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

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In autoimmune hemolytic anemia (AIHA), auto-antibodies (auto-Ab) directed against red blood cells (RBCs) lead to their breakdown through complement-dependent and -independent mechanisms. Multimeric IgG or IgM auto-Ab bind to RBCs and recruit C1, thereby triggering activation of the classical complement pathway. This results in the deposition of the opsonic fragment C3b on the RBCs and may, upon increasing opsonization, lead to the formation of membrane attack complexes (MAC). Complement-opsonized RBCs are cleared extravascularly via complement receptor-mediated phagocytosis mainly by liver macrophages, whereas IgG-opsonized RBCs are phagocytosed via *Fc-gamma* receptors by splenic macrophages. Additionally, intravascular hemolysis may occur due to complement-induced MAC formation on the RBCs membrane as a consequence of strong complement activation (by e.g. IgM auto-antibodies) causing direct hemolysis in circulation. A significant proportion of AIHA patients present IgM auto-Ab that are not detectable using the most common diagnostic techniques and complement activation accompanied by intravascular hemolysis¹. Intravascular hemolysis in turn directly relates to the disease course and severity².

Immunosuppressants are the first-line treatment in AIHA aiming to reduce auto-Ab production. However, they do not act immediately and a subset of patients is unresponsive³. In severe cases, symptomatic anemia in patients is corrected by RBC transfusion⁴. Yet, the efficacy of RBC transfusion is reduced since the RBC auto-Ab react with both recipient and donor RBCs causing the destruction of transfused cells⁵. Complement inhibition may be implemented to halt ongoing hemolysis in patients refractory to immunosuppressants and to improve the recovery of RBC transfusions by preventing hemolysis of donor RBCs. Currently, the only available therapeutic complement inhibitors are Eculizumab, used for the treatment of paroxysmal nocturnal hemoglobinuria (PNH), atypical hemolytic uremic syndrome (aHUS) and generalized myasthenia gravis, and C1 esterase inhibitor (C1-INH), approved for treatment of hereditary angioedema. Eculizumab inhibits complement activation at the level of C5 and blocks MAC formation, thereby preventing intravascular hemolysis; however, it does not halt opsonization or extravascular hemolysis, which renders this drug less suitable for AIHA patients⁶. Although complement inhibition at the C1 level using C1-INH has been shown to prevent MAC formation and complement-mediated extravascular hemolysis *in vitro* and *in vivo*^{7,8}, treatment of AIHA with C1-INH has limitations. Due to the low-affinity interaction with its substrates, high C1-INH doses are needed⁸. Moreover, C1-INH shows broad protease specificity and regulates different plasma cascade systems, which might be suboptimal for therapeutic use. Sutimlimab, a humanized monoclonal antibody directed against human C1s, specifically blocks the classical pathway of complement. Although Sutimlimab has been recently shown to be safe in a randomized first-in-human study in healthy volunteers, anti-drug antibodies were detected in some of the treated volunteers, which could compromise the inhibitory capacity of this antibody⁹. In the present study, we investigated the potential therapeutic effect of a small peptide of the Compstatin family (Cp40) in AIHA. Cp40 targets complement activation at the level of C3 and is therefore expected to block both extra- and intravascular hemolysis. Cp40 has been previously shown to inhibit C3 deposition on RBCs in an *in vitro* malaria model¹⁰ and complement opsonization and hemolysis of RBCs from PNH patients¹¹. Furthermore, Cp40 has been tested for safety in nonhuman primates¹² and is under clinical development for age-related macular degeneration treatment¹³.

First, to examine the effect of Cp40 on complement deposition, donor RBCs were incubated with sera from AIHA patients with a positive direct antiglobulin test (DAT) score for C3 deposition (see Methods in Online Supplementary Materials) and C4b and C3b deposition on the RBCs were analyzed using flow cytometry. Although complement deposition was observed with all tested sera,

