SUPPLEMENTAL MATERIAL

The MFHR1 Fusion Protein Is a Novel Synthetic Multitarget Complement Inhibitor with Therapeutic Potential

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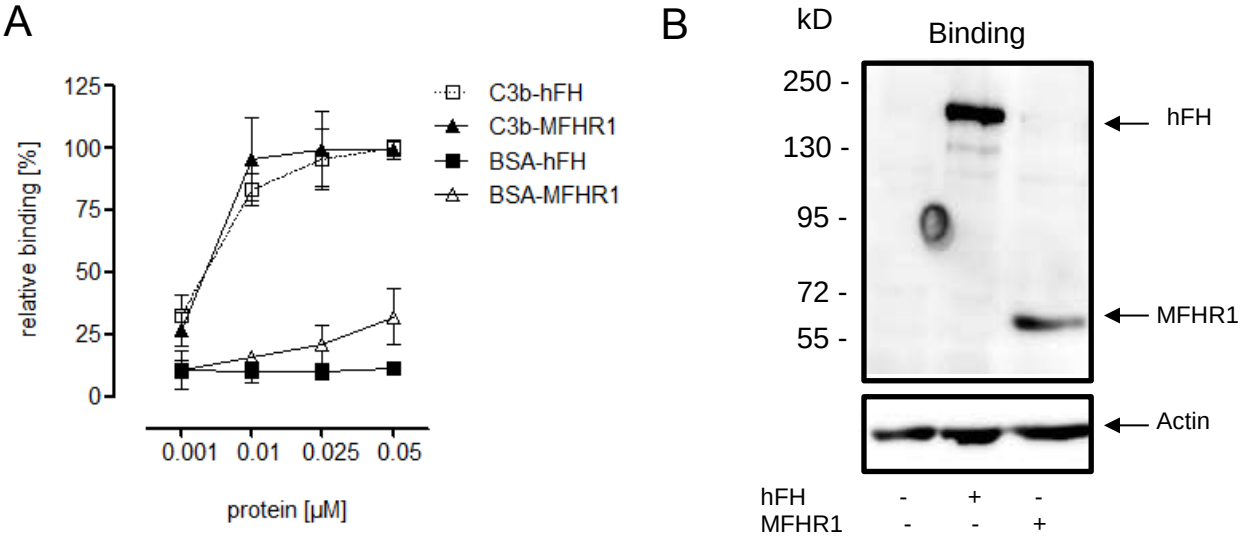
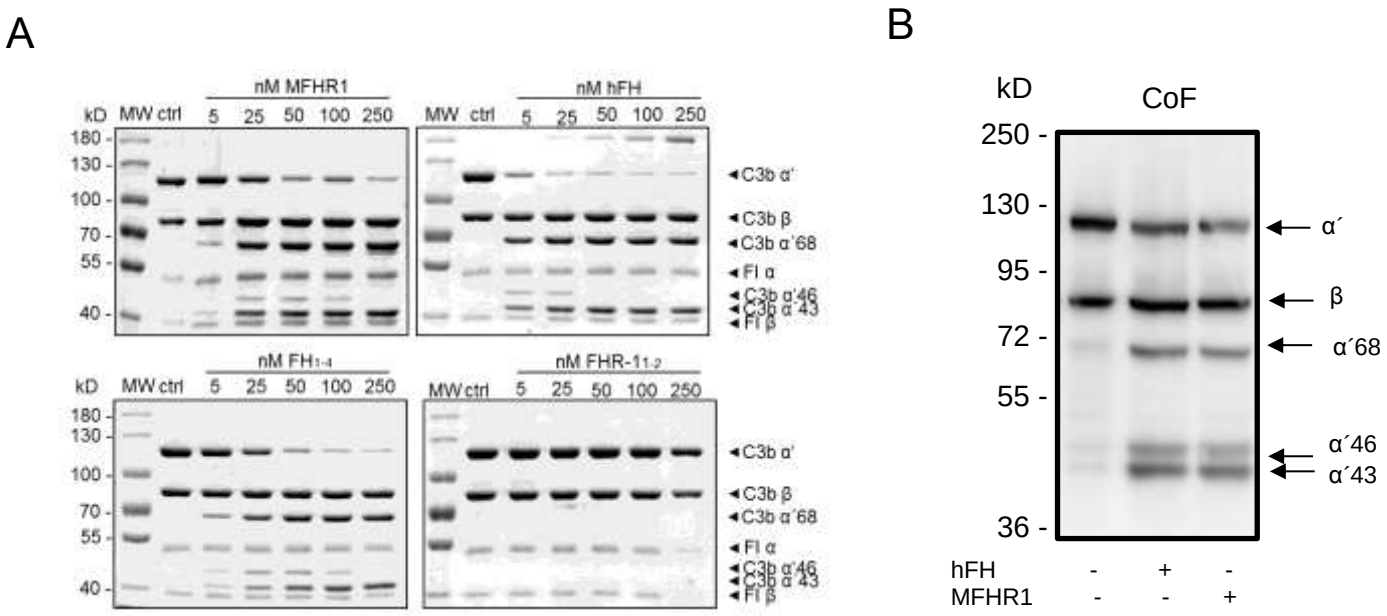
Supplemental Results

