FLOWCYTOMETRY-BASED PHAGOCYTOSIS ASSAY OF DAT+ ERYTHROCYTES (FPAD) IS IN-VITRO SURROGATE FOR IMMUNE-MEDIATED HEMOLYSIS

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Background

Extravascular immune hemolytic anemia (IHA) is triggered by antibodies (AB) against erythrocyte (EC) antigens and is executed by tissue macrophages (MP). Monocyte Monolayer Assay (MMA) has been proposed to assess the potency of AB to trigger IHA. However, experimental variability and microscopic read-out of MMA limit its reliability. Here, we offer a flowcytometry (FACS)-based approach to quantify complexes of activated human blood monocytes (aMP) with antibody-coated erythrocytes (ACE) - in short, FPAD - to predict the hemolytic potency of anti-EC-AB.

Results

More than 600 experiments were carried out using monoclonal and polyclonal AB of various blood group epitope specificity (Anti-D, -k, -K -M, -N, -s, -Jka, -Ge and autoantibodies) to induce in vitro phagocytosis by aMP (Figure 1). Latency and strength of aMP-ACE interaction proved to depend on experimental conditions (e.g. antigen-specificity of AB, incubation temperature, CO2 pressure, status and sex of MP donor, data not shown). In vitro hemolysis as by fHb corresponds with the quantitative formation of aMP/ACE complexes (Figure 2 + 3). Applying standardized experimental conditions, we received robust and reproducible data on aMP/ACE complex formation as a function of the antigen specificity of AB.

