SDC, Materials and methods

Cell ELISA for determination of complement deposition and endothelial cell activation

In a whole-cell ELISA, wild-type, hCD46-only and hCD46/HLA-E double transgenic PAEC were grown to confluence in 96-well plates and washed twice with phosphate-buffered saline (PBS) supplemented with calcium and magnesium (680 mM $CaCl_2$, and 490 mM $MgCl_2$). Pooled normal human serum (NHS) in gelatin veronal buffer with calcium and magnesium $(GVB)^{++}$ (1:10) was added to the cells and incubated at 37°C for 4h followed by washing. Cells were fixed in 3.7% formaldehyde for 20 min at room temperature (RT), washed, and blocked with PBS containing 1% bovine serum albumin (PBS-BSA) for 90 min at RT. Mouse anti-human C5b-9 (Diatec), mouse anti-human CD62E (LifeSpan Biologicals) were diluted in PBS-BSA and incubated for 1h at RT followed by three washes. Subsequently, biotin-conjugated goat anti-mouse IgG (Abcam) were used, diluted 1:500 in PBS-BSA, and incubated for 1h at RT. After washing, alkaline phosphatase conjugated streptavidin (GE Healthcare) diluted 1:1000 in PBS-BSA was incubated for 30 min at RT. After washing, 1 mg/ml p-nitrophenyl phosphate substrate (Sigma) was added. Color development was quantified at 405 nm, reference wavelength 490 nm, using an Infinite M1000 microplate reader (Tecan).

PAEC cytotoxicity

NHS-induced PAEC cytotoxicity was measured by calcein AM / ethidium homodimer-1 (EthD-1) staining. A Live/Dead Kit (Molecular Probes) was used according to the manufacturer's protocol. Briefly, confluent wild-type, hCD46-only and hCD46/HLA-E double transgenic PAEC in a 96-well plate were exposed to 1:10 diluted NHS for 120 min at 37°C. After washing with PBS, 100 µl of 2 µM calcein AM and 2 µM EthD-1 was added to the cells and incubated in dark for 20 min. The fluorescence of calcein

and EthD-1 was then measured at 485 nm (excitation) / 535 nm (emission) and 530/635 nm, respectively, using an Infinite M1000 microplate reader (Tecan).

Coagulation assay

Wild-type, hCD46-only, hCD46/HLA-E double transgenic PAEC were used to evaluate their coagulation-inhibiting effects in vitro in a microcarrier-based whole blood clotting assay. Briefly, PAEC were cultured on Biosilon microcarrier beads (Thermo Fisher Scientific) coated with 100 µg/ml of bovine collagen-I, in a magnetic stirrer flask (3 min at 100 rpm every 60 min) at 37°C. A volume of 2 ml microcarrier beads with confluent PAEC were then incubated with 8 ml of freshly withdrawn whole, non-anticoagulated human blood. Samples of the bead-blood mixture were incubated at 37°C with gentle rocking, and tubes were observed at regular intervals for occurrence of clotting.

Results

Regulation of complement deposition and endothelial activation by transgenic hCD46