SUPPLEMENTAL Figure 1



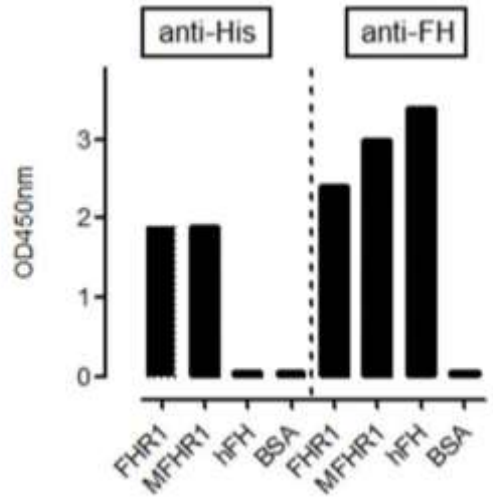
Supplemental Figure 1: MFHR1 binds to C3b and HUVEC cells A) Binding of MFHR1 to C3b was analyzed by ELISA. C3b was immobilized on microtiter plates and serial dilutions of MFHR1 or hFH were added and bound proteins were detected using anti-FH primary and HRP-labelled anti-goat secondary antibodies. BSA coated wells were used as controls. Data are mean ±SD from n=3 experiments. Maximum binding of hFH to C3b was set to 100%. The binding of hFH and MFHR1 to C3b was similar (two-way ANOVA) **B)** MFHR1 or hFH were incubated on HUVEC cells. Following incubation, cells were lysed and lysates were separated by SDS-PAGE. Bound MFHR1 and hFH were detected using anti-FH antibodies. Actin is shown as a control. The position of the markers is indicated on the left.

SUPPLEMENTAL Figure 2



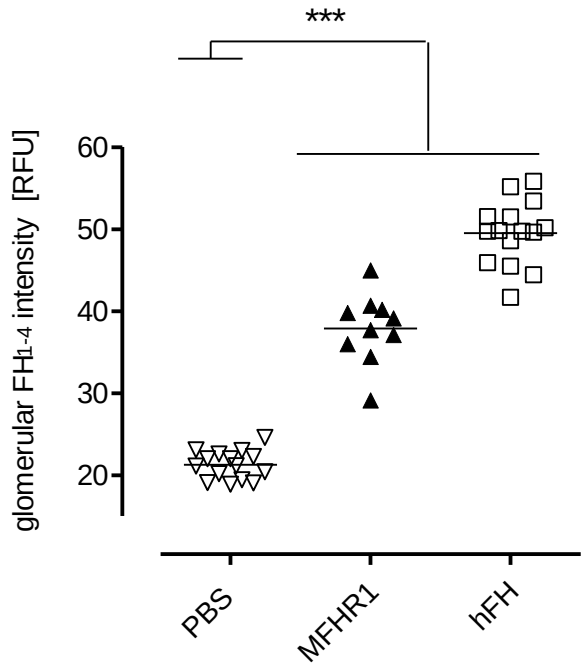
Supplemental Figure 2: MFHR1 displays cofactor activity in the fluid phase and if bound to HUVEC cells A) MFHR1, hFH and FH_{1-4} but not FHR-1_{1-2} display cofactor activity. Cofactor testing was performed by incubation of C3b and Factor I (FI) with increasing concentrations of test proteins (5-250 nM) for 30 min at 37°C. The reactions were separated by SDS-PAGE and Coomassie blue staining was used to visualise α-chain cleavage and α'68, α'46 and α'43 fragments. MFHR1, hFH and FH_{1-4} mediated a dose-dependent cleavage of the α'-chain in the fluid-phase cofactor assay into α'68 and α'46 fragments, while the ß-chain remained unaffected. In a second step the α'46 fragments were further cleaved to yield α'43 fragments in which for both cleavage steps hFH showed the highest activity **B)** The cofactor activity of HUVEC-bound MFHR1 or hFH, was measured after adding C3b and factor I. C3b cleavage was analyzed in the supernatants by western blotting.