Figure 1

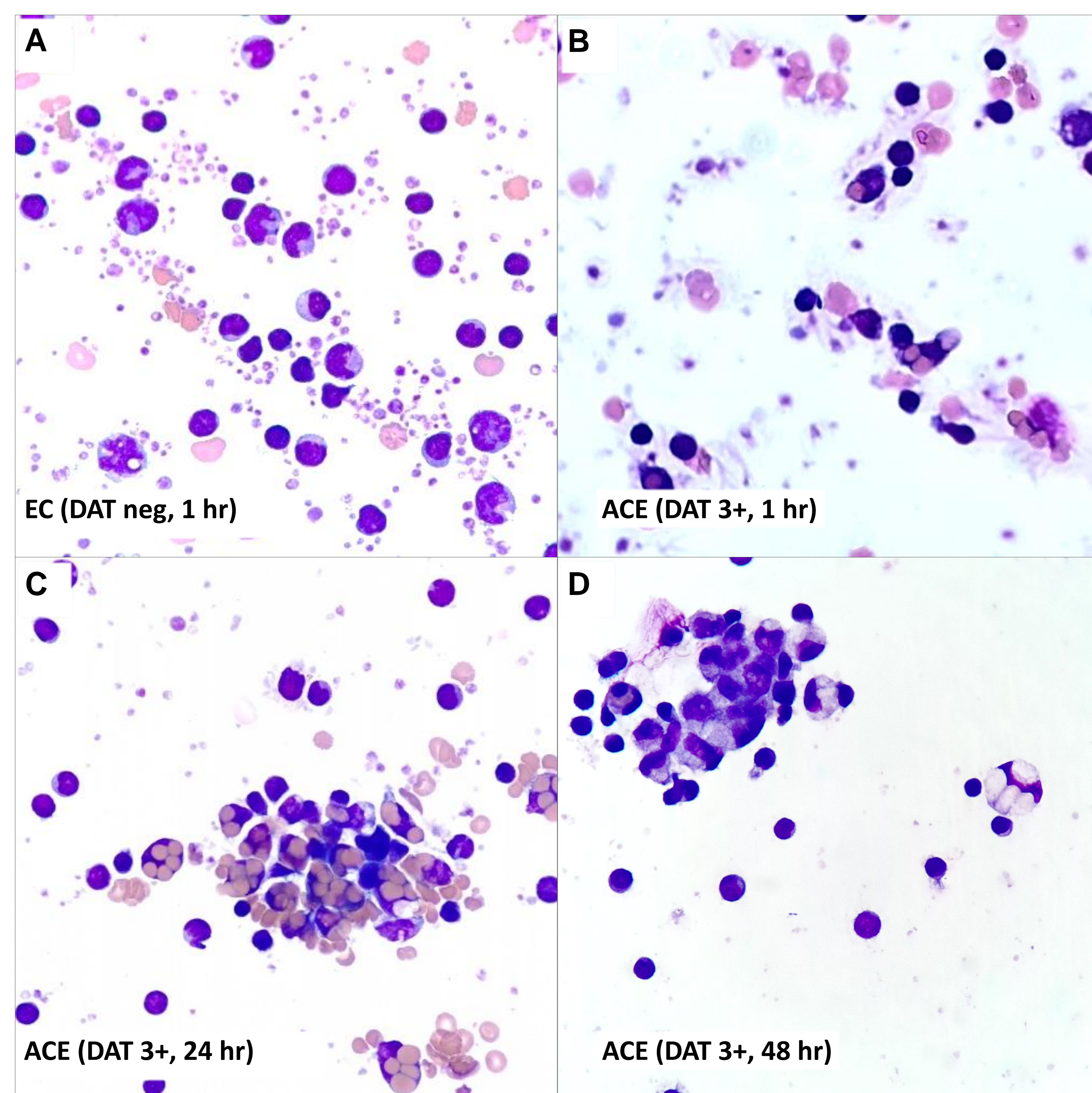

Figure 1: RhD negative (A) and RhD positive (B – D) EC were pre-incubated with monoclonal anti-D (BRAD-3) and exposed to aMP. In contrast to RhDneg EC, RhD+ EC (ACE) were phagocytosed by aMP starting upon 1 hr of co-incubation (B). Maximal phagocytosis was observed after 24 hr (C) and wean off thereafter (D).
(May-Grünwald-Giemsa-staining, 1'000x magnification)

Methods

CD14+ monocytes from human whole blood were activated with phorbol-12-myristate-13-acetate (PMA) to induce aMP. Subsequently, Direct Antiglobulin Test (DAT) positive ACE were co-incubated with aMP in a 1:6 ratio (ACE:aMP) at 37°C. Fc-receptor-mediated (FcR) aMP/ACE agglutinates were quantified by FACS upon staining with fluorophore-conjugated antibodies against CD14 and CD235a (Glycophorin A). The formation of agglutinates was inhibited by adding IgG (Privigen). Various culture conditions were assessed. Cytospin preparations and determination of free hemoglobin (fHb) were used to validate AB-mediated in vitro hemolysis.

