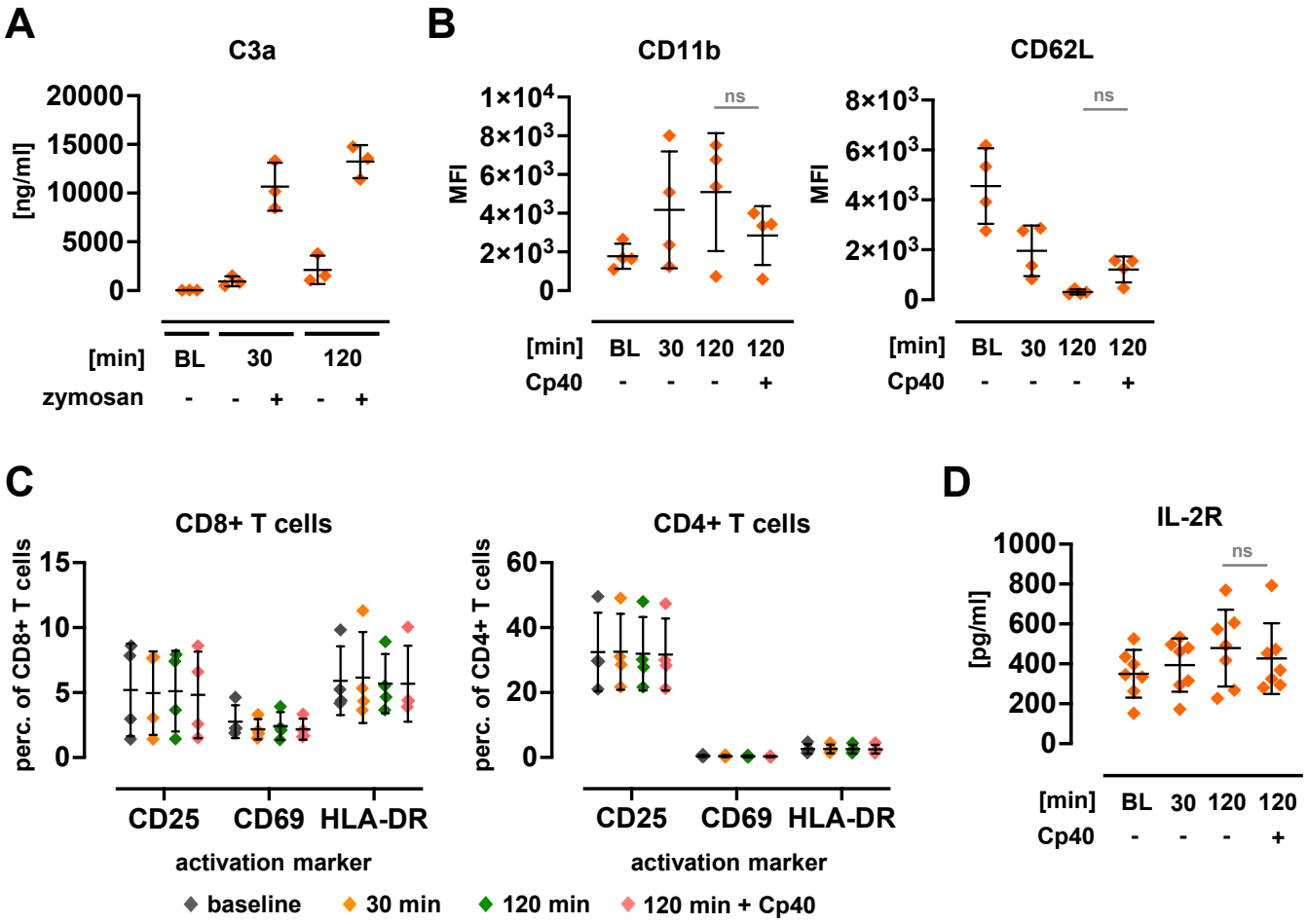
Supplemental Figures: **pages 1-7**

Supplemental tables: **pages 8-11**

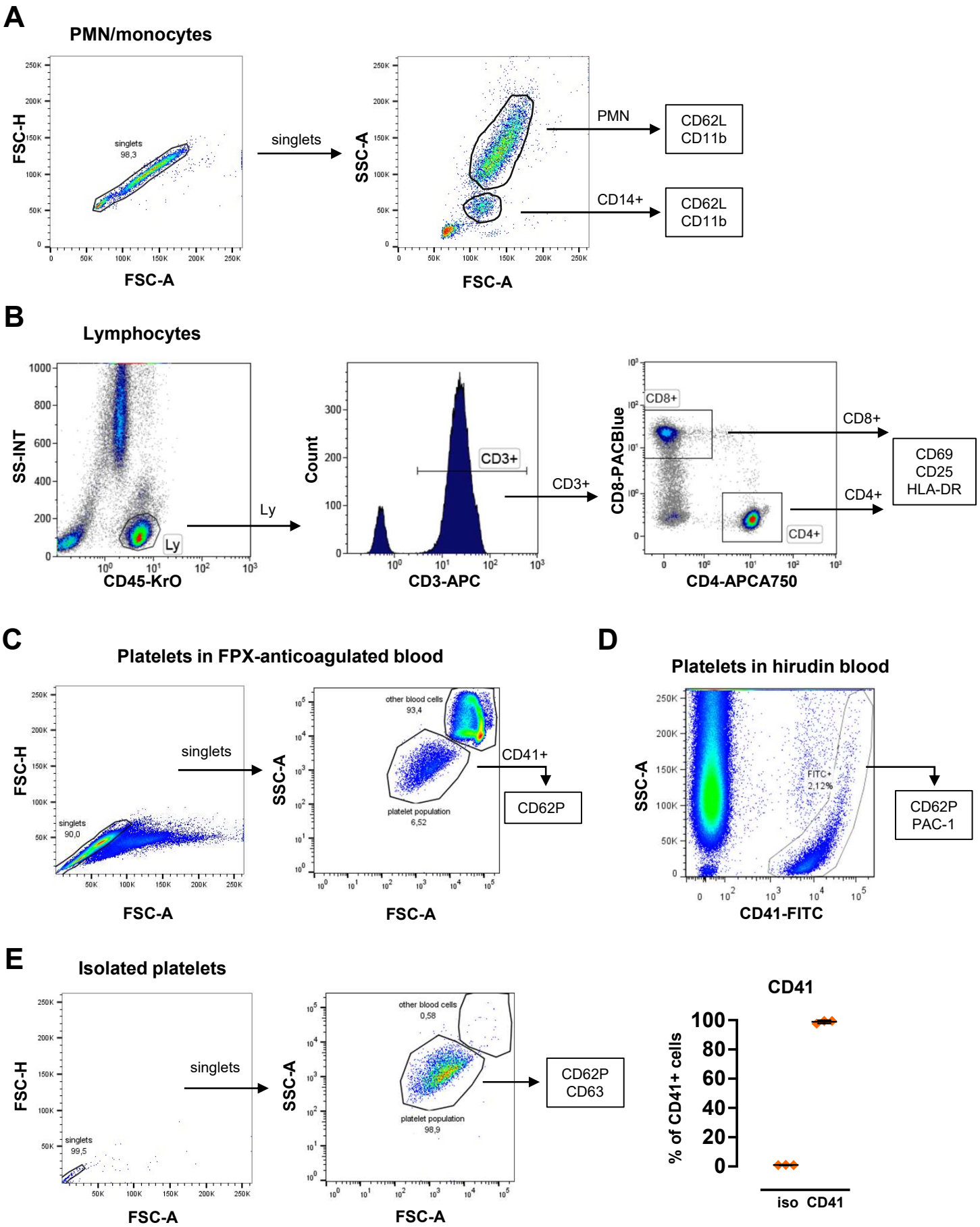
Supplemental material and methods: **pages 12-18**