opsonization levels differed among patients presumably due to variability in titer and the subclass of the opsonizing auto-Ab in the different patient sera (Fig 1A). Addition of Cp40 resulted in nearly complete reduction in C3b deposition on RBCs sensitized with AIHA sera (Fig. 1B-C). This reduction was stronger compared to C1-INH and similar to the levels observed when a monoclonal Ab against C1q was used and in the EDTA control (Fig. 1C), latter of which blocks all complement activity. No inhibition was observed with a sequence-scrambled Cp40 control peptide. As reported previously, higher levels of C4b were detected on the RBC membrane in the presence of Cp40 (Fig. 1D), probably due to enhanced detection of C4b in the absence of surrounding C3b¹⁵. Since Cp40 inhibits C3b deposition on RBCs incubated with AIHA sera, we next examined the effect of Cp40 on MAC formation that results in intravascular hemolysis. RBCs were incubated with patient serum in the presence or absence of Cp40. As expected, Cp40 inhibited lysis of RBCs opsonized with all tested sera (Fig. 1E-F). This inhibition was comparable to that observed in Eculizumab- and EDTA-treated sera, whereas the scrambled Cp40 control did not inhibit MAC formation. In conclusion, we show that Cp40 effectively prevents C3b deposition and MAC formation on RBCs opsonized with AIHA sera from patients with a DAT score positive for C3. Previous reports have shown that Cp40 blocks C3 deposition and hemolysis of RBCs in the context of malaria and PNH which are both antibody-independent diseases^{10,11}. Our results confirm these findings using AIHA sera to opsonize RBCs and suggest that Cp40 is a potential candidate for complement inhibition to prevent intravascular hemolysis, which has been associated with thrombosis and unfavorable prognosis³, in complement-driven AIHA.

In addition to complement-mediated intravascular hemolysis, extravascular hemolysis by phagocytosis of opsonized RBCs is a major cause of RBC breakdown in AIHA. We studied the effect of Cp40 on extravascular hemolysis by measuring the phagocytic uptake of opsonized RBCs by macrophages. We found that opsonization of the RBCs with AIHA serum resulted in different levels of internalization of the RBCs by macrophages (Fig. 2A). Patient sera were then divided into two groups based on results obtained in the DAT, either positive for complement deposition only (colored samples) or positive for complement deposition and IgG opsonization (black symbols), which was confirmed using flow cytometry (Fig. 2B). As expected, a clear reduction in phagocytosis of the RBCs by macrophages was observed for all tested sera when Cp40 was added during opsonization (Fig. 2C) while IgG opsonization levels remained unchanged (Online Supplementary Materials Fig. S1) indicating that inhibition of complement deposition on the RBCs by Cp40 diminishes internalization. Overall, the same degree of internalization inhibition was achieved using Cp40 and EDTA but Cp40 was more effective at preventing RBCs phagocytosis compared to α C1q-85 (Fig. 2C). The same samples were divided according to their DAT score for IgG (Fig. 2D). For samples with complement-activating capacity only, RBC internalization by Cp40 was reduced to the same extent as EDTA (dotted line; median, 22.45; range 25.8-8.97). As expected, phagocytosis of RBCs opsonized with complement and IgG-positive AIHA sera (Fig. 2D) was less well inhibited by Cp40 (median, 38.9; range 81.91-7.3) probably due to IgG binding to Fc-*gamma* receptors. This suggests that complement inhibition at the level of C3 with Cp40 is potentially beneficial in AIHA patients with chronic hemolysis caused by complement activation. Complement and Fc-*gamma* receptors are the two major classes of receptors involved in extravascular RBC breakdown in AIHA. Our results show that Cp40 can inhibit complement-mediated extravascular hemolysis of AIHA sera opsonized RBCs. Previous studies showed that complement inhibition at the C5 level has positive effects in AIHA patients with cold agglutinin disease¹⁶. Our findings are in line with these results and additionally show that complement regulation by Cp40 can be even more beneficial since both intra- and extravascular

hemolysis are prevented. Together, we conclude that Cp40 is a promising new treatment for complement-mediated AIHA. Future research should confirm these findings *in vivo*.

AUTHORSHIP CONTRIBUTIONS

Contribution: I.B. and L.D.N. performed experiments. S.S.Z., D.W., M.H., designed the research and wrote the paper. L.D.N., I.B. and I.J. analyzed the results and wrote the manuscript. P.L., C.B., C.F. collected samples and provided information about these samples. E.S.R, D.R., J.D.L. provided Cp40 and a Cp40 control peptide and contributed to the design, analysis and interpretation of the study.

