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Complement Factor H Binds to Human Serum Apolipoprotein E and Mediates Complement Regulation on High Density Lipoprotein Particles*

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Background: Factor H is the main complement alternative pathway regulator in plasma.
Results: Factor H binds to apoE leading to reduced complement activity against HDL particles.
Conclusion: Interaction of factor H with HDL particles is involved in complement regulation in plasma.
Significance: Complement regulation on HDL particles by factor H could be involved in disease processes caused by alternative pathway dysregulation.

The alternative pathway of complement is an important part of the innate immunity response against foreign particles invading the human body. To avoid damage to host cells, it needs to be efficiently down-regulated by plasma factor H (FH) as exemplified by various diseases caused by mutations in its domains 19–20 (FH19–20) and 5–7 (FH5–7). These regions are also the main interaction sites for microbial pathogens that bind host FH to evade complement attack. We previously showed that inhibition of FH binding by a recombinant FH5–7 construct impairs survival of FH binding pathogens in human blood. In this study we found that upon exposure to full blood, the addition of FH5–7 reduces survival of, surprisingly, also those microbes that are not able to bind FH. This effect was mediated by inhibition of complement regulation and subsequently enhanced neutrophil phagocytosis by FH5–7. We found that although FH5–7 does not reduce complement regulation in the actual fluid phase of plasma, it reduces regulation on HDL particles in plasma. Using affinity chromatography and mass spectrometry we revealed that FH interacts with serum apolipoprotein E (apoE) via FH5–7 domains. Furthermore, binding of FH5–7 to HDL was dependent on the concentration of apoE on the HDL particles. These findings explain why the addition of FH5–7 to plasma leads to excessive complement activation and phagocytosis of microbes in full anticoagulated blood. In conclusion, our data show how FH interacts with apoE molecules via domains 5–7 and regulates alternative pathway activation on plasma HDL particles.

A key player in the humoral innate immune response is the complement system that quickly attacks microbes and foreign particles invading the human body. The complement cascade can be activated through three pathways, the classical, alternative (AP),[2] and lectin pathways. Activation of any of the three pathways leads to cleavage of C3 and covalent surface deposition of the C3b fragment capable of forming an enzyme with factor B. C3b and its degradation fragments, inactive C3b (iC3b) and C3dg, act as important opsonins recognized by specific complement receptors on phagocytes. Upon activation of C3 and subsequently the terminal complement cascade, proinflammatory chemotactic and anaphylatoxic protein fragments C3a and C5a are also released, mediating their effects by binding to receptors on phagocytes.

AP is constantly active in plasma, leading to low grade challenge to all plasma-exposed particles. Activation proceeds rapidly through amplification on surfaces that are missing efficient regulators. Factor H (FH) is the main AP regulator in plasma. It is an elongated molecule composed of 20 domains. The N-terminal domains 1–4 are responsible for FH regulatory activity, whereas domains 19–20 on the C-terminal end are responsible for the self-surface recognition (1). Domain FH19 binds to surface-attached C3b, whereas FH20 interacts with sialic acids and glycosaminoglycans (GAGs) present on self-surfaces (2). In this way, FH discriminates host self-surfaces from non-self ones. Another important surface recognition site of FH is located within domains 6–7, and it mediates the interaction with ligands such as sulfated GAGs, heparin, and C-reactive protein (3, 4). When bound on C3b, FH acts as a cofactor for factor I in

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[2] The abbreviations used are: AP, alternative pathway of complement; iC3b, inactive C3b; AMD, age related macular degeneration; DDD, dense deposit disease; FH, factor H; GAG, glycosaminoglycan; HIS, heat-inactivated serum; NHS, normal human serum; MF, multiplication factor.

the inactivation of C3b to iC3b. Simultaneously, it inhibits binding of factor B to C3b (5, 6) and accelerates the decay of formed AP convertases, further preventing AP amplification (7).

Activation of AP in serum or plasma is spontaneous due to hydrolysis or mechanical activation of C3. FH is the main regulator keeping this spontaneous activation in check (8). This is obvious as depletion of plasma/serum from FH or blockage of FH by autoantibodies causes dysregulation of AP leading to loss of active complement by overconsumption within <30 min (9, 10). Although the AP activation is based on spontaneous low grade process without a need for a trigger, the activation can be enhanced by an imbalance between activation and regulation, *e.g.* by numerous C3b deposits on protein complexes in serum/plasma (11).

Recently, it has become clear that AP dysregulation is a central event in development of several complement-related diseases caused by mutations or polymorphisms in domains FH5–7 and FH19–20. Although mutations in FH19–20 are associated with atypical hemolytic uremic syndrome the Y402H polymorphism in domain 7 is associated with age-related macular degeneration (AMD) (12, 13) and dense deposit disease (DDD) (14).

The Y402H polymorphism has also been shown to alter binding of FH to M protein of *Streptococcus pyogenes* (15, 16). Several other microbial pathogens have also been shown to use FH for immune evasion via two regions, FH6–7 and FH19–20 (17). At least *Neisseria meningitidis* and most of the pathogenic *S. pyogenes* strains interact only with the FH5–7 region on FH, thereby increasing their immune evasion and survival in serum (18). The reasons why several microbes acquire FH particularly via FH5–7 or why mutations or polymorphisms in this region lead to complement related inflammatory diseases are not fully understood.

We started the current study by analyzing the importance of FH5–7 for microbial immune evasion. In those analyses we surprisingly found out that the addition of FH5–7 to anticoagulated full blood or plasma led to impaired survival of both the microbes that bind FH and those that do not. This led us to find that FH interacts via domains 5–7 with apoE (apolipoprotein E)-associated HDL particles. Incubation of FH5–7 in human plasma led to excessive AP activation and significant reduction in iC3b formation, suggesting that FH binding on HDL-associated apoE via domains 5–7 has an important regulatory role. We show that binding of FH to apoE on HDL particles prevents excessive AP activation on those particles and, therefore, in plasma in general. Therefore, it seems that binding of FH via FH5–7 to microbes mimics acquisition of FH onto the host HDL particles present in plasma.

Experimental Procedures

Proteins—Cloning and *Pichia pastoris* expression of the recombinant fragments FH5–7, histidine-tagged FH5–7, and FH19–20 has been described earlier (16). FH5–7 fragments were further purified by passing through HiLoad 16/60 Superdex 200 prep-grade gel filtration column (GE healthcare) in 300 mM PBS, pH 7.0, and concentrated using heparin affinity chromatography. FH and C3 proteins were isolated from human plasma as described earlier (16).

Isolation and Purification of ApoE3 Protein—ApoE DNA constructs were kindly obtained from Dr. Karl H. Weisgraber (Gladstone Institute of Cardiovascular Disease, San Francisco, CA). The cloning, expression of apoE3 in the *Escherichia coli* BL21 (DE3) bacterial system, and purification is described elsewhere (19). Thrombin digestion was performed at 22 °C for 30 min at a ratio of 1:500 (thrombin:apoE, w/w). Delipidation of apoE3 was carried out as described (20).