Figure 2

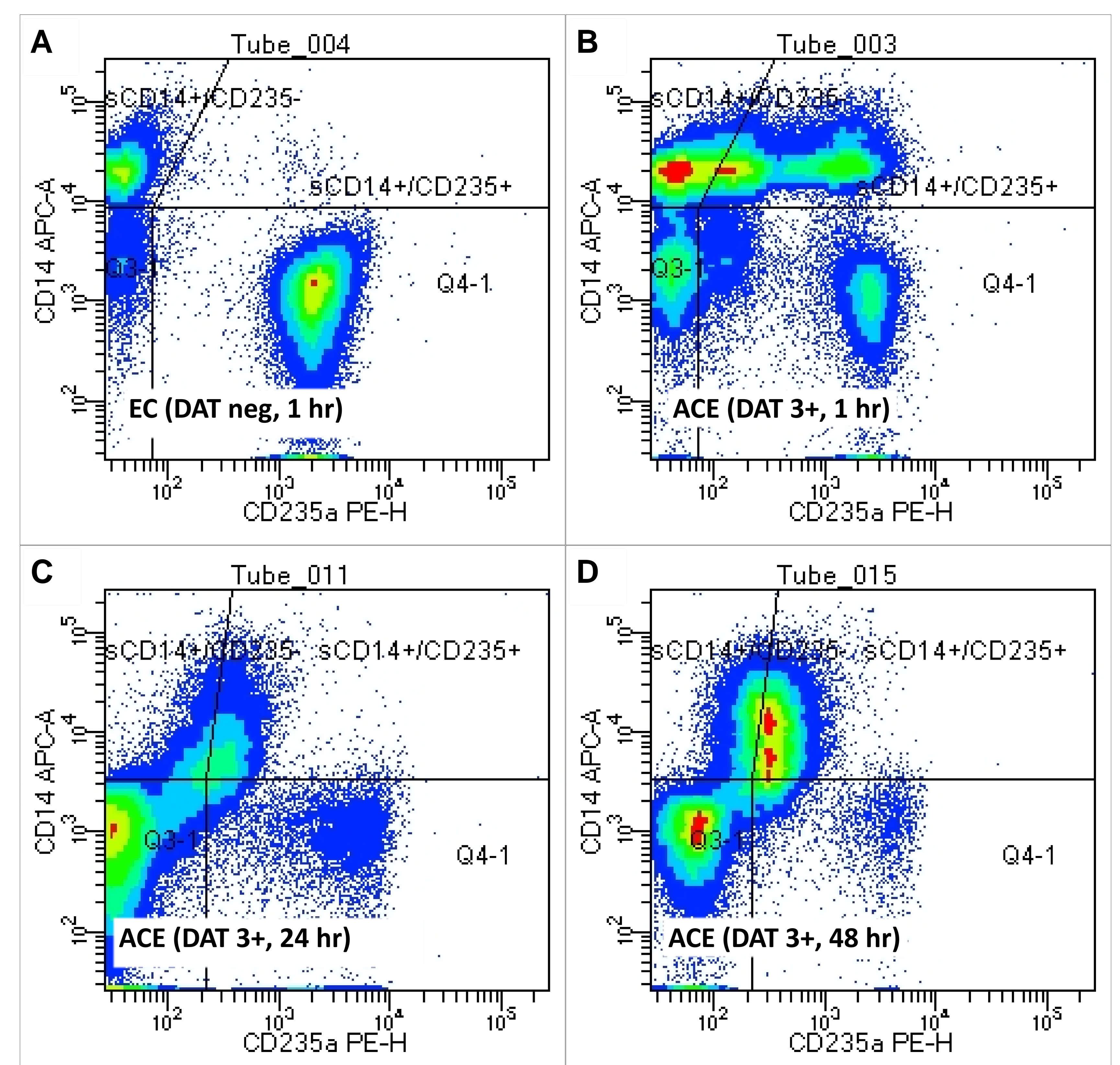

Figure 2: aMP/ACE immune complexes by FACS of the same preparations as in Figure 1: RhD positive (B – D) AEC engaged quantitatively with aMP depending on time of co-incubation. DAT negative EC (A) were not bound by aMP.

Figure 3

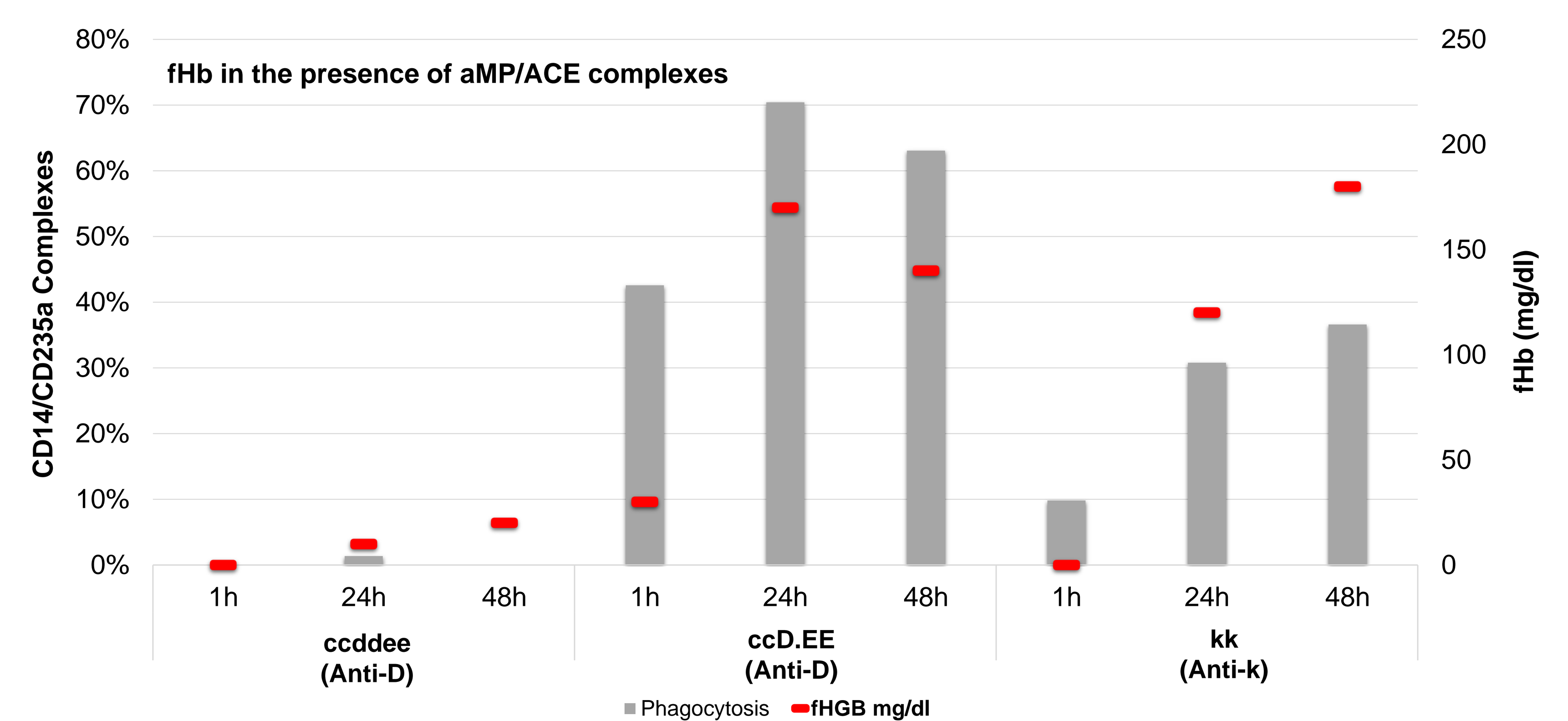

Figure 3: Monoclonal AB of different specificity (Anti-D, Anti-k) was loaded on EC carrying the cognate antigen (D, k) and co-incubated with aMP. In the presence of the antigen, ACE were complexed by aMP, and delayed in vitro hemolysis by fHb was observed.

Conclusion

FACS-based assessment of in-vitro interaction between aMP and ACE may serve as a surrogate for cellular IHA. Standardized experimental conditions and read-out of sizeable target events may enable FPAD to overcome the limitations of MMA. The diagnostic power of FPAD remains to be shown.

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