DISCLOSURE AND CONFLICTS OF INTEREST

J.D.L. is the founder of Amyndas Pharmaceuticals, which is developing complement inhibitors (including third-generation Compstatin analogs such as AMY-101) and inventor of patents or patent applications that describe the use of complement inhibitors for therapeutic purposes, some of which are developed by Amyndas Pharmaceuticals. J.D.L. is also the inventor of the Compstatin technology licensed to Apellis Pharmaceuticals (i.e., 4(1MeW)7W/POT-4/APL-1 and PEGylated derivatives). D.R. is a co-inventor on patents or patent applications describing Compstatin analogs and their clinical use. The remaining authors declare no competing financial interest.

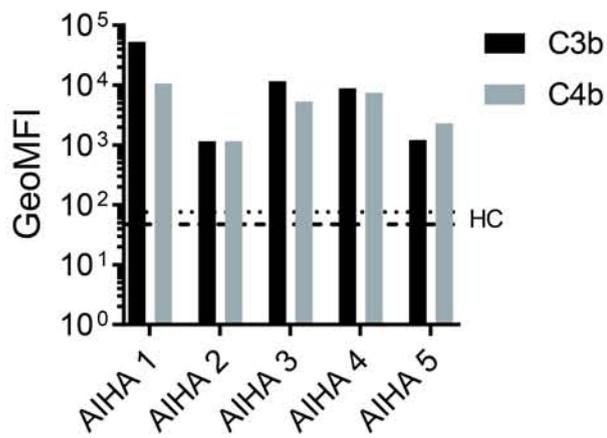
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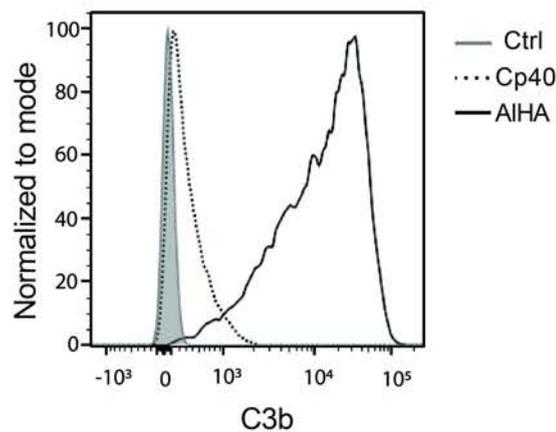
Figure 1. Inhibition of complement deposition and lysis of RBCs opsonized with AIHA sera by Cp40. RBCs were opsonized with sera of AIHA patients in the presence of recalcified human plasma from an AB blood group donor as a source of complement factors and C3b and C4b deposition on the RBCs was determined using flow cytometry. **(A)** Levels of C3b and C4b deposition on RBCs after incubation with sera from 5 individual AIHA patients. Dotted and dashed lines represent the C3b and C4b deposition levels obtained with RBCs opsonized with a healthy control sera (HC). **(B)** Representative flow cytometry histogram showing the effect of Cp40 on the C3b deposition on RBCs opsonized with one tested AIHA serum. **(C-D)** Detection of C3b and C4b on RBCs incubated with sera of the same 5 patients as shown in figure A in the presence of Cp40, scrambled Cp40 control peptide, α C1q-85, C1-INH or EDTA. **(E)** Hemolysis of RBCs after incubation with patient sera (n=10) in the presence of recalcified human plasma as source of complement factors with either Cp40, Cp40 scrambled peptide, α C1q-85, C1-INH or EDTA. Median and data points are shown. **(F)** Hemolysis induced by opsonization of RBCs with all tested patient sera with or without Cp40. Percentage lysis is normalized to a 100% lysis control that was obtained by incubating the RBCs with distilled water. Statistical differences were calculated using one-way ANOVA testing, ns=not significant.

Figure 2. RBCs opsonized with complement activating AIHA sera in the presence of Cp40 are not internalized by macrophages. RBCs labeled with PKH (yellow) were opsonized with AIHA sera in the presence of normal human serum as a source of complement factors and incubated with M1 differentiated HLA-DR labeled (red) macrophages to induce antibody- and/or complement-mediated RBC internalization. **(A)** Representative imaging flow cytometry image showing different levels of RBC internalization by macrophages. Panel 1, 2 and 3 show low, intermediate and high internalization of RBCs by the M1 macrophages respectively. **(B)** Sera from AIHA patients with a positive DAT score for complement deposition (n=4 colored symbols) or complement deposition in combination with the presence of IgG auto-Ab (n=6 black symbols) were incubated with healthy RBCs and IgG deposition was determined using flow cytometry in different experiments (symbol code) to confirm DAT results. IgG deposition induced by incubating RBCs with healthy control serum (HC) is depicted as dotted line. **(C)** Internalization of RBCs opsonized with the two different types of AIHA sera according to the DAT (C3 positive patients: colored symbols; C3 + IgG positive patients: black symbols) in the presence of Cp40, scrambled Cp40 control peptide, α C1q-85 or EDTA by M1 macrophages was determined using imaging flow cytometry. **(D)** Comparison of the phagocytosis levels of RBCs opsonized with IgG positive or negative AIHA patient sera (black vs colored symbols) in the presence of Cp40. Dotted line represents the percentage of phagocytosis when EDTA was added. Median and data points are shown. Statistical differences in phagocytosis were calculated using one-way ANOVA testing, ns=not significant.