Supplemental Figure 1



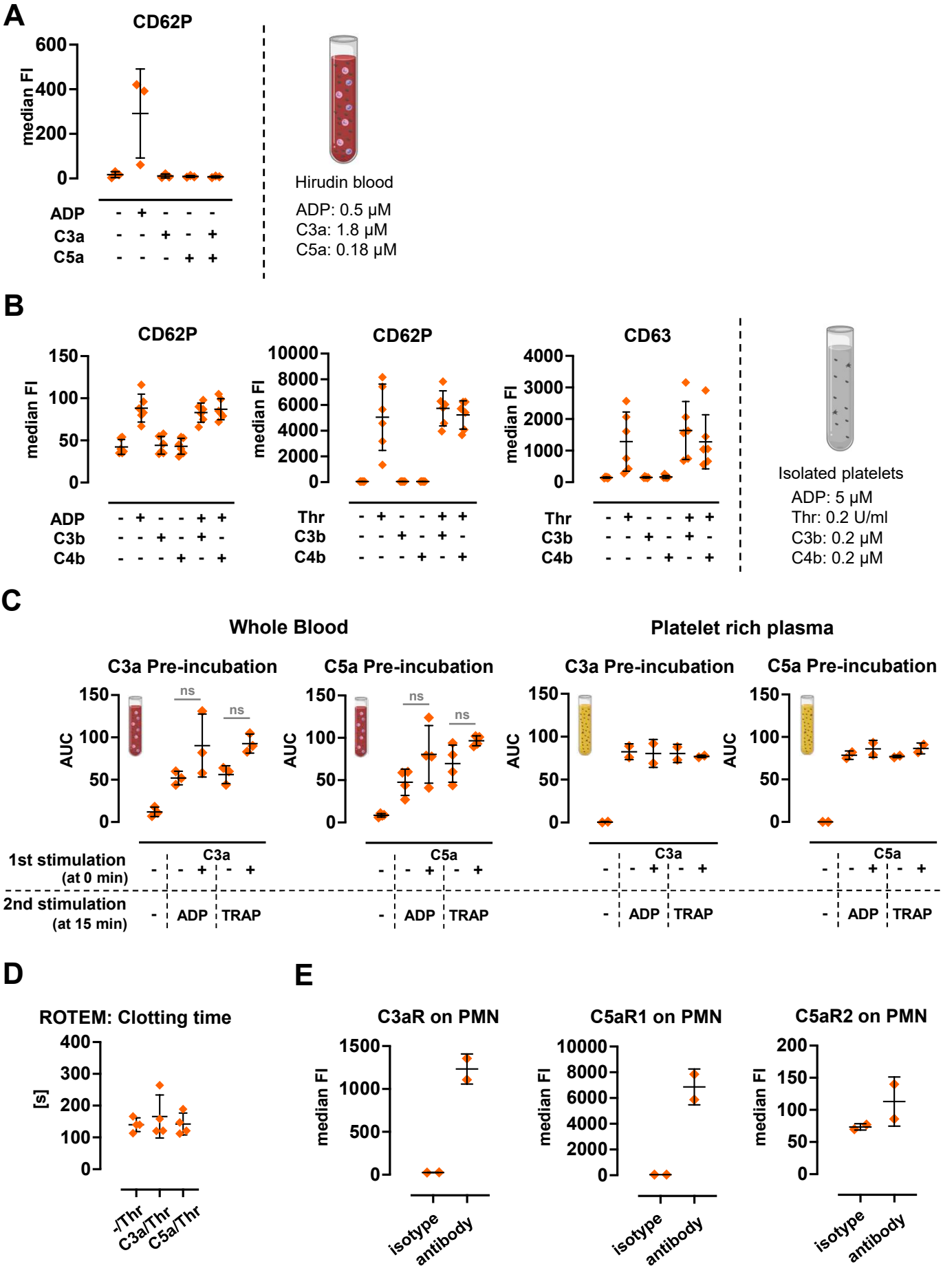
Supplemental Figure 1: Effects of complement activation on monocytes and T cells. (A) Zymosan-mediated complement activation. FPX-anticoagulated whole blood was incubated for 30 or 120 min in absence or presence of zymosan (2.5 mg/ml) at 37 °C under moving conditions and compared to baseline (BL; i.e. 0 min). Plasma samples derived from this whole blood model were analyzed for C3a generation by ELISA. **(B)** Activation of monocytes. Surface expression of CD11b and CD62L was measured by flow cytometry after incubation in the whole blood model (without addition of zymosan) at the specified time points. **(C)** Evaluation of CD8+ and CD4+ lymphocytes. FPX-anticoagulated blood was heparinized and CD4+ and CD8+ lymphocytes were analyzed for surface expression of CD25, CD69 and HLA-DR by flow cytometry. **(D)** Soluble activation markers. Plasma levels of IL-2R were measured in EDTA plasma derived form the whole blood model. All graphs show mean values with standard deviation derived from 3-5 independent assays.
Data sets were tested for outliers using the ROUT outlier test (Q=5 %). Data sets in (A,B) and (D) were analyzed using repeated measures one-way ANOVA. Experimental groups were post-hoc tested for statistical significance against the *120 min + Cp40* group with Dunnett`s correction for multiple comparisons (B,D) or against each other experimental group (Tukey's test with correction for multiple comparisons; (A)). For the sake of visibility, only statistical comparison of relevance for the experimental hypotheses are shown. ns: not significant.

