Complement C3 inhibition by compstatin Cp40 prevents intra- and extravascular hemolysis of red blood cells

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Supplemental information

Complement C3 inhibition by Compstatin Cp40 prevents intra- and extravascular hemolysis of red blood cells

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MATERIALS AND METHODS

AIHA samples

Serum samples from patients with suspected (n=6) or with proven AIHA (n=4) were sent in for pre-transfusion work-up in case of hemolysis or a positive result in the direct antiglobulin test (DAT) before planned surgery. Based on clinical characteristics shared with the reference laboratory 4 out of 10 patients had active hemolysis accompanied by the presence of auto-Ab. In 6 out of 10 of these patients, no detailed information on the presence of hemolysis has been shared with the reference laboratory. Residual material was anonymized and stored at -20°C until further use. Samples were included in this study based on a positive test result for complement C3d in the DAT. Samples were used according to the Dutch established codes of conduct for responsible use and approved by our institute. Healthy control serum, RBCs and plasma from a healthy donor with blood group AB were obtained from anonymous healthy volunteers. Written informed consent was given in accordance with Dutch regulations and the Declaration of Helsinki.

RBC isolation and bromelain treatment

Blood group O-typed RBCs from healthy donors were used in all experiments to avoid anti-blood group antibodies from the patient serum to react. EDTA blood was spun down and washed three times in phosphate buffered saline (PBS) to isolate RBCs. RBCs were used for an experiment the same day or stored at 4°C in 50% SAGM solution for up to a maximum of 2 weeks. To facilitate antibody binding and complement activation on RBCs, cells were treated with bromelain (Sanquin Reagents) on the day of the experiment. RBCs and bromelain were incubated in a 1:2 (v/v) ratio at 37°C for 10 minutes before the cells were washed twice in PBS and resuspended in the required buffer.

Complement deposition assay

Bromelain treated RBCs were resuspended in veronal buffer supplemented with 0.05% gelatin (VBG^{--}) and 1 $x10^6$ RBCs/well were added to a round bottom 96-well plate. Patient serum was diluted in veronal buffer supplemented with 0.05% gelatin, 10 mM $CaCl_2$ and 2 mM $MgCl_2$ (VBG^{++}) and added to the RBCs in a final concentration of 0.5%. Normal human EDTA plasma was recalcified by incubation with $CaCl_2$ (12mM) to induce clotting. The formed fibrin clot was removed by centrifugation (10 min at 1300g) and the recalcified plasma was stored at -80°C until use in the complement deposition assay. As a source of complement proteins, 25% (v/v) recalcified EDTA plasma from a donor with AB blood group was added, supplemented with 20 µg/mL Eculizumab (Alexion pharmaceuticals) to prevent MAC formation and hemolysis. Then either VBG^{-} or one of the inhibitors was added. Cp40 and a Cp40 control scramble peptide were used at 15 µM. αC1q-85 (50 ug/mL, Sanquin Research), C1 esterase inhibitor (C1-INH,

40µM, Sanquin) and EDTA (20 mM) served as controls. Samples were incubated for 30 minutes at 37°C while shaking. Next, cells were spun down and washed three times in PBS supplemented with 0.5% bovine serum albumin (BSA). RBCs were stained for 20 minutes at RT in PBS 0.5% BSA supplemented with FITC conjugated anti-C3dg (1 µg/mL, clone anti-C3-19, Sanquin Research) and APC conjugated anti-C4d (1 µg/mL, clone anti-C4-10, Sanquin research). The anti-C3 antibody used to detect C3 deposition was raised against C3dg and recognizes C3b, iC3b and C3d. All this deposition is termed C3b throughout the manuscript. For IgG opsonization experiments, either bromelain-treated or untreated donor RBCs were incubated with patient sera and a FITC conjugated anti-IgG (1ug/mL, clone M1025, Sanquin Research) was used to stain the RBCs. After staining, cells were washed three times and resuspended in PBS 0.5% BSA and measured on a FACS Canto. To exclude agglutinated RBCs upon analysis, single-cell gating was applied.

Hemolytic assay

Bromelain treated RBCs were resuspended in VBG^{--} and added to wells of a round bottom 96-well plate to a final concentration of 0.4% haematocrit. As a 100% lysis positive control, distilled water was added to the RBCs and PBS and VBG^{++} were used as negative controls. 10% (v/v) patient serum was added with 25% (v/v) recalcified plasma from a donor with AB blood group. The final concentration of Cp40 and the control peptide was 21 mM. The anti-C5 antibody Eculizumab was used at 28 µg/mL and EDTA at 28 mM final concentration. Cells were incubated for 90 minutes at 37°C, then remaining RBCs were pelleted and 100 µL supernatant was transferred to an ELISA maxisorp plate to measure extinction at 412/690 nm. Percentage hemolysis was calculated relative to the 100% water control.