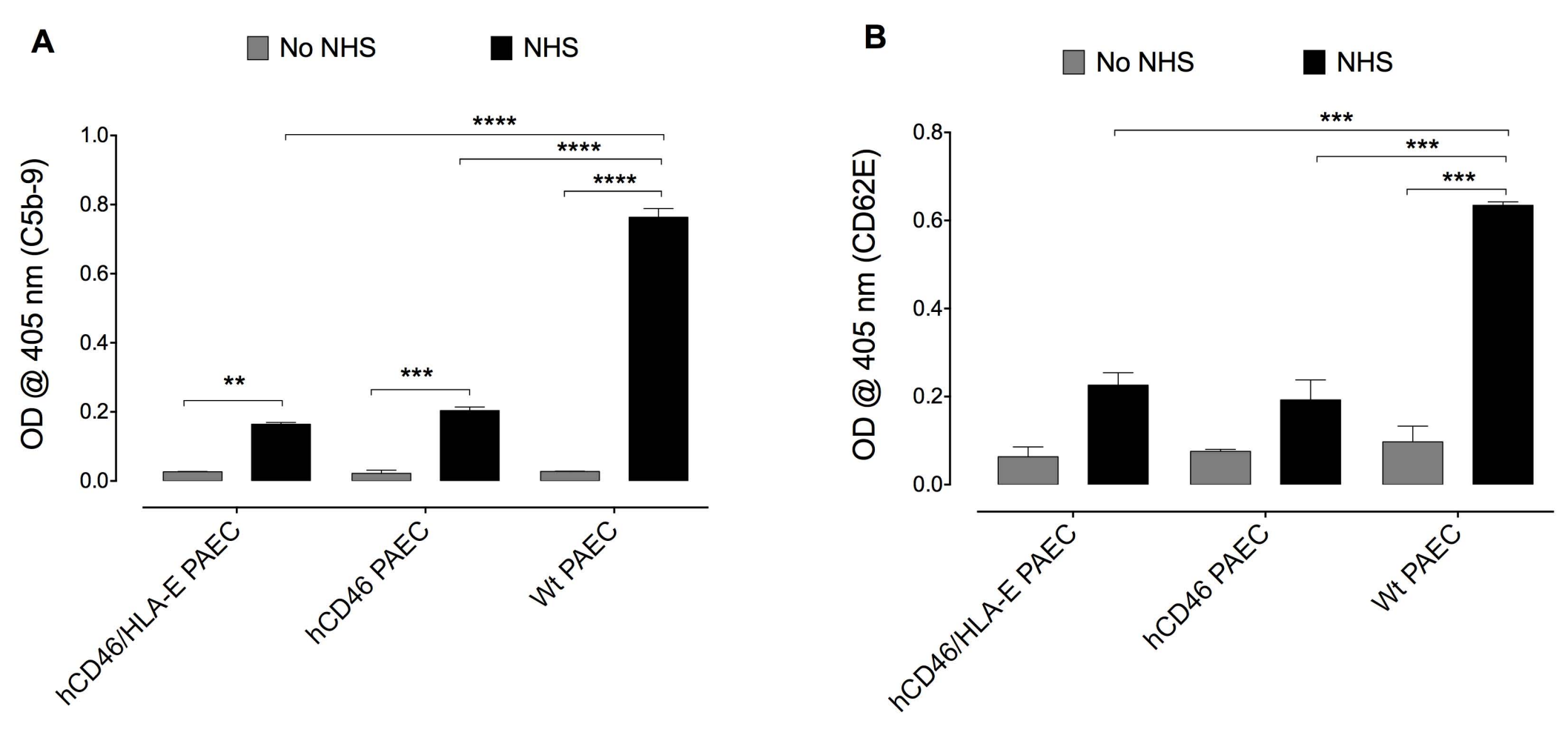
Wild-type, hCD46-only and hCD46/HLA-E PAEC were treated with 1:10 diluted NHS and assessed for complement deposition as well as complement mediated PAEC activation and cytotoxicity. Overexpression of hCD46 on hCD46-only or hCD46/HLA-E transgenic background significantly reduced (A) C5b-9 binding ($p<0.0001$) as well as (B) CD62E expression ($p<0.0001$) as compared with wild-type PAEC. (C) In addition, complement-mediated PAEC cytotoxicity was also significantly ($p<0.0001$) reduced by overexpression of hCD46 on transgenic PAEC as compared to wild-type. However, no difference between hCD46-only and hCD46/HLA-E PAEC was observed. The reduced complement deposition and endothelial activation on the genetically modified PAEC correlated well with reduced complement-mediated cytotoxicity.

Prolongation of clotting time by hCD46 single and hCD46/HLA-E double transgenic PAEC

Microcarrier beads covered with wild-type, hCD46 single and hCD46/HLA-E double transgenic PAEC were incubated with whole human blood. As shown in Supplementary Figure 1D, microcarriers with or without collagen coating, incubated with blood, showed a strong procoagulant property with a short clotting time. Microcarriers covered by confluent wild-type PAEC (52.3 ± 10.9 min) showed a significant anticoagulant effect compared to microcarriers without PAEC (6.2 ± 1.6 min, $p<0.0001$) and human blood alone (22.2 ± 3.1 min, $p=0.021$). The clotting time in the presence of hCD46, both as hCD46-only (80.0 ± 5.7 min, $p=0.0001$ vs. wild-type) or as hCD46/HLA-E double transgene (79.0 ± 4.5 min, $p=0.0002$) was significantly prolonged as compared to wild-type. Clotting time observed with the

hCD46 single and hCD46/HLA-E double transgenic PAEC was not different ($p>0.9999$).