Supplemental Figure 2



Supplemental Figure 2: Gating strategy for analysis by flow cytometry. (A) Innate immune cells. For determining polymorphonuclear cells or monocytes, singlets were gated via FSC-H/FSC-A plot and separated in the FSC-A/SSC-A plot. CD14+ monocytes and size-gated PMNs were analyzed for CD11b and CD62L surface expression. **(B)** Lymphocytes. CD45+ lymphocytes were stained for CD3 and further separated in CD4+ and CD8+ lymphocytes. These populations were then analyzed for CD69, CD25 and HLA-DR surface expression. **(C)** Platelets in FPX-anticoagulated whole blood. Singlets were identified in a FSC-H/FSC-A plot and a platelet population was identified by size in the FSC-A/SSC-A plot. CD41+ platelets were examined for CD62P (P-Selectin) expression. **(D)** Platelets in hirudin-anticoagulated whole blood. Platelet measuring in hirudin blood was performed by identifying CD41+ cells which were then stained for CD62P and PAC-1. **(E)** Isolated platelets. The gating strategy resembles the on in *(C).* Cells were stained with an anti-CD41 antibody as platelet marker to confirm reliability of platelet isolation.

Supplemental Figure 3

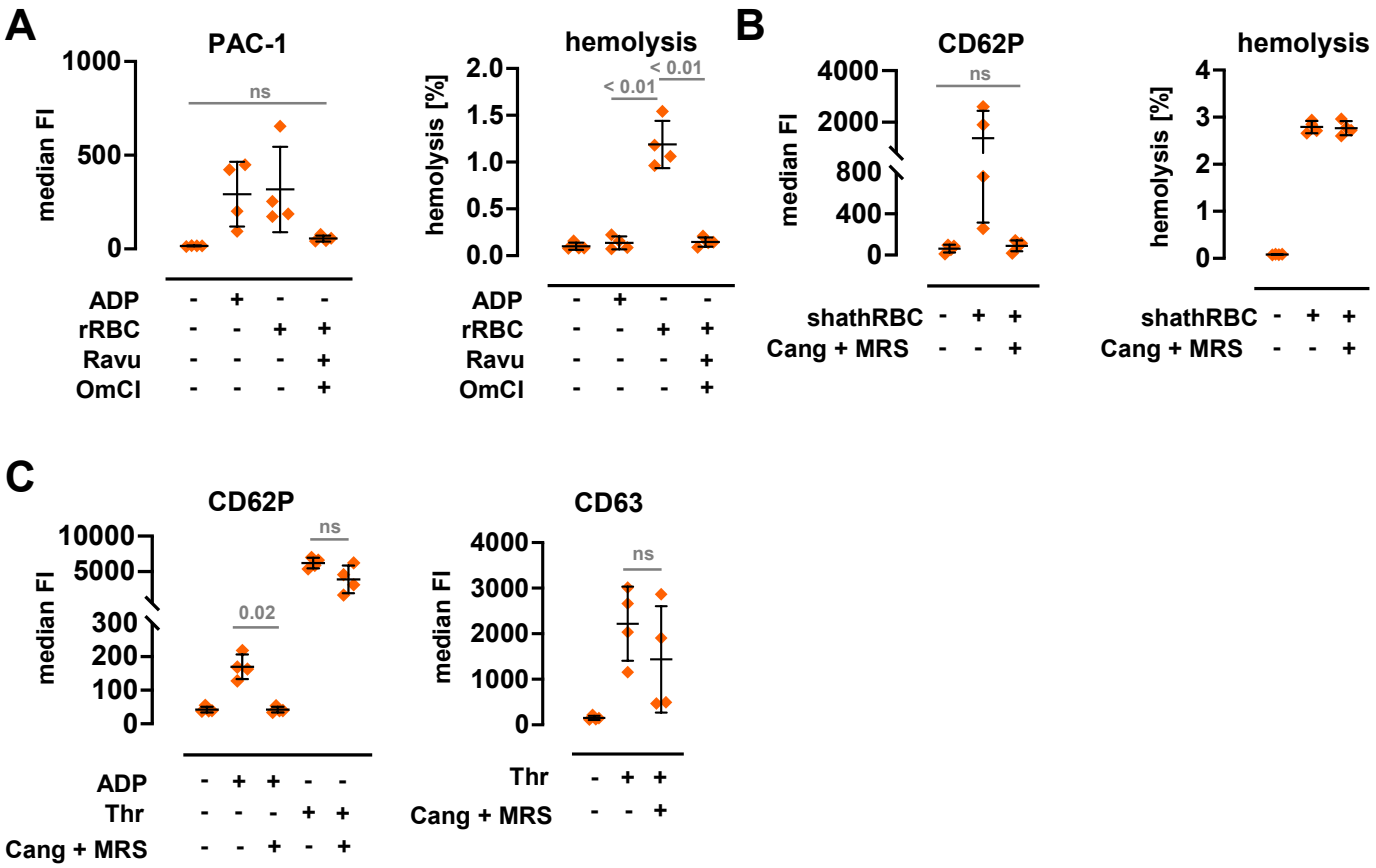


Supplemental Figure 3: Effects of complement activation products on platelet activation. (A) Stimulation of whole blood with anaphylatoxins. Hirudin-anticoagulated blood was exposed to either PBS (neg. Ctrl), ADP (0.5 µM), C3a (1.8 µM) and/or C5a (0.18 µM) and was analyzed for CD62P surface expression on CD41+ cells. Of note, the chosen C3a (1.8 µM) and C5a (0.18 µM) concentrations simulating a turnover of almost half of the native C3 and C5 in blood, respectively. Mean values with standard deviations are shown. **(B)** Stimulation of isolated platelets with soluble C3b or C4b. Isolated platelets were exposed to either PBS (neg. Ctrl), ADP (5 µM), Thrombin (Thr, 0.2 U/ml), C3b (0.2 µM) or C4b (0.2 µM) alone or in combinations as specified. CD62P and CD63 surface expression were measured as readouts. Mean values with standard deviation are shown **(C**) Multiplate aggregometry. Lepirudin-anticoagulated blood or platelet rich plasma was mixed with C3a (1.8 µM) or C5a (0.18 µM) for 15 min prior to ADP (3.25 µM) or TRAP (16 µM) exposure. NaCl (0.9 %) served as negative control. Mean of the area under the curve with standard deviation is shown from 3 experiments in whole blood and 2 in platelet rich plasma. **(D)** ROTEM analysis of pre-stimulated whole blood without addition of a specifc reagent that activates the intrinsic or extrinsic pathway. Citrated blood was incubated with PBS (neg. Ctrl), C3a (1.8 µM) or C5a (0.18 µM) for 15 min before being exposed to Thrombin followed by ROTEM analysis. Mean values with standard deviation are shown. **(E)** C3aR and C5aR1 expression on PMN. Citrated blood was stained with less antibody amount in comparison to the platelet experiment. The C3aR (hC3aRZ8), C5aR1 (S5/1) and C5aR2 (1D9-M12) antibodies were used at 0.16 µg/ml, 0.33 µg/ml and 2 µg/ml respectively.