Isolation of Human HDL with and without Apolipoprotein E—HDL with and without apoE was purified by heparin-affinity chromatography. A HiTrap heparin-Sepharose column (1 ml, Amersham Biosciences) was equilibrated with 5 mM Tris-HCl, 50 mM NaCl, pH 7.4, with a flow rate of 0.5 ml/min. Solid $MnCl_2$ was added to the HDL solution just before application to the column, to give a final Mn^{2+} concentration of 25 mM. The column was equilibrated, and 25 mM $MnCl_2$ and HDL were applied to the column. Non-bound fractions to heparin represented the apoE-free HDL particles. The heparin-bound apoE-containing HDL was detached via elution with 5 mM Tris-HCl, 100 mM NaCl, pH 7.4. ApoE was analyzed in isolated HDL pools by ELISA (21).

To estimate the amount of HDL particles in the fluorometric HDL binding assay, the concentrations of HDL samples are expressed as the equivalent concentration of cholesterol in μg/ml. Total cholesterol concentrations were determined by using Infinity™ cholesterol reagent (Sigma). The samples were stored at +4 °C before use.

Production and Purification of apoE Using the Adenovirus System—All plasmids and recombinant adenoviruses containing the wild-type apoE4, apoE2 and mutated human apoE4 genes were constructed as described previously (22). ApoE was purified from the culture medium of adenovirus-infected HTB-13 cells using dextran sulfate-Sepharose column fractionation (23).

Bacterial Strains and Growth Conditions—*S. pyogenes* strain st369 was obtained from the collection of the National Institute for Health and Welfare (Prof. Jaana Vuopio). and *emm*8 was from ATCC (ATCC catalogue number 12349). These strains were suitable controls for bacterial whole-blood multiplication and FH binding assays as shown earlier (18). The *Staphylococcus aureus* 2151 strain is a blood isolate collected from a blood culture of a septic patient with the permission of the Ethical Review board of the Hospital District of Helsinki and Uusimaa (448/13/03/00/09). *N. meningitidis* DE9686 strain was a kind gift from Dr. Hanna Jarva.

Bacterial Multiplication ex Vivo—Bacteria were inoculated in Brain-Heart infusion (*N. meningitidis*) or Todd-Hewitt broth media and incubated overnight at 35 °C in a shaking incubator (220 rpm) (*N. meningitidis*) or at 37 °C 5% CO_2 (other bacteria) atmosphere without agitation. Thereafter the cultures were diluted 1:20 in appropriate growth media, and the bacteria were grown without agitation to $A_{600} = 0.4$ (*N. meningitidis*) or 0.15 (other bacteria) and finally diluted in 150 mM PBS to obtain 1×10^4 (*N. meningitidis*) or 2×10^3 cfu/ml (other bacteria). 200–1000 cfu in 100 μl of PBS or PBS with 20 μg of FH5–7 were added in 1.2 ml of 40% (*N. meningitidis*) or 100% (other bacte-

ria) human blood supplemented with lepirudin (Refludan 50 μg/ml). The concentration of FH5–7 in the assay was chosen according to the previous study where the final concentration of FH5–7 was 0.7 mM, and the FH5–7/FH ratio was ~1:3 (18).The samples were incubated at 35 °C or 37 °C for 60 (*S. aureus*, *S. pyogenes*), 180 (*S. aureus*), or 90 min (*N. meningitidis*). At the 0 and 60-, 90-, 120-, and 180-min time points dilutions of the samples were cultured on agar plates for 17 h. The cfu were counted, and the multiplication factor (MF) at the final time point was determined by counting the ratio between final cfu present at each time point and the original cfu present in the inoculum as described previously (18).

Microscale Thermophoresis—ApoE2 protein was labeled with an *N*-hydroxysuccinimide-reactive Red dye (catalog no. L001) following the manufacturer's instructions (NanoTemper). Serial dilutions of unlabeled FH5–7 protein was mixed with the labeled apoE2 in PBS with 0.025% Tween 20 (PBS-T) and scanned in the premium-coated monolith NT capillaries (catalog no. K005) using a NanoTemper Monolith NT.115Pico Instrument (20–50% LED, 40% IR-laser power).

Direct ^{125}I-Labeled Factor H Binding and Radio Ligand Binding Assay—FH, FH5–7, and apoE were labeled with iodine (^{125}I; PerkinElmer Life Sciences) using the Iodogen method (24). The specific activities of the labeled proteins were 1–2 × 10^7 cpm/μg. Each bacterial strain (3 × 10^7 log phase bacteria/reaction) and/or neutrophils (1 × 10^5 cells/ml) were incubated with 30,000 cpm of radiolabeled protein in 100 μl of veronal-buffered saline containing 0.1% gelatin at 37 °C for 30 min in a shaking incubator (800 rpm). For preopsonization, bacteria were incubated with 20% normal human serum (NHS) pool obtained from six-eight healthy donors and washed three times with PBS before the assay. Non-opsonized *S. pyogenes* strains were used as a negative (*emm*8) and positive (st369) controls for FH/FH5–7 binding. After incubation the reaction mixtures were centrifuged 10,000 × *g* for 3 min through 20% sucrose, and the radioactivity of each pellet and supernatant was measured with a gamma counter. The ratios of bound (pellet) to total activity (pellet + supernatant) were calculated. The relative binding was counted as the percentage of the cpm obtained using the positive control strain (st369).

For the radio ligand binding assay (16), 96-well polysorp (Nunc, Thermo Scientific) plates were coated with 7 μg/ml FH5–7 or full-length FH and incubated with 0.9, 0.3, and 0.1 μM apoE, FH, and FH5–7 or BSA and 75,000 cpm of ^{125}I-labeled apoE protein.

Binding Assays Using ELISA—The 96-well maxisorp plates (Nunc) were coated with 7 μg/ml apoE4 fragments or BSA and blocked with 3% BSA in PBS-T. Wells were incubated with 10 μg/ml FH5–7 at room temperature for 60 min. Thereafter wells were incubated with goat anti-FH (Calbiochem) and with peroxidase-conjugated anti-goat IgG (Jackson ImmunoResearch). Between each step the wells were washed with PBS-T. After incubations TMB (3,3′,5,5′-tetramethylbenzidine) substrate was added, and the reaction was stopped with 1 M H_2SO_4. The absorbance A_{450} was measured.

Gel Filtration of FITC-FH and FITC-FH5–7 with Lipoprotein Particles—Full-length FH and the recombinant FH5–7 was labeled with FITC (Sigma) by incubating 10 μM concentrations of the protein with 10 μg/ml FITC in 1 M Na_2CO_3, pH 9.6, for 2 h at 4 °C followed with 1 h of incubation with 50 mM NH_4Cl. Labeled proteins were separated from unbound FITC using a desalting column. 50 μg of FITC-labeled FH or FH5–7 was incubated with 250 μl of lipoprotein particles for 1 h at 37 °C before loading the samples into a Superdex 200 10/300GL column (GE Healthcare). FITC-FH only was loaded to the column to separate the elution profile of the 155-kDa molecule from HDL. Protein contents of the eluted samples were analyzed at A_{280} using ÄKTA explorer (Amersham Biosciences), and the fluorescence of the collected fractions were measured in a fluorescence spectrophotometer (Fluostar Omega, BMG).