SUPPLEMENTAL Figure 3



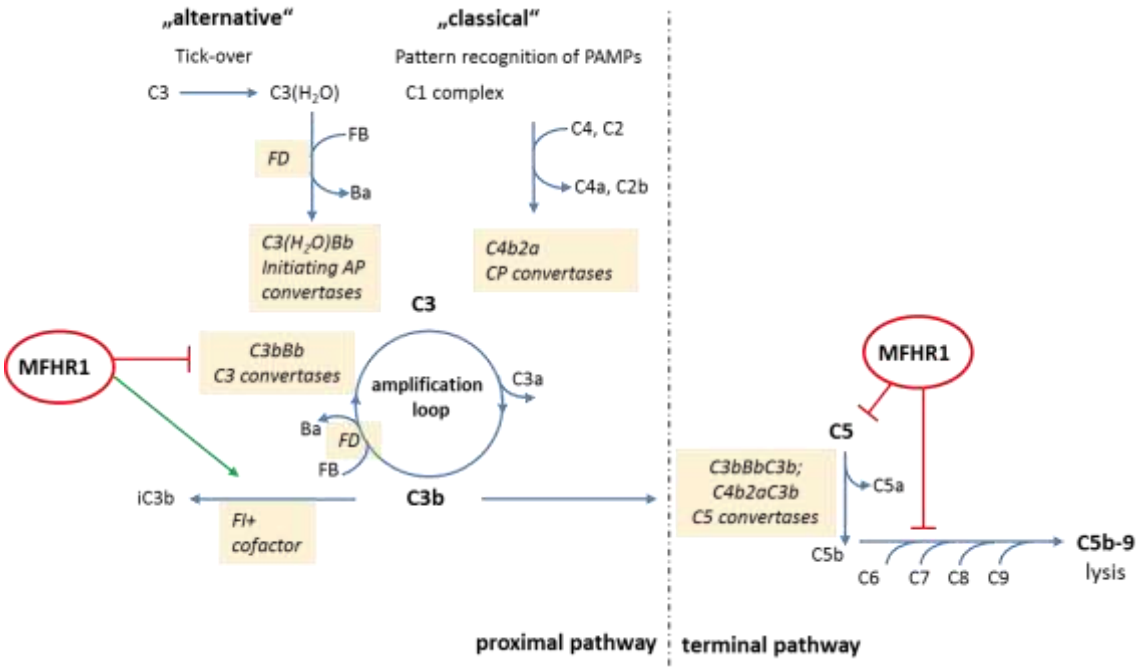
Supplemental Figure 3: Coating of inhibitors. Equimolar amounts of indicated protein (133 nM) were coated on Nunc plates and coating efficiency was analyzed using anti-His or anti-hFH antibodies and HRP-labelled secondary antibodies following HRP-detection.

SUPPLEMENTAL Figure 4



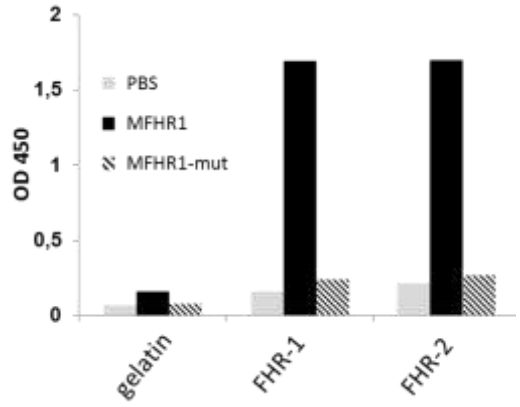
Supplemental Figure 4: MFHR1 is detectable in mouse glomeruli. Glomerular MFHR1 and hFH fluorescence immunostaining intensity was determined 24 hours after administration of MFHR1, hFH or PBS in treated *FH-/-* mice. Means are shown with plotted individual data points obtained from five glomeruli per section expressed as relative fluorescence units (RFUs). *** marks significant difference between the treatments and PBS group ($P<0.001$), analyzed by one-way ANOVA with Bonferroni test.

SUPPLEMENTAL Figure 5



Supplemental Figure 5: Mechanism of MFHR1 activity. Complement alternative pathway (detailed description is provided in the Introduction). MFHR1 is a fusion protein that combines the first 2 SCRs of FHR-1 with the first 4 SCRs of FH. The FHR-1 domain binds to C5 and C5b6 and inhibits C5 to be cleaved by C5 convertases, C5b-9 formation and lysis of host cells, while SCR1-4^19-20 of FH mediates cofactor activity and decay acceleration activity thereby blocking C3 convertase amplification resulting from AP and CP activation.

SUPPLEMENTAL Figure 6



Supplemental Figure 6: MFHR1 but not a dimerization mutant binds to FHR-1 and -2.