Legend to Supplementary Figure 1: Regulation of complement deposition and endothelial activation as well as cytotoxicity on PAEC using genetic modification strategies. (A) Deposition of C5b-9 and (B) CD62E expression on NHS (1:10, 4h) treated wild-type, hCD46 and hCD46/HLA-E transgenic PAEC. (C) Treatment of PAEC with 1:10 diluted NHS for 120 min at 37°C and assessment of cytotoxicity by calcein AM and EthD-1. (D) Measurement of the clotting time after incubation of freshly withdrawn, non-anticoagulated whole human blood with wild-type or genetically modified endothelial cells cultivated on microcarrier beads. Expression of the hCD46 transgene, either alone or in combination with HLA-E, led to a significantly prolonged clotting time as compared with wild-type PAEC. Cells expressing the HLA-E transgene in addition to hCD46 did not show a prolonged clotting time as compared with the latter. Four independent experiments were performed for each group. Significance was tested by one-way ANOVA with Bonferroni correction. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.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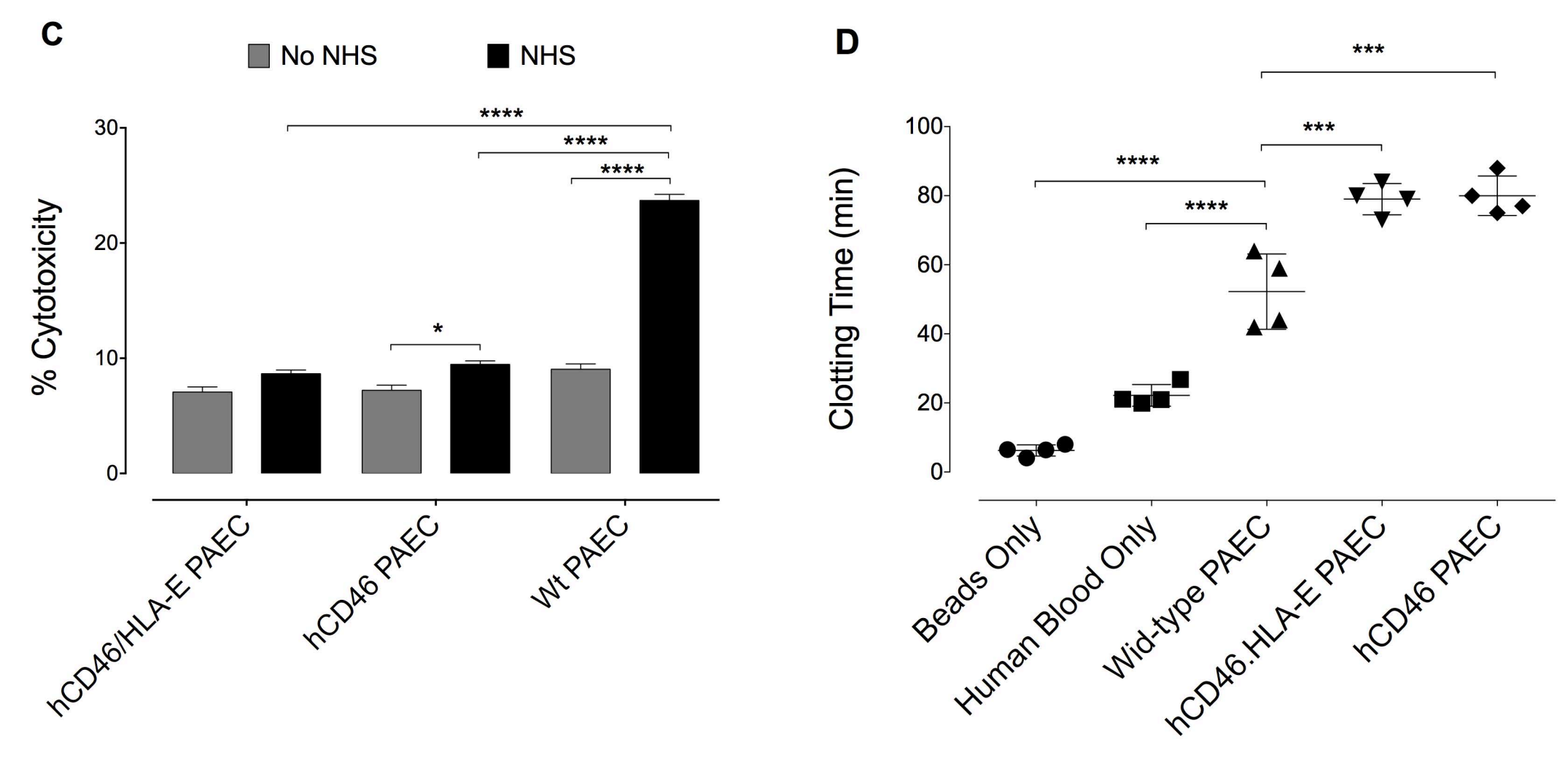
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Human Blood Only
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hCD46 PAEC