SUPPLEMENTARY APPENDIX

Complement deposition in autoimmune hemolytic anemia is a footprint for difficult-to-detect IgM autoantibodies

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Supplementary methods

Handling of patient samples

In general, RBCs were isolated from EDTA blood by centrifugation and subsequently washing them three times with PBS. They were stored up to one week as 50% solution in storage solution (SAGM; saline – adenine - glucose – mannitol). Serum or EDTA plasma was frozen at -20°C within one week after receiving the sample. EDTA plasma was recalcified in glass tubes by adding $CaCl_2$ to a final concentration of 10 mM, incubating 10 minutes at 37°C and then put on ice for 30 min, after which the fibrin clot was removed.

Routine diagnostic tests

The direct antiglobulin test (DAT) and indirect antiglobulin test (IAT) were used to identify anti-RBC autoantibodies in routine diagnostics as described in [4]. In short, the DAT is performed by incubating patient RBCs with polyspecific (anti-IgG and anti-C3; Sanquin reagents) or monospecific antiglobulin reagents (anti-IgG or anti-IgM or anti-IgA or anti-C3; Sanquin reagents). The resulting agglutination is subsequently detected by visual inspection using reagent tubes ("tube method") or using small sephadex columns ("column method"; Biorad). Agglutination is semi-quantitatively scored on a scale of 0 to 4. For the IAT, patient serum or plasma is incubated with healthy donor RBCs and the resulting agglutination is assessed. IgM autoantibodies lead directly to agglutination of the RBCs in the IAT and IgG autoantibodies generally need addition of anti-IgG reagent for agglutination. Additives such as PEG, albumin or LISS are used to enhance sensitivity.

FACS-based Indirect Antiglobulin Test

Healthy donor O-typed RBCs were washed three times with PBS, resuspended in PBS supplemented with 0.5% BSA and added to a well of a round-bottom plate to a final concentration of 0.5%. Next, AIHA patient serum or EDTA plasma was added to a final concentration of 10% or 50%. In case serum was used, EDTA was added to a final concentration of 10 mM to prevent *in vitro* complement activation. For testing the setup, IgG anti-k (Millipore) and IgM anti-RhD (Sanquin Reagents) were used. The volume was adjusted with PBS with 0.5% BSA to a final volume of 50 µl. Samples were then incubated for 1 h at room temperature with gentle shaking. Next, RBCs were washed three times with PBS with 0.5% BSA. The RBCs were then stained by adding 75 µl fluorescently labeled antibody dilution to the RBC pellet and incubating 30-45 min at room temperature with gentle shaking. Detection antibody dilutions of 1/100 were seen to provide the appropriate amount of excess for the anti-isotype antibodies and were thus used in the rest of the study. The following antibodies were used: FITC-labeled anti-human IgA (RbHu14F, Sanquin Reagents) or FITC-labeled anti-human IgG (RbHu16F, Sanquin Reagents) or FITC-labeled F(ab')2 anti-human IgM (Rb26Hu15F, Sanquin Reagents) or 1 µg/ml FITC-labeled anti-human C3 (clone anti-C3-19, Sanquin Research) or 1 µg/ml FITC-labeled anti-human IL6 as isotype control (clone anti-IL6-8, Sanquin Research) or no antibody. Anti-IgM-Fab2-FITC yielded more symmetrical peaks than anti-IgM-FITC from the same source, hence we used the Fab2 fragments. After incubation with the fluorescently labeled antibodies, RBCs were washed three times with PBS with 0.5% BSA and resuspended in 150 µl PBS with 0.5% BSA. FACS analysis was performed on a BD FACS Canto flow cytometer. FSC-A versus FSC-W plots were used to gate on single RBCs. Results were analyzed by overlaying histograms of samples with patient serum with those without patient serum in FlowJo. Samples that had not been stained with antibodies or with an irrelevant antibody (anti-IL6) were used as additional negative controls. Signal to background was not improved by using additives such as PEG and LISS during antibody incubation, nor by using bromelain-treated RBCs, and hence these additives were not used in our study.