Data sets were tested for outliers using the ROUT outlier test (Q=5 %) In data set (A) one outlier was removed prior to statistical testing. Data sets in (A) were analyzed using Prism's Mixed-Effects Model (due to missing values) and (B, C) were analyzed via repeated measures one-way ANOVA. Experimental groups were post-hoc tested for statistical significance with correction for multiple comparisons (Sidak (B) or Tukey's test (A,C)).

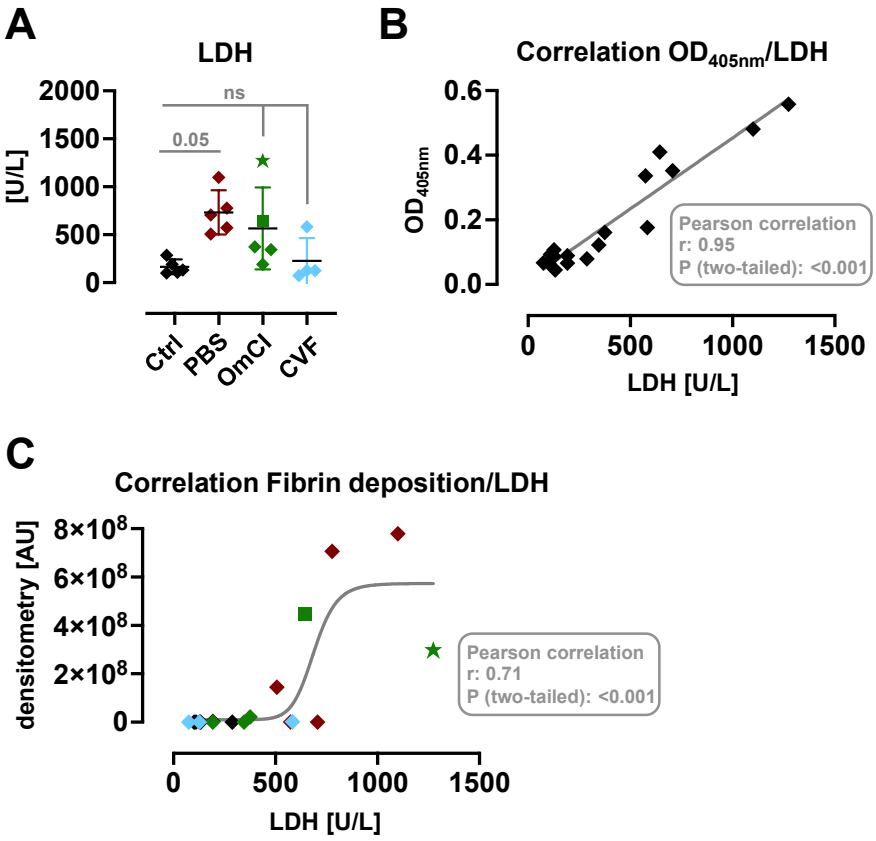
Data sets in (D) and (E) served as reference data and were not tested for statistical significance. For the sake of visibility, non-significant p-values were omitted from graphs, unless they were of relevance for the experimental hypotheses. ns: not significant.

Supplemental Figure 4



Supplemental Figure 4: Lysis of rRBC and associated platelet activation. (A) PAC-1 expression. In addition to the analysis of CD62P or CD63 surface expression, platelet activation was also assessed by the integrin IIb/IIIa status using the PAC-1 antibody which specifically recognizes an activation epitope of integrin IIb/IIIa. Blood was either treated with PBS (neg. Ctrl), rRBC in presence or absence of C5 double inhibition with Ravulizumab (Ravu, 0.4 µM) and OmCI (1.2 µM). Hemolysis was determined by measurement absorbance of the supernatant at 405 nm. **(B)** Inhibitory effects of ADP receptor antagonists on shattered human red blood cells. CD62P surface expression was assessed in hirudin blood after exposure to shattered human red blood cells in presence or absence of ADP receptor antagonists Cangrelor (1 µM) and MRS2179 (10 µM). **(C)** ADP receptor antagonists inhibits ADP-mediated but not Thr-mediated activation. Isolated platelets were exposed ADP (5 µM) or Thr (0.2 U/ml) in absence of presence of Cangrelor (1 µM) or MRS2179 (10 µM). All graphs show mean values with standard deviation.
Data sets were tested for outliers using the ROUT outlier test (Q=5 %) and analyzed using repeated measures one-way ANOVA. Experimental groups were post-hoc tested for statistical significance with correction for multiple comparisons via Tukey's test.

Supplemental Figure 5



Supplemental Figure 5: Rat model of acute intravascular hemolysis. (A) Lactat dehydrogenase (LDH) levels. End-point LDH levels were measured in peripheral blood. Mean values with standard deviation are shown. **(B)** Correlation between OD_{405nm} and LDH levels. **(C)** Correlation between Fibrin deposition and LDH levels.
Data sets were tested for outliers using the ROUT outlier test (Q=5 %). Data set in A was analyzed with one-way ANOVA and experimental groups were post-hoc tested for statistical significance with correction for multiple comparisons via Tukey's test. In (B) and (C), data sets were tested for correlation using Pearson's test. For the sake of visibility, non-significant p-values were omitted from graphs, unless they were of relevance for the experimental hypotheses. ns: not significant.

SUPPLEMENTAL Tables

Supplemental Table 1: ROTEM analysis after pre-incubation of whole blood. FPX-anticoagulated blood was citrated after indicated incubation time, recalcified and stimulated for either extrinsic (EXTEM) or intrinsic (INTEM) pathway activation. Mean values with standard deviations are shown from 8 subjects. ROUT outlier (Q=5 %) testing was performed and average values for which outliers had been excluded were compared to the '120 min + Cp40' sample by one-way ANOVA. A p-value below 0.05 was considered as statistically significant.