Bacterial Phagocytosis ex Vivo—Overnight-grown bacterial culture diluted to a concentration of A_{600} = 1.0 (1 × 10^9 cfu/ml) were incubated with 0.2 mg/ml *N*-hydroxysuccinimide-fluorescein (Thermo scientific) in 0.1 M Na_2CO_3 buffer, pH 9.6, for 2 h at 4 °C in the dark, where after the labeled bacteria were washed twice. 1 × 10^6 cfu bacteria were incubated in 1 ml of lepirudin anticoagulated blood or in lepirudin anticoagulated blood cells where the plasma was replaced with heat-inactivated serum (HIS) by centrifugation. Each sample contained either PBS, 1.9 μM FH5–7, or FH19–20. For analyzing the dose dependence, FH5–7 concentrations were 0–15 μM. The samples were incubated for 60 min at 37 °C in polystyrene round-bottom tubes (Falcon, BD Biosciences) under vigorous shaking. Phagocytosis was stopped on ice, and the cells were pelleted by centrifugation for 5 min at 5000 × *g*. The erythrocytes were lysed twice with 900 μl of ice cold water for 45 s after which 100 μl of 1.5 M PBS was added. Thereafter, the cells were washed and fixed with 1% paraformaldehyde in PBS before counting the fluorescence of 2,000–10,000 neutrophils in each sample using FACS. Cells were gated by their characteristic forward and side scatter profiles.

Bacterial Phagocytosis in Vitro—Neutrophils were isolated from hirudin-anticoagulated peripheral blood samples (MP 0600 Hirudin Blood tubes Multiplate; Verum Diagnostica GmbH) by using a histopaque gradient as described previously (25). 1 × 10^6 cfu/ml of *N*-hydroxysuccinimide-fluorescein-labeled bacteria were mixed with 2–4 × 10^4 neutrophils in 0.05% human serum albumin, RPMI with or without 20% NHS containing either PBS, 0.9 μM FH5–7, FH19–20, or FH. The samples were incubated for 1 h at 37 °C in polystyrene round-bottom tubes under vigorous shaking. The samples were washed and fixed similarly as in the *ex vivo* assay before counting the fluorescence of 2000 neutrophils in each sample.

Detection of C3b Deposition on the S. aureus Surface—C3b deposition assay was done as previously described (16). Briefly, 2 × 10^8 bacteria and ^{125}I-labeled C3 (200 000 cpm per reaction) were incubated in 40% NHS or HIS at 37 °C for 90 min. The reaction was stopped with 10 mM EDTA, and the radioactivity of both the pellet and supernatant was counted with a gamma counter (Wallac).

Detection of Complement Activation and C3b Inactivation in Plasma—PBS or various concentrations of FH5–7, FH19–20, or BSA were incubated with 65% hirudin-anticoagulated blood obtained from healthy volunteers for 30 min at 37 °C under continuous rotation, where after the reactions were stopped with 10 mM EDTA. The samples were centrifuged, and the

plasma was separated from the pellet. Plasma samples were analyzed immediately or stored at −70 °C before the assays.

The formation of soluble C5b-9 was analyzed by ELISA as described earlier (18). The 96-well plates (Maxisorp, Nunc) were coated with 2 μg/ml anti-C9 neoepitope antibody WU13-15 (Hycult Biotech) in 100 μl of 0.2 M Na_2CO_3, pH 10.6. After blocking with 0.5% BSA, the 1:400-diluted plasma samples were added. After incubation the wells were incubated with goat anti-C7 (Organon Teknika) and HRP-conjugated donkey anti-goat IgG (Jackson ImmunoResearch). Wells were washed with PBS-T between each step. Finally, o-phenylenediamine dihydrochloride substrate (Dako) was added, the reaction was stopped with 0.5 M H_2SO_4, and absorbance of each sample was detected at 490 nm. Formation of C3a was analyzed according to the manufacturer's instructions using 1:2500 diluted plasma samples (MicroVue C3a Plus EIA, Quidel). The amount of iC3b was analyzed by ELISA. The 96-well maxisorp plates were coated with 3 μg/ml goat anti-C3 (Jackson ImmunoResearch) in PBS and incubated for 1 h at 37 °C with the plasma samples. iC3b was detected with iC3b neoantigen (Serotech) and HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch). The experiments were performed two or three times using two or three parallel samples, and the relative formation of complement activation marker in each sample was counted as the percentage of the highest value obtained in the assay.

Serum Pulldown Assays and Proteomics—0.2 mg of purified histidine-tagged FH5–7 was immobilized on HisTrap HP 1-ml column (GE Healthcare). After washing the column with 5 ml of 75 mM PBS, 10 ml of 10% NHS was run through the column. The column was then washed with 5 ml of 75 mM PBS and eluted with a 75 mM PBS, 75 mM PBS/2 M NaCl gradient. An assay without FH5–7 coupling was used to control nonspecific binding of serum proteins to the column. Next the column was equilibrated with 5 ml of 50 mM imidazole followed by elution with a 50–300 mM imidazole gradient. The collected fractions were run into SDS-PAGE gels under reducing conditions, and the eluted proteins were detected by silver staining. The band was cut off the gel and reduced, alkylated ,and in-gel trypsin-digested (PMID: 17406544). The resulting peptides were purified by reversed-phase chromatography columns (C18 material, eluted with 50% CH_3CN, 0.1% TFA) (PMID: 23602568). The dried peptides were reconstituted (1% CH_3CN, 0.1% TFA), and the MS analysis was performed on an Orbitrap Elite ETD mass spectrometer (Thermo Scientific) using Xcalibur Version 2.7.1 coupled to a Thermo Scientific nLCII nanoflow HPLC system. Peak extraction and subsequent protein identification was achieved using Proteome Discoverer software (Thermo Scientific). Calibrated peak files were searched against the human component of the UniProt database by SEQUEST search engine. Error tolerances on the precursor and fragment ions were ±15 ppm and ±0.8 Da, respectively. For peptide identification, a stringent cut-off (1% false discovery rate) was used.