FACS-based Direct Antiglobulin Test

AIHA patient RBCs were washed three times with PBS and resuspended in PBS with 0.5% BSA to a final concentration of 0.1% using a volume of 150 µl per sample in a well of a round-bottom plate. The plate was spun down and the RBCs were stained and analyzed as described above in the protocol for the FACS-based Indirect Antiglobulin Test.

Comparison FACS-bases IAT and DAT with agglutination-based IAT and DAT

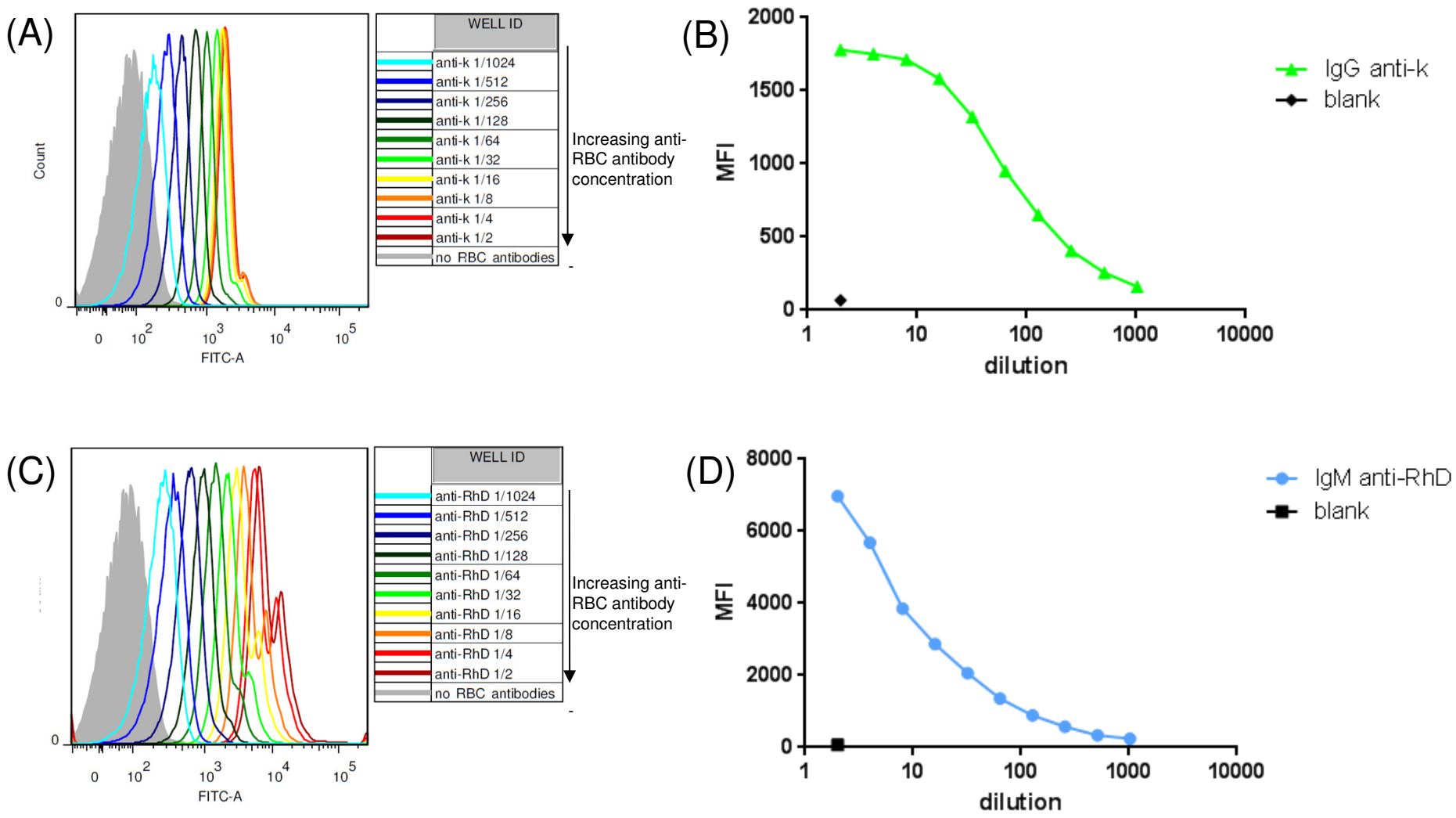
Results of the FACS-based DAT were directly compared to the results of the conventional column method DAT unless otherwise stated. The conventional IAT does not directly have a read-out in isotype. FACS-based results were therefore compared to the conclusion from the different conventional IAT results (e.g. at different temperatures, results before and after DTT treatment etc.). This might cause a bias in favor of the golden standard (agglutination-based IAT), which we preferred since this would allow only true improvements of our new assay to stand out in the comparison.