EXTEM	**Experimental group**				**statistics**
Parameter	**baseline**	**30 min**	**120 min**	**120 min + Cp40**	
Clotting time [s]	94.33 (± 18.99)	94.17 (± 8.73)	93.14 (± 9.03)	90.86 (± 8.82)	ns
Clot formation time [s]	91.83 (± 15.48)	92.67 (± 19.77)	87.14 (± 24.20)	87.00 (± 17.08)	ns
Maximum clot firmness [mm]	62.67 (± 4.13)	60.83 (± 4.58)	62.43 (± 5.22)	62.00 (± 4.55)	ns
Maximum clot firmness time [s]	1430 (± 210.9)	1374 (± 147.7)	1525 (± 245.5)	1446 (± 233.4)	ns
alpha [°]	72.33 (± 3.27)	71.50 (± 3.62)	72.57 (± 4.61)	72.43 (± 3.46)	ns
INTEM	**Experimental group**				**statistics**
Parameter	**baseline**	**30 min**	**120 min**	**120 min + Cp40**	
Clotting time [s]	260.8 (± 37.40)	229.4 (± 6.19)	203.4 (± 16.29)	194.3 (± 22.11)	*
Clot formation time [s]	88.50 (± 26.46)	76.80 (± 13.16)	69.29 (± 21.36)	69.83 (± 19.95)	ns
Maximum clot firmness [mm]	60.17 (± 5.42)	61.80 (± 3.35)	63.29 (± 6.42)	64.17 (± 6.21)	ns
Maximum clot firmness time [s]	1211 (± 157.6)	1220 (± 175.6)	1223 (± 187.2)	1246 (± 242.7)	ns
alpha [°]	72.17 (± 5.04)	74.20 (± 2.68)	76.14 (± 3.81)	75.83 (± 3.98)	ns

Supplemental Table 2: ROTEM analysis under C3 inhibition. Freshly drawn citrated blood was supplemented with either PBS-/- (neg. Ctrl) or Cp40 (20 µM) and measured in according to the manufacturer's instructions for intrinsic or extrinsic pathway activation. The addition of cytochalasin D to exclude a platelet contribution (FIBTEM) was either started by the extrinsic or intrinsic activation reagent. Mean values with standard deviation are shown from 5 subjects (FIBTEM with extrinsic activation start n=4). A p-value below 0.05 was considered as statistically significant.

EXTEM	**Experimental group**		**Paired t-test**
Parameter	**PBS-/-**	**Cp40**	
Clotting time [s]	78.40 (± 9.18)	79.60 (± 6.51)	ns
Clot formation time [s]	104.8 (± 12.13)	96.60 (± 12.42)	ns
Maximum clot firmness [mm]	61.00 (± 1.58)	60.80 (± 1.64)	ns
Maximum clot firmness time [s]	1574 (± 330.1)	1541 (± 262.5)	ns
alpha [°]	69.20 (± 2.77)	71.00 (± 2.91)	ns
INTEM	**Experimental group**		**Paired t-test**
Parameter	**PBS-/-**	**Cp40**	
Clotting time [s]	227.2 (± 49.14)	284.8 (± 80.29)	0.03
Clot formation time [s]	100.2 (± 22.73)	117.20 (± 33.09)	ns
Maximum clot firmness [mm]	58.20 (± 2.17)	57.20 (± 2.95)	ns
Maximum clot firmness time [s]	1383 (± 227.6)	1432 (± 187.7)	ns
alpha [°]	70.00 (± 4.47)	67.20 (± 5.93)	ns
FIBTEM (with EXTEM start)	**Experimental group**		**Paired t-test**
Parameter	**PBS-/-**	**Cp40**	
Clotting time [s]	71.25 (± 16.36)	75.25 (± 19.21)	ns
Maximum clot firmness [mm]	15.00 (± 3.83)	16.00 (± 2.94)	ns
Maximum clot firmness time [s]	1259 (± 311.5)	1424 (± 334.2)	ns
alpha [°]	70.50 (± 0.71)	66.67 (± 5.68)	ns
FIBTEM (with INTEM start)	**Experimental group**		**Paired t-test**
Parameter	**PBS-/-**	**Cp40**	
Clotting time [s]	257.6 (± 73.67)	294.2 (± 127.7)	ns
Maximum clot firmness [mm]	20.60 (± 5.55)	19.80 (± 4.76)	ns
Maximum clot firmness time [s]	1434 (± 739.5)	1622 (± 549.4)	ns

Supplemental Table 3: ROTEM analysis under C5 inhibition.
Citrated blood was supplemented with the C5 inhibitor OmCI (2 µM) or PBS-/- (neg. Ctrl) and activated by the INTEM reagent. Mean values with standard deviation are shown from 5 subjects. A p-value below 0.05 was considered as statistically significant.

INTEM	**Experimental group**		**Paired t-test**
Parameter	**PBS-/-**	**OmCI**	
Clotting time [s]	312.0 (± 90.09)	353.8 (± 138.8)	ns
Clot formation time [s]	145.8 (± 37.04)	135.2 (± 33.00)	ns
Maximum clot firmness [mm]	55.40 (± 6.11)	55.80 (± 6.34)	ns
Maximum clot firmness time [s]	1532 (± 241.1)	1494 (± 242.9)	ns
alpha [°]	62.40 (± 5.77)	64.00 (± 5.70)	ns

Supplemental Table 4: ROTEM analysis under C3 inhibition in platelet rich plasma.
Citrated blood was processed to platelet rich plasma which was exposed to either PBS-/- (neg. Ctrl) or Cp40 (20 µM). Mean values with standard deviation is shown from 6 experiments.

INTEM	**Experimental group**		**Paired t-test**
Parameter	**PBS-/-**	**Cp40**	
Clotting time [s]	296.2 (± 184.4)	275.7 (± 130.7)	ns
Clot formation time [s]	67.50 (± 54.94)	60.33 (± 37.10)	ns
Maximum clot firmness [mm]	74.50 (± 4.32)	75.17 (± 3.87)	ns
Maximum clot firmness time [s]	1269 (± 209.1)	1216 (± 162.7)	ns
alpha [°]	77.67 (± 8.80)	78.67 (± 6.77)	ns

Supplemental Table 5: Raw data of the rat model of acute intravascular hemolysis.

There are no absorbance (OD_{405nm}) values for the first two PBS animals since no citrate plasma was collected at the final bleed. Missing neutrophil counts in the OmCI and CVF group were due to erroneous sample handling at the clinical chemistry analysis department. The missing lactate dehydrogenase value in the CVF animal is due to a detection problem. The measurement was disturbed by the massive hemolysis in the sample. The green and blue stars and the green square relate to specific animals in inhibitor groups showing very high hemolysis levels.