Interaction of FH Fragments with ApoE on HDL Particles—In the fluorometric assay 96-well maxisorp plates (Nunc) were coated with FH5–7, FH19–20, or BSA in PBS. 15 μg/μl cholesterol concentration of each HDL sample (HDL-apoE, HDL, and HDL + apoE) was added to the wells. The apoE concentrations

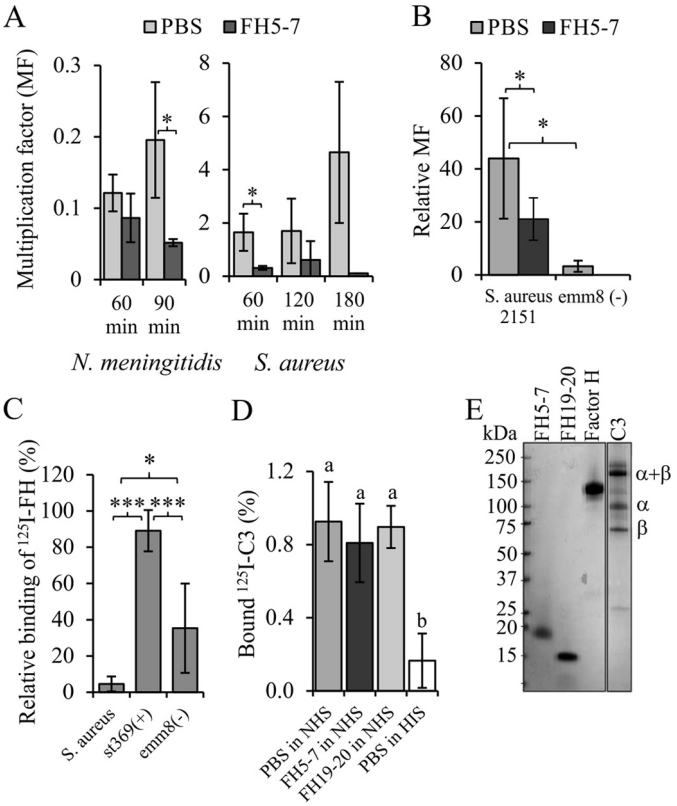

FIGURE 1. **FH5–7 impaired survival and increased phagocytosis of bacteria independently from factor H and C3 binding.** *A*, effect of FH5–7 on multiplication of *N. meningitidis* (*left*) and *S. aureus* (*right*) in blood was tested by incubating the bacteria for 90–180 min in lepirudin-anticoagulated blood with FH5–7 or PBS, where after the MF was counted using the pour-plate method. *B*, effect of FH5–7 on multiplication of *S. aureus* on the 60-min time point. The relative MF was counted as the percentage of the maximal MF obtained in each assay. A Group A streptococcal strain *emm*8 was used as a negative control for multiplication in blood ($n = 2$). *C*, binding of ^{125}I-labeled FH on *S. aureus*. The values indicate relative binding (percentage of the maximal cpm obtained in each assay). Non-opsonized *S. pyogenes* strains were used as negative (*emm*8) and positive (st369) controls for FH binding ($n = 2$). *D*, the effect of FH5–7 on *S. aureus* susceptibility to complement-mediated opsonization was tested by incubating the bacteria in NHS or HIS. A trace amount of fresh ^{125}I-labeled C3 in the sera was used to indicate the relative amount of C3b depositions onto *S. aureus* with or without FH5–7 fragment ($n = 2$). *E*, SDS-PAGE and Coomassie staining of the complement proteins used in the assays. C3 ($\alpha+\beta$) consists of two chains, α and β. Molecular mass marker (kDa) are indicated on the *left*. *Error bars* show the ± S.D. *D*, a statistically significant difference (p value below 0.05) is annotated with different letters (between *a* and *b*) using one-way analysis of variance supplemented with Tukey's multiple comparison test. *Asterisks* (* and ***) indicate a p value below 0.05 or 0.001 obtained using Student's t test.

of the samples were 10 (HDL-apoE), 300 (HDL), and 700 (HDL+apoE) ng/ml. Wells were washed with PBS between each step, and the cholesterol concentration of each well was counted according to manufacturer's instructions (Amplex® Red Cholesterol Assay kit, Molecular Probes®). The relative cholesterol concentrations were counted as the percentage of the maximal cholesterol concentration obtained in each assay.

Statistical Methods—Statistical analyses were performed using one-way analysis of variance supplemented with the Tukey-HSD multiple comparison test, or the difference between the means of two independent samples was compared using Student's t test (SPSS for Windows, Analytical Software).