Preparation of eluates

Eluates were prepared from AIHA RBCs using the Gamma EluKit II (Immucor) following the manufacturer's instructions.

Quantitative hemolysis assay

For some fractionations, quantitative hemolysis assays were used as an alternative way to test for complement activation. These quantitative hemolysis assays were performed as described previously[17]. In brief, patient sera are incubated for 2 hours at 37°C with bromelain-treated O-typed RBCs and AB serum in veronal buffer supplemented with 10 mM $CaCl_2$, 2 mM $MgCl_2$ and 0.05% gelatin (VBG^{++}). After incubation, the plate with samples is spun down and the supernatant is transferred to a new plate. Lysis is quantified by measuring the absorbance of hemoglobin (A414/690) in the supernatants using a spectrophotometer. Lysis is expressed as percentage of lysis of RBCs with milliQ water.



Supplementary Figure 1: Optimization of IAT by FACS.

The feasibility of IAT by FACS was assessed by incubating different concentrations of monoclonal anti-RBC antibodies with healthy donor RBCs and then staining with anti-IgG-FITC or anti-IgM-FITC. Titration of such an antibody (IgG anti-k) gave nicely shaped peaks (A) with a regularly increasing MFI (B), that reaches a plateau. Titration of an IgM anti-RBC monoclonal antibody (IgM anti-RhD) is shown in (C) and (D).

2 (A)