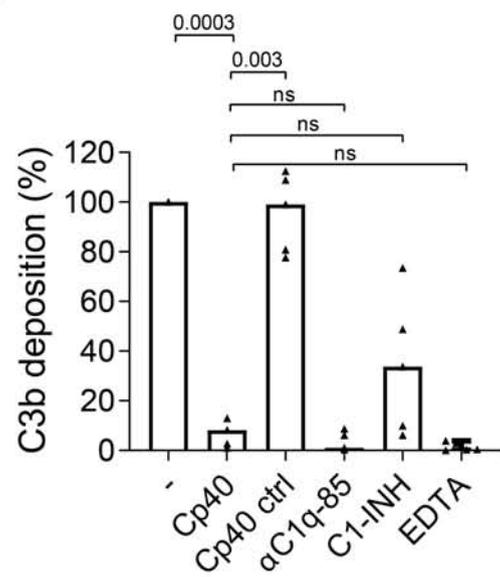
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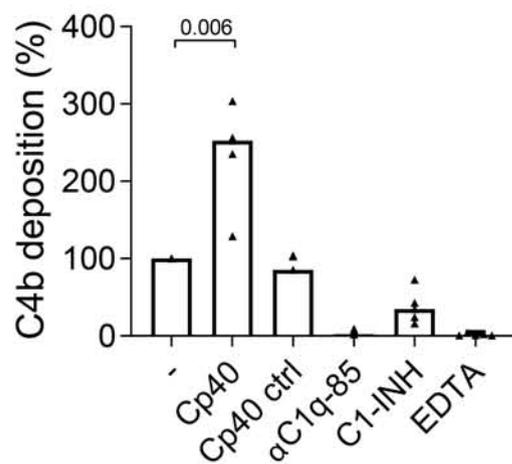
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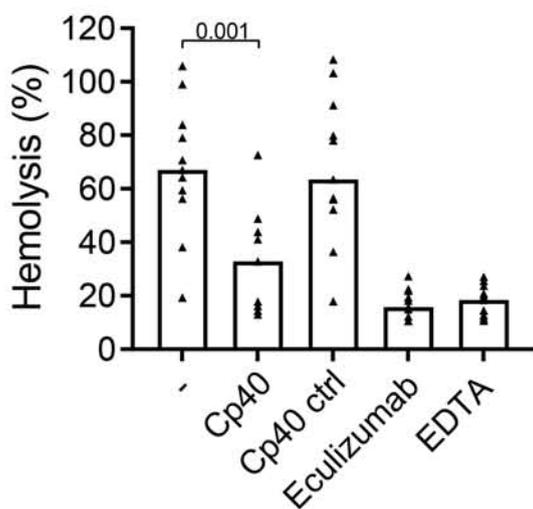
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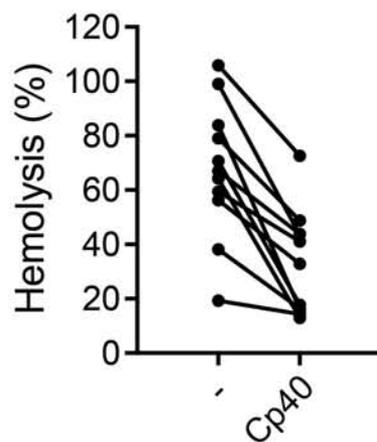
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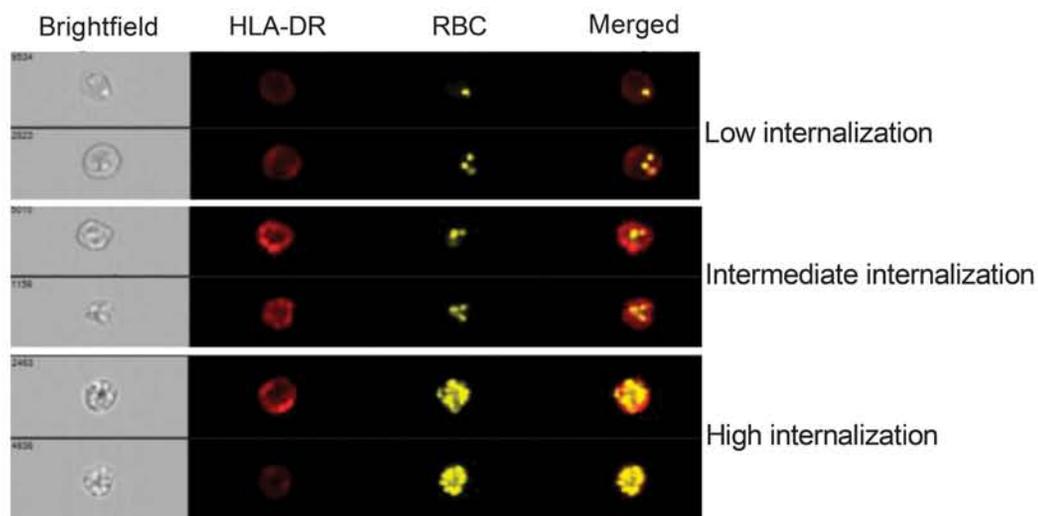