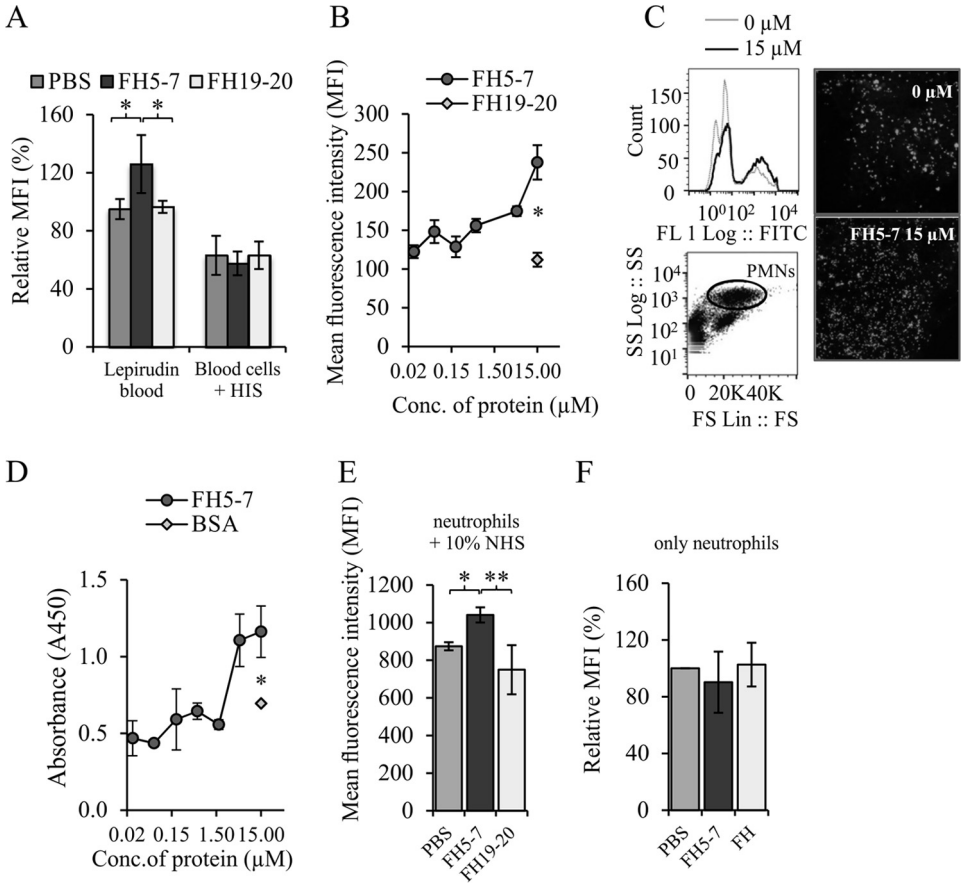

FIGURE 2. *A*, phagocytosis of fluorescein-labeled bacteria by neutrophils was tested *ex vivo* in lepirudin-anticoagulated blood and lepirudin-anticoagulated blood with HIS ($n = 2$). *B*, dose-dependent effect of FH5–7 on *S. aureus* phagocytosis *ex vivo* ($n = 3$). *C*, analysis of fluorescence-labeled (*FL*) bacteria associated with blood cells (*right panel*) and neutrophils (*left panel*) in the *ex vivo* assay. Gated neutrophils (*left panel above*) were analyzed for fluorescence intensity, and the distribution of the fluorescent cells in the presence (15 μM) and absence (0 μM) of FH5–7 were visualized in a histogram (*upper left panel*). Blood cells (*right panel*) under confocal microscopy in the (15 μM) presence and (0 μM) absence of FH5–7. *D*, dose-dependent effect of FH5–7 on plasma C3a formation *ex vivo*. After incubating the bacteria in blood the formation of C3a on plasma was calculated ($n = 2$). Phagocytosis of fluorescein-labeled bacteria *in vitro* is shown. The labeled bacteria were incubated with neutrophils both in the presence (*E*) or absence (*F*) of fresh 10% serum ($n = 3$). In the assays, FH19–20, BSA, or FH were used as a negative controls. Mean fluorescence intensity (*MFI*) was counted from 10,000 (blood cells in NHS or HIS) or 2,000 neutrophils using flow cytometry. The relative mean fluorescence intensity is counted as the percentage of the mean fluorescence intensity of PBS sample obtained in repetitive assays. *Error bars* show ±S.D. (*B*, *D*, and *E*) or 95% (*A*) confidence intervals. The *asterisks* (* and **) indicate a *p* value below 0.05 or 0.01 obtained using Student's *t* test (*B* and *D*) and one-way analysis of variance (*A* and *E*) supplemented with Tukey's multiple comparison test.

Results

FH5–7 Impairs Survival and Increases Phagocytosis of Bacteria Independently from Factor H and C3 Binding—We have previously shown that *ex vivo* survival of a bacterium that uses FH binding for immune evasion is reduced in the presence of FH5–7 lacking the active domains 1–4 of FH (18). To detect which of the main human pathogens has impaired survival in the presence of FH5–7 fragment, we also studied *N. meningitidis* and *S. aureus*. The addition of FH5–7 significantly reduced multiplication of both bacterial species in lepirudin-anticoagulated blood (Fig. 1*A*). For *N. meningitidis* this result was expected because it can evade host complement by binding FH via domain 6. Surprisingly, multiplication of *S. aureus* was also reduced, although it did not bind FH (Fig. 1, *A–C*). The multiplication was not, however, completely blocked when compared with *S. pyogenes* strain *emm*8 that was used as a negative control for bacterial multiplication in blood (16). Although *S. aureus* was able to survive and multiply in human blood (Fig. 1, *A* and *B*), a clear ^{125}I-labeled C3b opsonization on the microbial surface could be detected when the bacterium was incubated in NHS (PBS in NHS *versus* PBS in HIS $p < 0.05$) (Fig. 1*D*). Because the addition of FH5–7 in this assay did not cause reduction in C3b deposition (PBS *versus* FH5–7 added to NHS $p > 0.05$; Fig. 1*D*) it became probable that impaired multiplication of *S. aureus* in the presence of FH5–7 was not due to inhibition of FH binding to the bacteria.

Phagocytosis of S. aureus Is Enhanced by FH5–7 Only in the Presence of Active Serum—Our results above showed that FH5–7 reduces bacterial multiplication *ex vivo* independent of bacterial FH binding or C3b opsonization. Therefore, we next studied if reduction in bacterial survival could be caused by enhanced neutrophil activity. Fluorescein-labeled *S. aureus* bacteria were incubated in lepirudin-anticoagulated blood in the presence of FH5–7. When compared with the values obtained with FH19–20 or PBS, a significant and dose-dependent increase ($p < 0.05$) in mean fluorescence intensity (*MFI*) of neutrophils could be detected in the presence of FH5–7 (Fig. 2, *A–C*). In addition, in the same samples FH5–7 caused a significant and dose-dependent increase in plasma C3a formation (Fig. 2*D*). In contrast, no increase in bacterial phagocytosis by

FH5–7 could be detected when the blood cells had been separated from plasma and the plasma was replaced with heat-inactivated serum (*Blood cells + HIS*) (Fig. 2*A*). This indicates that the effect of FH5–7 can be detected only in the presence of active complement. Next we showed that *in vitro* phagocytosis of preopsonized fluorescein-labeled bacteria by blood-isolated neutrophils was detected only when both FH5–7 and serum were present (Fig. 2*E*). In a direct binding assay we could not detect any binding of ^{125}I-labeled FH5–7 to isolated neutrophils even after activating neutrophils with bacteria (Fig. 3). No binding of FH5–7 to serum-opsonized and non-opsonized *S. aureus* was detected.

FH5–7 Enhances Complement Activity by Reducing Complement Inhibition in Plasma—When FH5–7 without bacteria was added to hirudin-anticoagulated blood in the presence or absence of FH5–7, it was noticed that C3a generation in plasma was significantly increased in the presence of FH5–7 (Fig. 4*A*). This indicated that the addition of FH5–7 led to enhanced complement activation at or before the C3b level. No differences in plasma C5b-9 concentration could be detected after adding FH5–7 to hirudin-anticoagulated blood (Fig. 4*B*). The reduction in iC3b formation by FH5–7 fragment (Fig. 4*C*) indicated that the FH5–7 fragment interferes with FH-mediated complement regulation in plasma, and this was found to be dose-dependent (Fig. 4*D*). The reduction of iC3b concentration was ~20% in the presence of 0.5 μM of FH5–7, equal to ⅓ that of the concentration of FH in the 65% plasma samples.

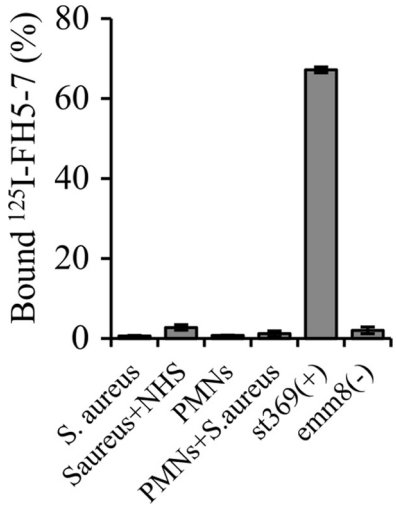

FIGURE 3. **Binding of FH5–7 on blood isolated neutrophils (*PMNs*) was tested by incubating the cells for 30 min with ^{125}I-labeled-FH5–7.** In the same experiment, binding of FH5–7 on non-opsonized (*S. aureus*), preopsonized bacteria (*S. aureus + NHS*), and bacteria activated neutrophils were tested (*PMNs + S. aureus*). Non-opsonized *S. pyogenes* strains were used as negative (*emm8*) and positive (*st369*) controls for FH5–7 binding ($n = 1$).

HDL Binds FH Fragment FH5–7 via ApoE in Human Serum—Because the data suggested that FH5–7 inhibits complement regulation in plasma, we next searched for a plasma component that interacts with FH5–7 by affinity chromatography and mass spectrometry. A clear ~34-kDa protein was seen in the fractions eluted with 0.8, 0.9, and 1.8 M NaCl from FH5–7-coupled HisTrap column but not from the control column (Fig. 5*A*). This protein was identified as apoE using liquid chromatography-mass spectrometry (LC-MS) analysis (Table 1). The other proteins identified with two or more unique peptides were not studied further because of lower confidence (score <20). The interaction of FH5–7 with apoE2 protein was confirmed by microscale thermophoresis that showed ~200 nM affinity (Fig. 5*B*).