Group		Neutrophil [Giga/L]	Platelet [Giga/L]	OD_{405nm}	LDH [U/L]	Fibrin deposition [AU]
Ctrl	1	1	938	0.079	288	4.3e5
	2	0.6	945	0.066	101	3.3e5
	3	0.8	1076	0.066	192	3.7e6
	4	0.7	1052	0.093	110	1.1e6
	5	0.7	1155	0.045	133	5.3e5
PBS	1	2	960	n/d	507	1.4e8
	2	3.3	902	n/d	778	7.1e8
	3	2.4	847	0.481	1100	7.7e8
	4	3	963	0.336	574	1.4e6
	5	2.5	883	0.352	706	3.4e5
OmCI	1 ★	2.2	789	0.558	1274	2.9e8
	2	n/d	944	0.122	346	2.4e5
	3	1.4	937	0.089	192	4.3e5
	4	3.7	1024	0.161	375	2.2e7
	5 ■	n/d	834	0.410	644	4.4e8
CVF	1 ★	n/d	913	0.691	n/d	7.6e7
	2	2	993	0.087	127	1.2e6
	3	1.1	831	0.176	583	2.1e6
	4	1.7	1162	0.067	75	2.1e5
	5	1.6	883	0.107	127	1.4e5

SUPPLEMENTAL Material and Methods

In vitro whole blood models

Artificial surface-induced complement activation

Freshly drawn FPX-anticoagulated blood was briefly inverted and transferred into 3 % (w/v) 2-methacryloyloxethyl phophorcholine polymer (MPC)-coated reaction tubes. Samples were incubated for 0, 30 or 120 min at 37 °C under rotatory conditions (10 rpm) on a rotating wheel. To suppress complement activation, another sample was supplemented with Cp40 (final concentration 20 µM) and incubated for 120 min. After the respective incubation time, blood was either citrated (9:1 with 3.2 % sodium citrate) or supplemented with PBS-EDTA (10 mM final concentration) and further processed as indicated in the following sections.

Foreign cell-induced complement activation

20 µl of hirudin-anticoagulated blood were exposed to 80 µl PBS-/- supplemented with stimulants, including ADP (A2754, Sigma-Aldrich, Germany), rRBC or shatRBC. Optionally, the complement inhibitors Cp40 (4 µM), Eculizumab (0.4 µM), Ravulizumab (0.4 µM), OmCI (1.2 µM) or ADP receptor antagonists Cangrelor (Cang, 1 µM, SML2004, Sigma-Aldrich, Germany) and MRS2179[26,27] (MRS, 10 µM, M3808, Sigma-Aldrich, Germany) were added. Samples were incubated for 20 min at 37 °C and subsequently fixed with paraformaldehyde (PFA, 0.5 % final concentration, P6148, Sigma-Aldrich, Germany). In the case of staining with the PAC-1 antibody, the fixation with paraformaldehyde was performed 20 min after staining. In all other cases staining was performed after fixation. After a centrifugation step at 300 g for 5 min, the supernatant was collected, and the absorbance was measured at 405 nm. Cells were resuspended in 500 µl PBS-/- and processed as described in the following sections.

Platelet isolation

Blood was drawn into a neutral monovette spiked with citrate-phosphate-dextrose buffer (16 mM citric acid, 90 mM NaCl, 16 mM Na_2HPO_4, 142 mM glucose, pH 7.4) and immediately centrifuged at 150 g with the lowest possible adjustments for acceleration and brake. The upper plasma part was mixed with HEP buffer (140 mM NaCl, 2.7 mM KCl, 3.8 mM HEPES, 5 mM EGTA, pH 7.4) at a 1:1 ratio and supplemented with Iloprost (10 µM final concentration, SML1651, Sigma Aldrich, Germany). After another centrifugation step at 100 g for 15 min, plasma was removed and cell pellet was rinsed with platelet-wash-buffer (10 mM sodium citrate, 150 mM NaCl, 1 mM EDTA, 1 % (w/v) glucose, pH 7.4) and eventually resuspended in Tyrode's buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4).

Rotational thromboelastometry (ROTEM)

Citrated blood or platelet rich plasma were assayed for their coagulative functionality by rotational thromboelastometry (ROTEM® delta, Werfen GmbH, Germany). After recalcification using star-tem20 (000503-10, Werfen GmbH, Germany), the extrinsic and intrinsic coagulation pathways were stimulated specifically by the addition of ex-tem (000503-05, Werfen GmbH, Germany) or in-tem (000503-02, Werfen GmbH, Germany) reagents, respectively. To exclude thrombocytes from the reaction, cytochalasin D was added via fib-tem reagent (000503-06, Werfen GmbH, Germany). The impact of complement inhibition was assayed by the addition of Cp40 (20 µM) or OmCI (1 µM) in comparison to PBS-/- (negative control).

Beside this basic ROTEM investigation with fresh citrated blood, citrated FPX blood was analyzed after the respective incubation time in accordance to the manufacturer's instructions. For analyzing anaphylatoxins, citrated blood was exposed to C3a (1.8 µM), C5a (0.18 µM), thrombin (1 U/ml) or PBS-/- (neg. Ctrl), recalcified using star-tem20 reagent but not specifically activated via extrinsic or intrinsic stimulation. Besides, citrated blood was pre-incubated with C3a and C5a for 15 min at 37 °C prior to thrombin exposure and recalcification.

ELISAs

If not stated otherwise, all commercially obtained ELISAs were performed in accordance with the manufacturer's instructions. Complement activation products C3a, C5a and sC5b-9 were measured in EDTA-plasma via the C3a ELISA kit (A031, Quidel, USA), the C5a ELISA kit (EIA-3327, DRG international, USA) and the sC5b-9 ELISA kit (558315, BD Biosciences, USA), respectively. Levels of thrombin/anti-thrombin (TAT) complexes (ab108907, abcam, USA), the cytokines IL-6 (555220, BD Biosciences, USA) and IL-8 (DY208, R&D systems, USA), CD40L (DY617, R&D systems, USA), vWF (DY27664-05, R&D systems, USA), MMP-9 (DY911, R&D systems, USA) and IL-2R (DY233, R&D systems, USA) were measured in EDTA-plasma as well.

For determination of native, non-activated C5 in rat plasma, a self-assembled sandwich ELISA was established. A 96-well plate (MaxiSorb, 446612, ThermoFisher Nunc) was coated overnight at 4 °C with 200 µg/ml recombinant FH8-15-OmCI diluted in PBS-/- in a total volume of 50 µl/well. FH8-15-OmCI is fusion protein of the factor H domains 8-15 and OmCI. The fusion was constructed because coating of the relatively small protein OmCI (17.3 kDa) onto the microtitre plate resulted in the loss of binding activity for C5 which was maintained for the FH8-15-OmCI (71.8 kDa) fusion protein. OmCI is known to bind to rat as well as human C5[28]. The next day, wells were washed twice with 200 µl PBST (PBS + 0.05 % Tween20) and blocked with PBS-BSA (PBS-/- + 1 % (w/v) BSA) for 1 h at RT. Afterwards, 1:25-diluted rat plasma was incubated in a total volume of 50 µl for 45 min at RT. After three washing steps with PBST, 50 µl of a 1:100-diluted polyclonal rabbit anti-rat C5a antibody (PA5-78891,

Invitrogen, Germany) was added for 1 h. Followed by three washing steps with PBST, 50 µl of a donkey anti-rabbit HRP-linked F(ab')2 fragment (NA9340, GE Healthcare) were added at a final dilution of 1:500 and incubated for 30 min at RT in darkness. Finally, antibody was washed away and 100 µl of TMB substrate solution (cat.: 555214, BD Biosciences, USA) were added to each well for 15 min in darkness before 50 µl of 2N H_2SO_4 solution was added to stop the reaction. The absorbance was measured at 450 nm.