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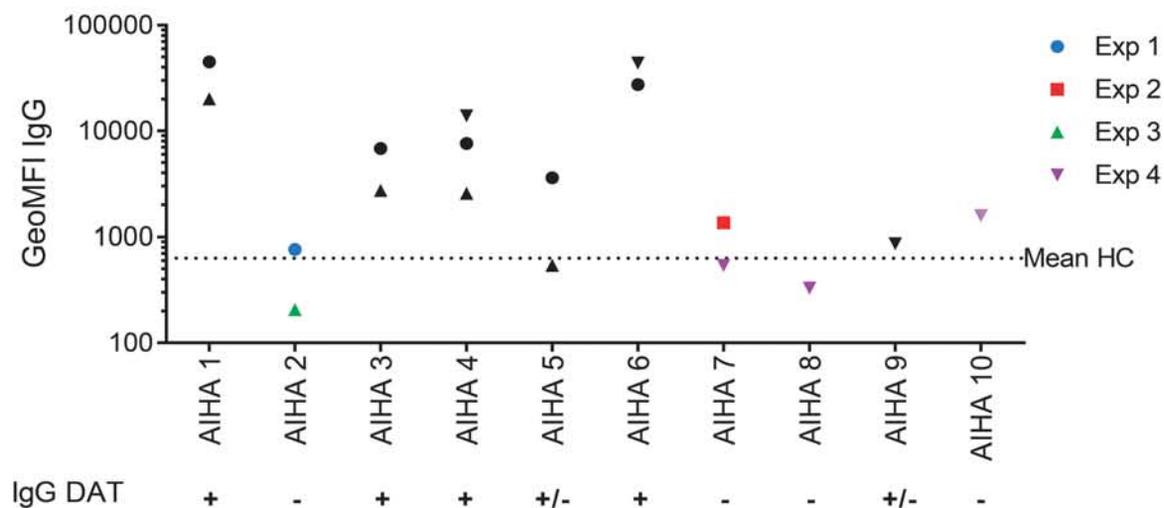
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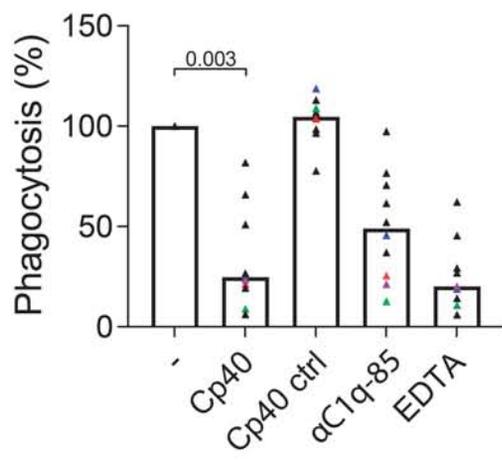
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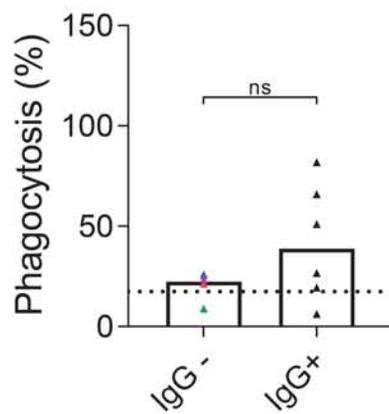
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Supplemental information