Because apoE is a secretory lipoprotein-bound protein in human plasma/serum and the majority of these molecules are associated with lipoprotein particles, binding of FH and FH5–7 to HDL particles was analyzed. HDL isolated from human serum was preincubated with FITC-FH or FH5–7, and gel filtration of the mixture revealed that the fluorescence signal of the collected FH (Fig. 6*B*) and FH5–7 (Fig. 6*C*) fractions followed exactly the elution positions of HDL particles. Next, to demonstrate that binding of FH via FH5–7 onto HDL particles is mediated via apoE, isolated HDL particles with different apoE concentrations were prepared. When these particles were incubated with FH5–7, FH19–20, or BSA, only FH5–7 bound to HDL dose-dependently as a function of apoE concentration (Fig. 6*D*). This indicated that binding of FH5–7 onto the HDL particles is mediated via apoE.

Our data suggested that FH5–7 could cause complement activation and reduction in iC3b formation in plasma by inter-

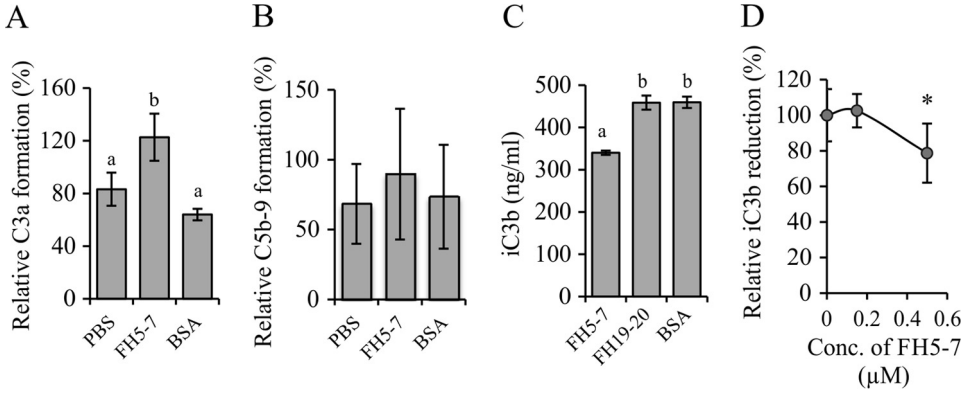

FIGURE 4. **FH5–7 enhanced C3a formation and reduced iC3b formation in plasma.** The effect of FH5–7 in plasma was analyzed by incubating the fragment in hirudin anticoagulated blood. Thereafter, formation of C3a ($n = 2$) (*A*), C5b-9 ($n = 3$) (*B*), and iC3b (a representative from two individual experiments) (*C*) from the isolated plasma samples was analyzed using enzyme immunoassay and ELISA. In the assays BSA (*A* and *B*) and/or FH19–20 (*C*) was used as a negative control. *D*, to quantify the inhibition of iC3b formation by FH5–7, different concentrations of the fragment were added to the blood ($n = 3$). *Error bars* show the ± S.D. Statistically significant difference (*p* value below 0.05) from other values is annotated with an *asterisk* (*) using Student's *t* test (*D*) or different letters (*e.g.* between *a* and *b*) using one-way analysis of variance supplemented with Tukey's multiple comparison test (*A* and *C*) .

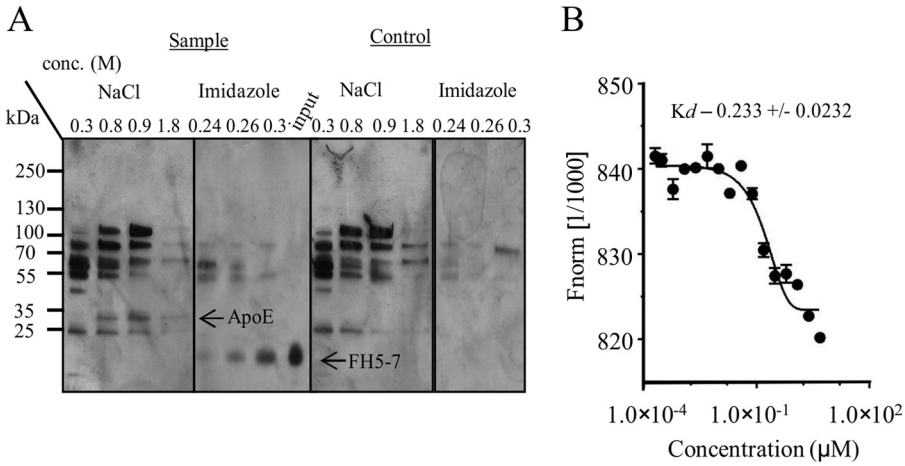

FIGURE 5. **Factor H fragment FH5–7 interacted with serum apoE.** *A*, in a pulldown assay, 0.2 mg of purified histidine-tagged FH5–7 protein (*input*) was first coupled on a HisTrap HP 1-ml column. After washing, the column was eluted with a salt gradient. An assay without FH5–7 coupling was used to control nonspecific binding of serum proteins to the column (*Control*). The collected fractions were run into SDS-PAGE gels under reducing conditions. An ~34-kDa band in fractions eluted from the FH5–7 coupled column is seen in the sample but not in the control assay (0.8–1.8 M NaCl). This protein was identified as apoE by mass spectrometry analysis. *B*, for microscale thermophoresis analysis FH5–7 was incubated with labeled apoE2. The concentration of labeled ligand was 0.0013 μM, and the starting concentration for the unlabeled FH5–7 was 5 μM. The average of two experiments suggested a K_d value of 0.202 μM.

TABLE 1
Protein identification

Description	Accession	Score[a]	Coverage	Unique peptides	M_r	Sequence
					kDa	
Apolipoprotein E	P02649	22.38	23.97	6	36.1	VQAAVGTSAAPVPSDNH AATVGSLAGQPLQER SELEEQLTPVAEETR LEEQAQQIR LAVYQAGAR LGADMEDVcGR
Vimentin	P08670	14.09	6.22	2	53.6	LLQDSVDFSLADAINTEFK ILLAELEQLK
Ig κ chain C region	P01834	10.23	35.85	2	11.6	VDNALQSGNSQESVTEQDSK TVAAPSVFIFPPSDEQLK
Ig λ-2 chain C regions	P0CG05	6.42	23.58	2	11.29	SYSCQVTHEGSTVEK AGVETTTPSK

[a] Indicates relevance of the protein. Calculated by Proteome Discoverer software according to the score thresholds.

fering with binding of FH to apoE. Therefore, we next studied whether binding of full-length FH to apoE could be detected. We found that binding of ^{125}I-labeled apoE3 to FH could be dose-dependently inhibited by non-labeled apoE3 but not BSA (Fig. 7*A*). As expected, non-labeled apoE also inhibited binding of ^{125}I-labeled apoE3 to FH5–7 (Fig. 7*B*). Finally, we could demonstrate that FH5–7 could dose-dependently compete with FH in binding to ^{125}I-labeled apoE3 (Fig. 7*C*). To localize the FH5–7 binding site on apoE, we analyzed binding of FH5–7 to three different apoE4 fragments (amino acid regions 1–185, 1–229, and 1–259). We did not observe any differences in the binding of these fragments to FH5–7 (Fig. 7*D*), indicating that the FH5–7 binding site is located in the N-terminal region of apoE. These data indicate that FH binds specifically to apoE via its domains 5–7.