Blood cell analysis by flow cytometry

For analysis of monocytes, 50 µl of citrated FPX-blood were stained with an anti-CD14 (1.5 µg/ml, BV785-conjugated, 367142, Biolegend, USA; isotype: 400169, Biolegend, USA), anti-CD62L (62.5 ng/ml, PE-conjugated, 304806, Biolegend, USA; isotype: 400112, Biolegend, USA) and an anti-CD11b (7.5 ng/ml, APC-conjugated, 101212, Biolegend, USA; isotype: 400612, Biolegend, USA) antibody while incubating for 15 min in the water bath at 37 °C. Afterwards, 1.8 ml of a 1:10-diluted FACS™ lysing solution (349202, BD Biosciences, USA) were added. After incubation for 10 min at RT the samples were centrifuged at 500 g for 5 min. The supernatant was discarded and cells were washed once with 500 µl PBS-/-. Finally, cells were resuspended in 500 µl PBS-/- and kept in the fridge until measurement. The detailed gating strategy is shown in Supplemental Figure 2A.

Similar to that, polymorphonuclear cells (PMNs) were analyzed from citrated blood. After incubation with an anti-CD11b (7.5 ng/ml, APC-conjugated, 101212, Biolegend, USA; isotype: 400612, Biolegend, USA) and anti-CD62L (62.5 ng/ml, PE-conjugated, 304806, Biolegend, USA; isotype: 400112, Biolegend, USA) antibody, cells were transferred to 1 ml of a 1:10 diluted FACS™ lysing solution and incubated at RT for 30 min in darkness. After centrifugation at 340 g for 5 min at RT, the supernatant was discarded. Cells were resuspended in 200 µl PBS-/- + 1 % BSA and stored in fridge until measurement. Detailed gating strategy is shown in Supplemental Figure 2A.

Surface activation markers on peripheral lymphocytes were detected in heparinized full blood after the indicated time of stimulation by using anti- CD3 APC (IM2467), CD4 APC-AF750 (A94682), CD8 Pacific Blue (B49182), CD45 Krome Orange (B36294), CD69 PE-Cy7 (310912), HLA-DR PE (IM1639) (all Beckman Coulter, Krefeld, Germany) and anti- CD25 (R0811) (Dako/Agilent, Santa Clara, CA) antibodies. After staining of the cells in 100 µl full blood via incubating for 15 min at RT in darkness, erythrocytes were lysed by adding 500 µl of Optilyse™ (Beckman Coulter) for another 15 min. Cells were then washed once with 500 µl IF-Medium (PBS (Gibco/ Thermo Fisher Scientific, Waltham Massachusetts)/ 0,5% BSA (PAN Biotech Aidenbach, Germany)/ 0,1% NaN_3 (Merck, Darmstadt, Germany)) and centrifuged for 5 min at 350 g. Samples were measured in 500µl IF-Medium with a Navios™ Flow Cytometer

(Beckman Coulter) and analyzed using the KALUZA Analysis 2.1. Software (Beckman Coulter). Detailed gating strategy is shown in Supplemental Figure 2B.

For platelet measurement in FPX-anticoagulated blood, blood was citrated and diluted 1:12.5 in pre-warmed HBSS-/- buffer solution. 40 µl of this solution were mixed with ADP (5 µM final concentration, A2754, Sigma Aldrich, Germany) or PBS-/- (negative control) to reach a final volume of 50 µl and incubated for 10 min in the water bath at 37 °C. Afterwards, an anti-CD41 (250 ng/ml, 303710, Biolegend, USA; isotype: 400122, Biolegend, USA) and an anti-CD62P (2 µg/ml, 304904, Biolegend, USA; isotype: 400108, Biolegend, USA) antibody were added and incubated for another 5 min in the water bath. Finally, samples were transferred into 2 ml HBSS-/-, inverted and again 1:2 diluted with HBSS-/- in the FACS tube. The detailed gating strategy is shown in Supplemental Figure 2C.

For investigation of platelets in hirudin blood, cells were stained with an anti-CD41 antibody (0.4 µg/ml, FITC, MCA467F, BD Bioscience; isotype: FITC mouse anti-IgG1 kappa isotype controls, 33814) for platelet identification and a biotinylated anti-CD62P (1 µg/ml, ab239233, abcam) or anti-human PAC-1 (0.3 ng/ml, FITC, 340507, BD Bioscience; isotype: FITC mouse IgM kappa isotype control, 553474, BD Bioscience) as activation markers for 20 min in darkness. Afterwards, cells were centrifuged at 300 g for 5 min before being resuspended in 500 µl PBS-/-. In case of using a biotinylated antibody, cells were exposed to APC-labelled streptavidin (SA1005, Invitrogen) for another 30 min in darkness. Finally, cells were centrifuged and resuspended in 500 µl PBS-/- and measured by flow cytometry.

Besides measurement in whole blood, platelets were also analyzed after isolation. 40 µl of isolated platelets were treated with ADP (5 µM), Thrombin (0.2 U/ml, 605190, Merck, Germany), C3a (1000 ng/ml, A118, CompTech, USA), C4a (1000 ng/ml, A106, CompTech, USA), C5a (100 ng/ml, A144, CompTech, USA), C3b (0.2 µM, A114, CompTech, USA), C4b (0.2 µM, A108, CompTech, USA) or PBS-/- (negative control) for 10 min at 37 °C in the water bath and stained for platelet activation with an anti-CD62P antibody (2 µg/ml, 304904, Biolegend, USA; isotype: 400108, Biolegend, USA) or an anti-CD63 antibody (1 µg/ml, 353004, Biolegend, USA) for another 5 min before being fixed with 0.5 % PFA for another 10 min. Cells were centrifuged at 300 g for 10 min and resuspended in 500 µl PBS-/-. Additionally, isolated platelets were investigated for anaphylatoxin receptor expression by using an anti-C3aR antibody (2 µg/ml, 345808, Biolegend, USA; isotype: 400326, Biolegend, USA), an anti-C5aR1 antibody (1 µg/ml, 344310, Biolegend, USA; isotype: 400222, Biolegend, USA) and an anti-C5aR2 antibody (4 µg/ml, 342404, Biolegend, USA; isotype: 400212, Biolegend, USA) without fixation process. Afterwards, samples were transferred to 500 µl PBS-/- provided in FACS tubes. The gating strategy resembles that of FPX-anticoagualted whole blood and is shown in figure 2E.