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

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*** both authors contributed equally.**

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 x10⁶ 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 CaCl₂ and 2 mM MgCl₂ (VBG⁺⁺) and added to the RBCs in a final concentration of 0.5%. Normal human EDTA plasma was recalcified by incubation with CaCl₂ (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 µg/mL, Sanquin Research), C1 esterase inhibitor (C1-INH,

40 μ M, 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 μ g/mL, clone anti-C3-19, Sanquin Research) and APC conjugated anti-C4d (1 μ g/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 (1 μ g/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 μ g/mL and EDTA at 28 mM final concentration. Cells were incubated for 90 minutes at 37°C, then remaining RBCs were pelleted and 100 μ L 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 10⁶ 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 μ M PKH26 (Sigma). 3 x 10⁶ 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, α C1q-85 or EDTA at concentrations described previously. Macrophages were co-cultured with 2 x 10⁶ 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 S1. 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

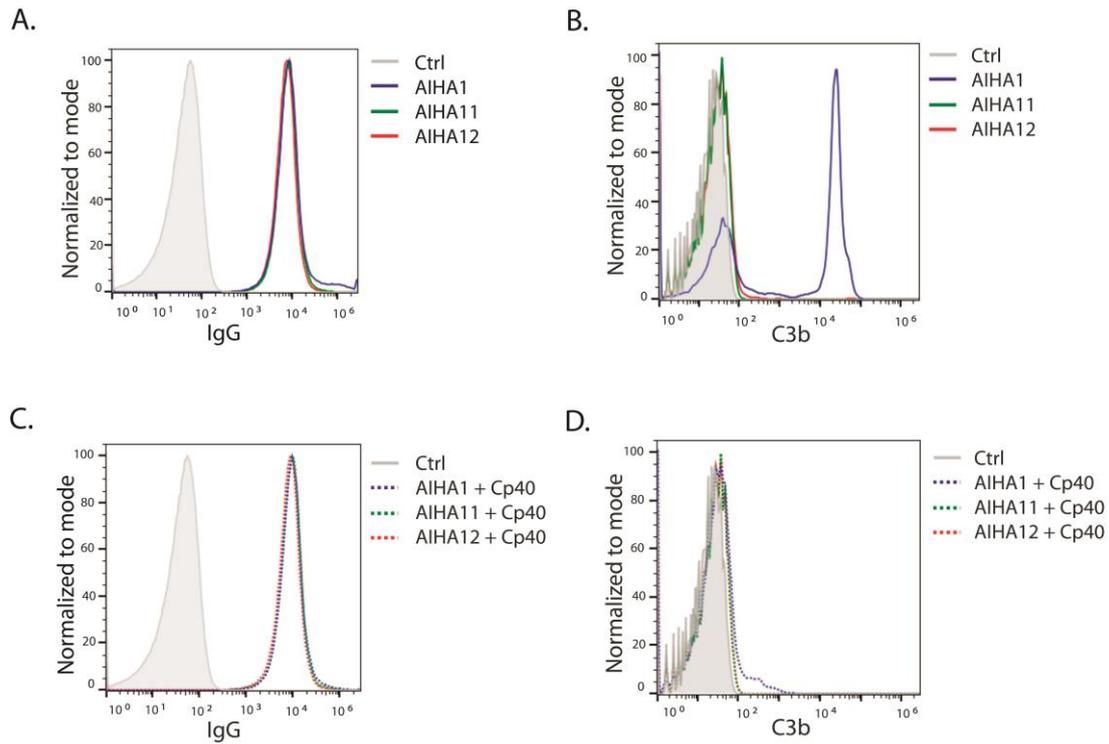


Fig. S1 Effect of Cp40 on IgG opsonization. RBCs were opsonized with sera of AIHA patients classified according to their DAT score as positive for C3 and IgG (AIHA1) or IgG only (AIHA11 and 12) in the presence of recalcified human plasma from an AB blood group donor as a source of complement factors. 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).