Discussion

The AP of complement is easily activated on any surface where hydroxyl or amino groups are present and regulation is inefficient. This occurs mainly on non-self surfaces such as microbial surfaces or necrotic cells that lack surface sialic acids or GAGs that are ligands for FH. The role of FH in regulation of AP activation on lipoprotein particles has been, however, largely unexplored. In this study we observed that the addition of FH5–7 in anti-coagulated blood reduces survival of *S. aureus* (Fig. 1, *A* and *B*) caused by excessive C3a release in plasma (Fig. 2*D*). We showed that the significant increase in plasma C3a levels (Fig. 4*A*) is due to inhibition of FH-mediated complement regulation of HDL particles by FH5–7. In the current manuscript we show to our knowledge for the first time, protection of HDL particles in plasma against the complement attack is mediated by binding of FH to apoE via domains 5–7 (probably the GAG/heparin site on domain 7) (Fig. 6*D*).

Initially the effect of FH5–7 in enhancing killing of FH-non-binding staphylococci was the hint to lead to the finding of FH-apoE interaction (Fig. 3). In those assays we observed that FH5–7 specifically inhibits regulatory function of FH at the C3b level. In principle this could be caused by interference of FH-mediated regulation on plasma C3b, but we failed to observe that. Instead, it became obvious that the excessive activation of AP in serum by FH5–7 was due to inhibition of the regulatory action of FH on HDL particles.

HDL and to a lesser extent LDL can provide protection against the complement terminal pathway partly due to the plasma terminal pathway regulator clusterin (apoJ) or by acting as an acceptor for glycosylphosphatidylinositol lipid-anchored complement regulator CD59 (26, 27). On the basis of various *in*

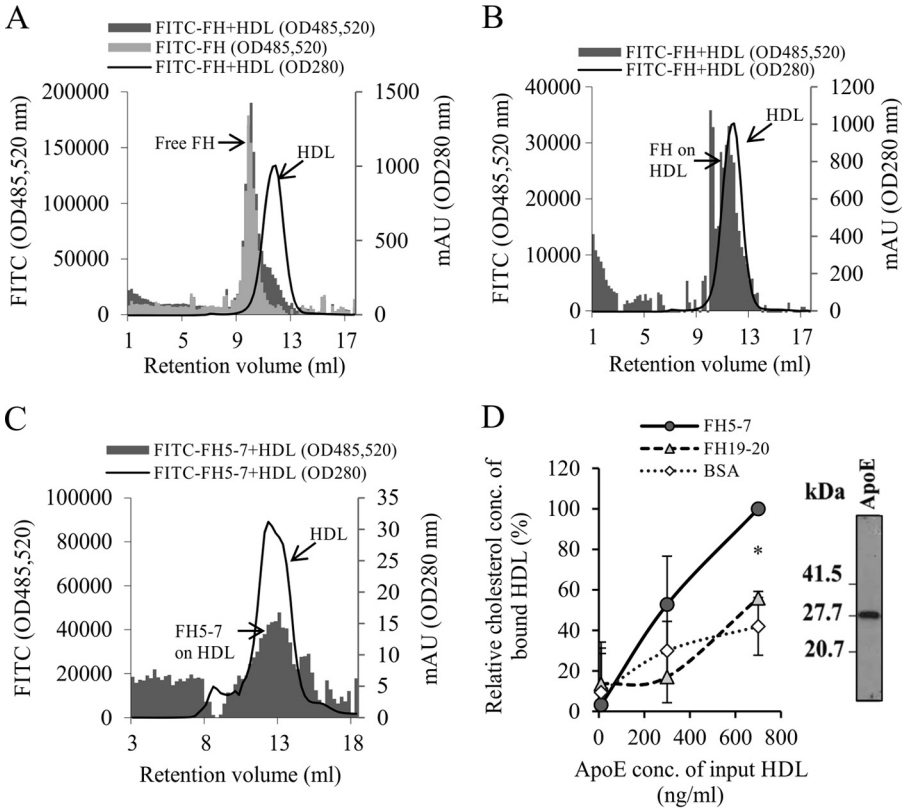

FIGURE 6. **Factor H and FH5–7 interacts with apoE serum protein on HDL particles.** Isolated HDL was incubated for 30 min at 37 °C with FITC-labeled FH (*A* and *B*) or FH5–7 (*C*) and loaded into Superdex 200 10/300GL gel filtration column, and the elution profile was analyzed by ÄKTA explorer at $A_{280\ nm}$ for HDL proteins (HDL peak is indicated with the *black line*) and fluorescence (A_{485} and A_{520}) of FITC-FH and FITC-FH5–7 fractions. *B*, the peak of HDL-bound FH from free FH was visualized by subtracting the fluorescence counts of a control sample where only FITC-FH was loaded to the column ((FITC-FH (A_{485} and A_{520})) from the HDL-incubated FITC-FH sample ((FITC-FH + HDL (A_{485} and A_{520})). *D*, the cholesterol concentration of the HDL fractions with different apoE concentrations (10, 300, and 700 ng/ml) were counted, where after a cholesterol concentration of 15 μg/ml of each fraction was incubated in 96-well plates coated with FH5–7, FH19–20, or BSA. After washing, the cholesterol concentration of bound HDL was counted by fluorometry. The values indicate the relative cholesterol concentration (percentage of the maximal cholesterol concentration was obtained in each assay, $n = 3$). *Error bars* show the ± S.D. Statistically significant difference (*p* value below 0.05) from other values was obtained from the value annotated with the *asterisk* (*) using Student's *t* test.

vitro and *in vivo* studies, it is evident that HDL has an important role in reducing inflammation (28). This is possibly at least partially due to binding of complement regulators FH and clusterin to HDL particles. The strict complement regulation on HDL could be especially important when foreign bacterial molecules such as lipopolysaccharide, teichoic acids, and *S. aureus* phenol soluble modulins are captured on the lipoprotein particles as those molecules might activate complement (29). The observed interaction between the apoE and FH (Fig. 7*A*) is logical because inhibition of only the terminal pathway may not be sufficient to reduce the inflammatory response and opsonization caused by activation at the C3 level.

Certain mutations and polymorphisms of FH are associated with AP dysregulation-mediated diseases such as AMD, DDD, and atypical hemolytic uremic syndrome. AMD and DDD are characterized by formation of deposits in the eye (drusen) or kidney (glomerular basement membrane) that are rich in complement activation products and, most importantly, also contain high amounts of apoE (30, 31). Therefore, pathogenesis of at least AMD and DDD could be linked to the novel results on FH interaction with apoE on HDL particles. FH-like protein 1 (FHL-1) is identical to FH for the first seven domains and is, therefore, most likely also interacts with apoE, although this was not shown in the present study. Next to FH, FHL-1 is a subject to the AMD-associated Y402H polymorphism and may, therefore, also contribute in AMD pathogenesis (32). It remains, however, to be studied whether the mutations or polymorphisms of FH affect binding to apoE.