IgG	agglutination +	agglutination -
FACS +	17	1
FACS -	4	12

IgM	agglutination +	agglutination -
FACS +	10	3
FACS -	12	9

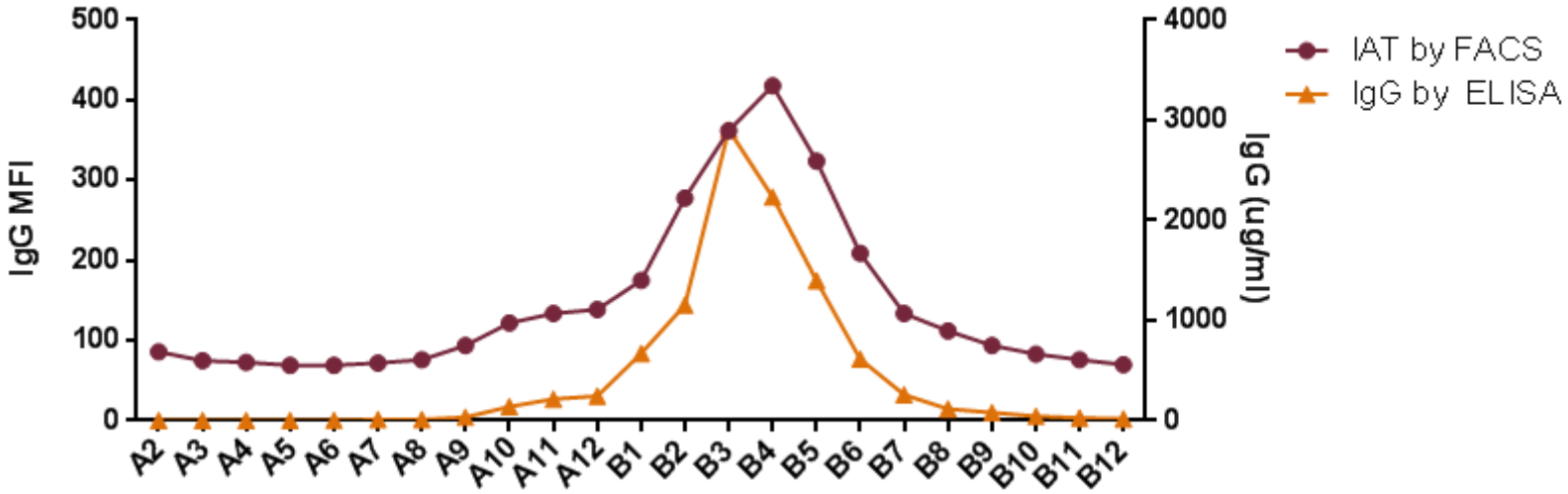
(B)

IgG	agglutination +	agglutination -
FACS +	39	1
FACS -	3	6

IgM	agglutination +	agglutination -
FACS +	10	5
FACS -	0	35

C3	agglutination +	agglutination -
FACS +	24	1
FACS -	0	25

Supplementary figure 2: Comparison of IAT and DAT by FACS versus IAT and DAT by agglutination.
(A): Comparison of the results of IAT by FACS to the IAT by agglutination in routine diagnostics for IgG and IgM, showing that the FACS-based method is less sensitive than the agglutination-based method especially for IgM.
(B): Comparison of the results of the FACS-based DAT versus the routine diagnostic DAT (column method) for IgG, IgM and C3, showing high similarity between the two techniques.



Supplementary Figure 3: Demonstration that anti-RBC IgG is not negatively influenced by the fractionation procedure.

Representative result of IAT by FACS executed on a fractionated AIHA patient sample verifying that anti-RBC IgG is still present and able to bind to RBCs after size exclusion chromatography.

Supplementary tables

Table 1: patient information (samples from fractionation/ depletion experiments). Ab = antibodies

Patient	DAT IgG	DAT IgM	DAT IgA	DAT C3d	Hemolysins	Eluate	Serum
1	-	-	-	3+	Cold and warm	negative	Aspecific cold auto-Ab
2	-	-	-	3+	Cold and warm	ND	Aspecific cold auto-Ab
3	-	-	-	3+	Cold on warm	ND	Aspecific cold auto-Ab
4	-	-	-	4+	Warm	aspecific	Aspecific and anti-e warm auto-Ab
5	+	(+)	-	4+	Warm	aspecific	Aspecific cold and warm auto-Ab, auto-anti-C,D,e
6	3+	(+)	-	2+	Warm	aspecific	Aspecific and anti-e warm auto-Ab
7	3+	(+)	-	2+	Warm	aspecific	Aspecific warm auto-Ab
8	+	-	-	+	Warm	aspecific	Aspecific warm auto-Ab
9	2+	-	-	2+	ND	aspecific	Aspecific cold and warm auto-Ab
10	2+	-	-	2+	ND	aspecifc	Aspecific cold and warm auto-Ab
11	2+	-	-	3+	None	aspecific	Aspecific and anti-e warm auto-Ab
12	3+	-	3+	3+	ND	ND	ND
13	3+	-	-	+	ND	ND	ND
14	-	-	-	(+)	Warm	ND	Negative
15	-	-	-	2+	Warm	Positive	Warm auto-Ab
16	2+	-	-	2+	Warm	aspecific	Aspecific warm auto-Ab