Multiplate aggregometry analysis

Multiplate aggregometry (Multiplate®, Roche Diagnostics) was performed in whole blood and PRP anticoagulated with 50 µg/ml lepirudin. Both were diluted with saline (0.9 % NaCl) at a ratio of 1:1. Test substances including thrombin receptor activating peptide (TRAP, 32 µM), ADP (6.5 µM) and the anaphylatoxins C3a (2 µM) and C5a (0.2 µM) were added and incubated for 2 min prior to measurement start in accordance with the manufacturer's instructions. Besides, whole blood and PRP were pre-incubated with C3a and C5a for 15 min at 37 °C before being exposed to TRAP (16 µM) or ADP (3.25 µM).

Rat model of acute intravascular hemolysis

The experimental procedure is in accordance to previously performed experiments (39, 40) with some modifications and was approved by the regulatory authority (Regierungspräsidium Tübingen TVA1542). In brief, male Wistar rats received a single dose of 500 mg/kg bodyweight buprenorphine prior to instrumentation. Rats were anesthetized with Sevofluorane (3.5-4 % initially, thereafter, level was adjusted depending on the animal behavior), a catheter was placed in the *vena femoralis* and animals were infused with either PBS-/-, or OmCI at a final blood concentration of 2 µM assuming a total blood volume of 14 ml. For the first two animals receiving PBS-/- as analyte, the catheter had been placed in the *vena jugularis*. However, after the first two animals the procedure was optimized to facilitate a faster catheterization via placing the catheter in the *vena femuralis*. All other procedures remained identical. After 5 min, 1 ml of human AB red blood cells at hematocrit of 80% were transfused simulating a 15% transfusion mismatch. Blood was drawn at 0.5, 5, 20, 60 and 120 minutes via tail vein puncture and anticoagulated by adding the same volume of PBS-EDTA (containing 10 mM EDTA dissolved in PBS). Finally, animals were euthanized. Blood was collected via the abdominal aorta and was anticoagulated with citrate, heparin or EDTA depending on the sampling. Organs were extracted at necroscopy for investigation of the various end-point effects.

Immunohistochemical fibrin staining of rat lungs

Post-mortem, lung samples were fixed in 3.5 % formalin for 2 days, then dehydrated and embedded in paraffin blocks. Paraffin sections (3-5 µM) were placed on glass slides and dried for one hour at 50 °C and overnight at 37 °C. After deparaffinization in xylene, slides were rehydrated in a graded series of ethanol to deionized water. Heat-induced antigen retrieval was performed in sodium citrate buffer at pH 6. Slides were blocked with 10 % normal goat serum (cat.: 005-000-121, Jackson Immuno Research, USA) before being incubated with the primary antibody (rabbit monoclonal anti-fibrinogen beta chain antibody, clone [EPR18145-84], ab227063, abcam, USA) at a 1:4000 dilution for 1 h at RT. After washing, slides were incubated with a 1:100-diluted alkaline phosphatase-labeled goat anti-rabbit antibody (cat.:

111-055-144, Jackson Immuno Research) and visualized with the Dako real detection system (cat.: K5005, Agilent Dako, USA) followed by counterstaining with hematoxylin (cat.: 51275, Sigma Aldrich, Germany). To quantify IHC signal intensities of fibrin depositions in the lung, 5 representative images (100x) were taken from each section with a Zeiss Axio imager A1 microscope and analyzed for signal density using the ZEN microscopy software (version 3.0, ZEISS, Germany) and median values of signal density were calculated for each animal.

Platelet activation in AB/O mismatch model

EDTA-anticoagulated blood was drawn from a donor of blood group AB and centrifuged for 5 min at 2200g, 4 °C. Plasma and white blood cells were removed and 50 µl of packed RBC were washed twice with 1 ml PBS-/-. In parallel, hirudin-anticoagulated blood was drawn from a donor with blood group O and supplemented either with PBS or a mix of Ravulizumab and OmCI (to reach a final concentration of 0.8 µM each). 5 µl of PBS (blank) or washed and packed AB-RBCs were added, and samples were incubated for 30 min under circulating conditions (10 rpm). Afterwards, 400 µl of each reaction was supplemented with EDTA to stop any complement activity. To obtain plasma the 400 µl aliquotes of the reactions were centrifuged at 2200 g for 3 min. For measuring cell activation levels 5 µl of the original whole blood reaction (from above) were added to 25 µl of pre-diluted antibodies to assess platelet activation (anti-CD41 Ab [final conc.: 0.5 µg/ml, clone: HIP-8, APC-conjugated, 303710, Biolegend] with isotype [final conc.: 0.5 µg/ml, clone: MOPC-21, APC-conjugated, 400120, Biolegend] and anti-CD62P Ab [final conc.: 2 µg/ml, clone: AK4, FITC-conjugated, 304904, Biolegend] with isotype [final conc.: 2 µg/ml, clone: MOPC-21, FITC-conjugated, 400108, Biolegend]. Then, cells were fixed by adding 25 µl of 1 % PFA, transferred to 500 µl of 1 % PBS-BSA solution and diluted 1:4 before measurement.

Statistics

For descriptive statistics, statistical testing and graphical depiction, Prism9 (GraphPad Software, USA) was used. Descriptive statistics were performed on all data sets, prior to further analysis. Due to small sample sizes, determination of data distribution was rationally based on descriptive statistics, visual analysis of scatter plots and cumulative analysis of preliminary data. Following descriptive statistics and determination of distribution, a ROUT outlier test with a false discovery rate set to 5%, was performed were applicable and cleaned data was tested for statistical significance. All eliminated outliers are referenced to in detail in the respective

figure legends. Data sets are plotted with individual values of replicants with group means and standard deviations.

Data sets with two experimental groups were tested for statistical significance using either paired or unpaired t-test, depending on the experimental set-up. Data sets with more than two experimental groups were tested employing either the repeated measures (RM) one-way ANOVA or the Prism 9 mixed-effects model with correction for unequal variability of differences (Geisser-Greenhouse method). Post-hoc tests with correction for multiple comparisons were performed as referenced in detail in the respective figure legends. Results were considered statistically significant for p-values <0.05. Note that, for the sake of visibility, depiction of statistically non-significant p-values was omitted if not of relevance for the experimental hypotheses.