Phagocytic assays with macrophages

Isolation of human monocytes from blood of a healthy donor was performed with the the Elutra Cell Separation System (Gambro, Lakewood, CO, USA). Purity of monocytes (>90%) was checked with flow cytometry before cells were frozen until further use. 4 x 10^6 monocytes per well were cultured in 12 well plates for 8 or 9 days in the presence of 10 ng/mL GM-CSF (Cellgenix) to obtain M1-like macrophages. Bromelain treated blood group O rhesus D-positive typed RBCs were fluorescently labelled with 0.5 µM PKH26 (Sigma). 3 x 10^6 labelled RBCs were incubated with 5% (v/v) patient serum, 25% (v/v) recalcified plasma from a healthy AB blood group donor and Cp40, scrambled Cp40 peptide, αC1q-85 or EDTA at concentrations described previously. Macrophages were co-cultured with 2 x 10^6 pre-treated RBCs for 2 hours at 37°C. After incubation, macrophages were washed with PBS 0.5% BSA and detached with 125 mM lidocaine (Sigma) with 10 mM EDTA. Non-phagocytosed RBCs were lysed (0.15 M NH4Cl, 0.01 M KHCO3, 0.1 mM EDTA in distilled water) and macrophages were stained with FITC-conjugated anti-HLA-DR antibodies (BD Biosciences). RBCs sticking to the outside of the

macrophages were stained with APC-conjugated anti-Rhesus D antibodies (Sanquin). Cells were fixed in 4% paraformaldehyde, resuspended in PBS 0.5% BSA and measured using imaging flow cytometry (ImageStream® X Mark II).

Statistical analysis

Graphical presentation and statistical analyses were performed using GraphPad Prism version 7.03 (GraphPad software, la Jolla, CA, USA). Median and data points or median and range are shown. A non-Gaussian distribution was assumed for the results and statistical tests were performed accordingly. Differences between groups were determined using one way ANOVA followed by appropriate post-test. $p<0.05$ was considered significantly different.

SUPPLEMENTAL FIGURES AND TABLES

Table SI. Patient characteristics and DAT score

Serum nr.	Sex	IgG	IgM	IgA	C3d	W/C/M AIHA
1	V	3	-	-	3	W
2	V	-	-	-	2	C
3	V	3	1	-	3	M
4	M	3	-	-	1	M
5	V	1	-	-	1	M
6	V	3	1	-	2	M
7	M	-	-	-	3	C
8	M	-	-	-	1	C
9	V	1	-	-	3	M
10	V	-	-	-	2	C
11	-	3	-	-	-	W
12	-	3	-	-	-	W

W= Warm AIHA
C= Cold AIHA
M= Mixed/Atypical AIHA

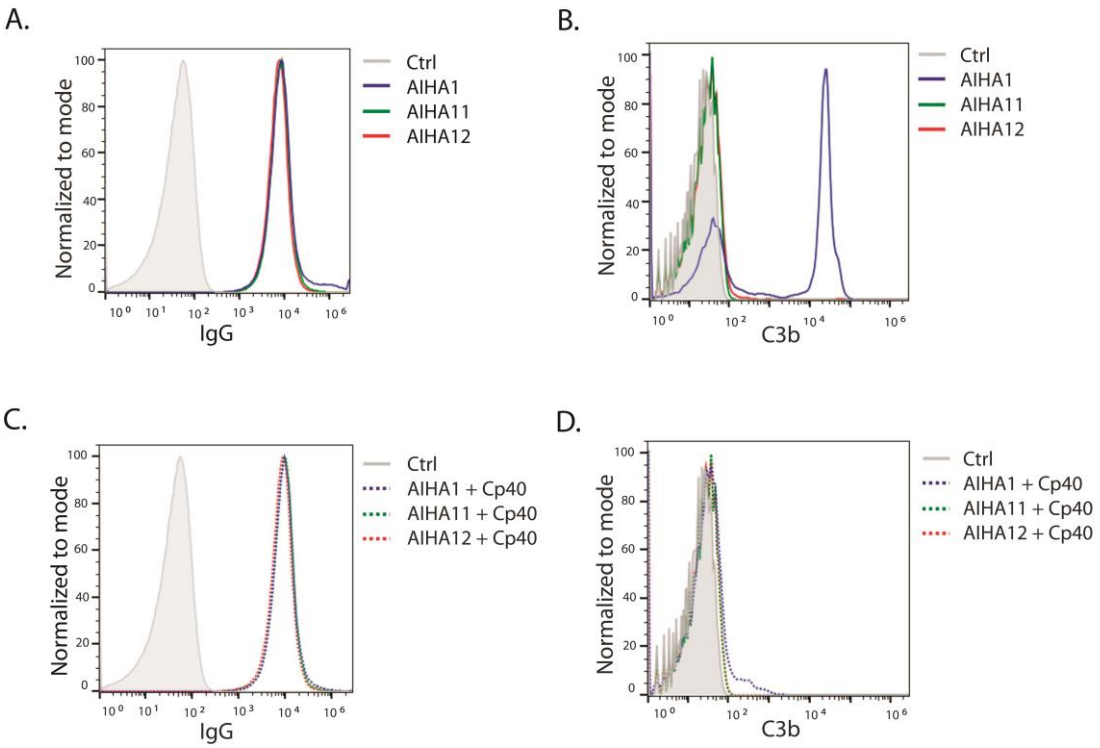

Fig. S1 Effect of Cp40 on IgG opsonization. RBCs were opsonized with sera of AIHA patients classified according to their DAT score as positive for C3 and IgG (AIHA1) or IgG only (AIHA11 and12) in the presence of recalcified human plasma from an AB blood group donor as a source of complement factors. C3b and IgG deposition on the RBCs was determined using flow cytometry. **(A and C)** Representative flow cytometry histograms showing IgG staining on the RBCs in absence **(A)** and presence of Cp40 **(C)** (n=3). **(B and D)** Representative flow cytometry histograms of C3b deposition on RBCs after incubation with sera from AIHA patients in the absence **(B)** or presence **(D)** of Cp40 (n=3).