No difference was seen between binding of FH5–7 to the three studied N-terminal fragments of apoE, indicating that the binding site on apoE is located in the N-terminal domain and not in the "hinge region" between amino acids 186 and 259 or the C-terminal domain containing the major lipid binding region. The three allelic isoforms apoE2, apoE3, and apoE4 differ at positions 112 and 158 at the N-terminal domain where apoE2 has two cysteines, apoE4 has two arginines, and apoE3 has one cysteine (Cys-112) and one arginine (Arg-158). These particular residues were not included in the MS peptide identification. These amino acid differences have a clear impact on the structure and function of the apoE isoforms: the presence of cysteine at position 158 lowers the affinity of LDL receptor for apoE2. As a result, apoE2 is associated with type III hyperlipoproteinemia (33), and apoE4 is associated with, for example, Alzheimer disease (34). But apoE polymorphism is also associated with AMD, characterized by strong association with FH polymorphism Y402H within the domain 7, and these two polymorphisms could have an additive effect (35, 36). It remains,

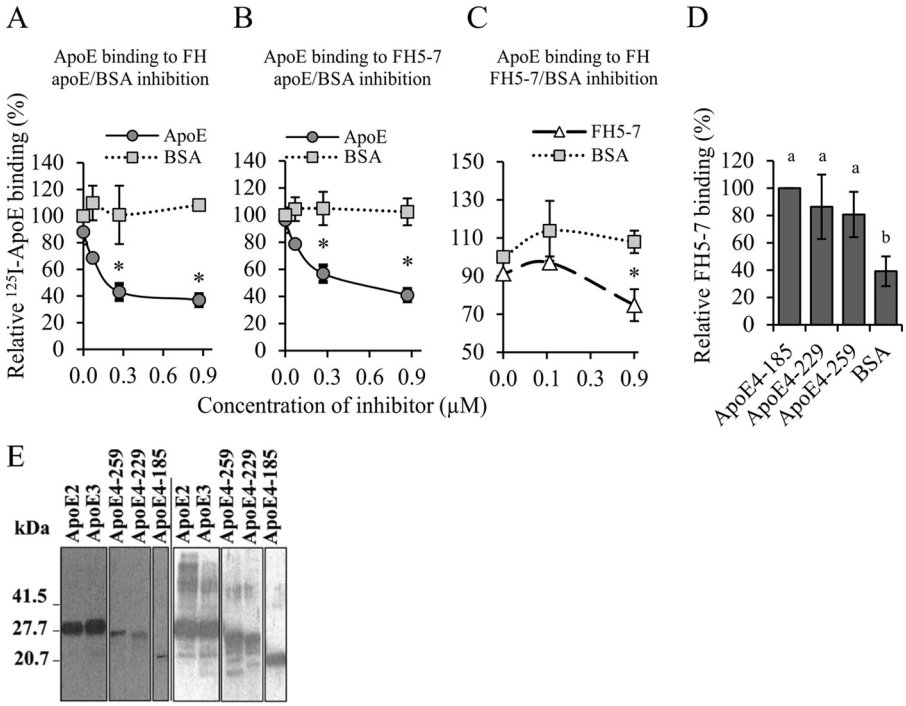

FIGURE 7. **FH bound to apoE via domains 5–7.** Factor H (*A* and *C*) and FH5–7 (*B*) were coated on 96-well plates and incubated with ^{125}I-labeled apoE3. Increasing concentrations of non-labeled apoE (*A* and *B*) or FH5–7 and BSA (*C*) were used in the competition ($n = 3$). *D*, binding of FH5–7 on 96-well plates coated different C-terminal fragments of apoE4 was analyzed by ELISA using anti-FH antibody ($n = 3$). *E*, SDS-PAGE, Coomassie staining (*left*) and Western blot (*right*) of apoE proteins used in the assays. Molecular mass marker (kDa) is indicated on the *left*.

however, to be studied whether the various apoE isoforms affect binding to FH. Our results provide a link between these two molecules by showing, for the first time, the interaction between apoE and FH.

It is widely accepted that factor H recognizes self-surfaces with two GAG binding sites located on domains 7 and 20. In addition, several microbes are known to bind FH using domains FH19–20 and FH6–7. A recent study showed that microbial proteins form tripartite complexes with FH and C3b by binding to a common site on FH domain 20 (37). Therefore, it is likely that bacteria use the complex to enhance inactivation of C3b on their surface. A similar tripartite complex has not been shown for any microbes or microbial proteins binding to domains 5–7, although a crystal structure of the complex between FH6–7 and FH-binding protein of *N. meningitidis* suggests that the bacterium uses molecular mimicry in binding to domains 6–7 (38). Our data on the interaction between FH5–7 and apoE provides a possible explanation as to why it is particularly beneficial for microbes to bind to these domains of FH. It is namely possible that by binding FH5–7, bacteria mimic plasma particles such as HDL and thereby increase their survival in blood. Although not yet studied, it is also possible that the FH-binding proteins on the microbial surface form a tripartite complex with FH and HDL particles, thereby obtaining surface-bound anti-inflammatory HDL particles that could also serve as a camouflage against the acquired immunity. This kind of binding of FH would be especially beneficial for the microbes causing septic infections such as *N. meningitidis* and *S. pyogenes* and could explain why nearly all the FH5–7 binding microbes are those that cause septicemia.

In summary, this study demonstrates how FH functions as a complement regulator on HDL particles and highlights the importance of domains 5–7 in the specific interaction between FH and apoE. These data provide a new aspect in pathogenesis of AMD and DDD and a novel explanation for the wide use of FH5–7 in microbial immune evasion. It remains also possible that the FH-apoE complex formation could be utilized in the treatment of excessive AP activation observed in complement-associated inflammatory diseases.

Author Contributions—K. H. and T. S. J. designed the study. K. H. wrote the paper with help of T. S. J. K. H. isolated the FH and C3 proteins and cloned, expressed, and purified the FH5–7 constructs. K. H. designed and performed the bacterial multiplication and phagocytosis experiments, direct binding and radioligand experiments, complement activation experiments, ELISA experiments, and serum pulldown experiments. K. H. performed the statistical analysis. K. v K designed the FH-HDL interaction experiments. E. N. designed and performed the microscale thermophoresis experiments. J. M. isolated the human HDL particles. T. J. performed bacterial multiplication experiments. S. M. and M. V. performed the MS analysis. T. S. J., K. v K, E. N., M. J., J. K., A. C., P. K., and J. v S. revised the manuscript critically for important intellectual content. A. C. produced WT and mutant apoE forms using infrastructure that is associated with the Greek National Research Infrastructure in Structural Biology, Instruct-EL. All authors analyzed the results and approved the final version of the manuscript.

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