MFHR1 comprises a dimerization motif. Binding of MFHR1 to FHR-1 or -2 was shown using purified recombinant MFHR1 or a dimerization mutant of MFHR1 (MFHR1-mut), labeled with biotin and added to immobilized FHR-1 and FHR-2. After washing bound MFHR1 or MFHR1-mut was detected with avidin. Binding of MFHR1 to FHR-1 and -2 was detected while the dimerization mutant did not bind to FHR-1 or FHR-2. Gelatin was used as control.

Supplemental Methods

Cloning, expression, purification and characterization of recombinant proteins

For expression of recombinant proteins, a modified baculovirus expression vector was generated to allow efficient cloning, production, secretion and purification of recombinant proteins. The *gp67* secretion signal sequence was amplified from pAcGP67-A (BD Biosciences, San Jose, USA) and extended with a 5´ADLGS(H_{10})DYDRS spacer containing an *Age*I site using appropriate primers by phusion DNA polymerase based-PCR (phusion PCR). Flanking *EcoRI* and *KpnI* sites were added to clone the 206 bp fragment directly into pFastBac^{TM}1 (Thermo Scientific, Rockford, USA) resulting in pFastBac-*gp67*-10His which allows directed cloning of gene fragments via *AgeI* and *KpnI*. The full-length human FHR-1 encoding sequence (Nextprot identifier: NX_Q03591, residues 19–330) or FHR-1_{1-2} fragment (Nextprot identifier: NX_Q03591, residues 19–142) were amplified by phusion-PCR reaction from pJet-FHR-1 using FHR-1-*Age*I-fw (5´- GATCACCGGTCCGAAGCAACATTTTGTGATTTTCC-3´) and FHR-1-*Kpn*I-rev (5´-GATCGGTACCCTATCTTTTTGCACAAGTTGGATATC-3´) primers or FHR-1-*Age*I-fw and FHR-1_{1-2} -*Age*I-rev (5´-GATCGGTACCCTAGGACCTGCATTTGGGAG-3´), respectively. To create pJet-FHR-1, human liver cDNA was amplified with the primers (5'-GGAGAAGCAACATTTTGTGAT-3') and (5'-AGGTGTGCATTTTATGATTGA-3') and cloned into pJet (Thermo Fisher Scientific). MFHR1 was generated in two steps. First, mini-FH[30] was generated by phusion-PCR amplification of the FH_{1-4} and FH_{19-20} fragments from pFH (Nextprot identifier: NX_P08603, residues 19–265 for FH_{1-4} and residues 1104–1231 for FH_{19-20}) using the following primers: FH_{1-4}-fw (5´-GAAGATTGCAATGAACTTCCTCC-3´), FH_{1-4} -rev (5´-TTCACATGAAGGCAACGG-3´), FH_{19-20}-fw (5´-CCTTCATGTGAAGATTCTACAGGAAAATGTGGGC-3´) and FH19-20-rev (5´-CTATCTTTTTGCACAAGTTGGATACT-3´). Hybridization of FH_{1-4} 3´ end and FH_{19-20} 5´ end was performed during 8 annealing and elongation steps followed by 36 cycles of phusion-PCR amplification using primers mini-FH-*Age*I-fw (5´-GATCACCGGTCCGAAGATTGCAATGAACTTCCTCC-3´) and mini-MFH-KpnI-rev (5´-GATCGGTACCCTATCTTTTTGCACAAGTTGGATACTC-3´). In a second step FHR-1_{1-2} (Nextprot identifier: NX_Q03591, residues 22–142) was amplified from pFHR-1 using MFHR1-*Age*I-fw (5´-GATCACCGGTCCTTTTGTGATTTTCCAAAAATAAACC-3´), MFHR1-rev (5´-CATTGCAATCTTCGGACCTGCATTTGGGAG-3´), mini-MFH -fw (5´-GAAGATTGCAATGAACTTCCTCC-3´) and mini-MFH -*Kpn*I-rev (5´-

GATCGGTACCCTATCTTTTTGCACAAGTTGGATACTC-3´). Hybridization of FHR-1_{1-2} 3´ and mini-MFH 5´ was performed as described above after the addition of MFHR1-*Age*I-fw and mini-MFH-*Kpn*1-rev. The FHR-5 sequence (Nextprot identifier: NX_Q9BXR6, residues 19–569) was amplified from pJet-FHR-5 using FHR-5-*Age*I-fw (5´-GATCACCGGTCCGAAGGAACACTTTGTGATTTTCC-3´) and FHR-5-*Kpn*I-rev (5´-GATCGGTACCTCATTCACATATAGGATATTCAAATTT-3´) primers. To create pJet-FHR-5, human liver cDNA was amplified with the primers (5'-GCATGTTGCTCTTATTCAGTG-3') and (5'-TTCAGGAAAATTATGCTTGC-3') and cloned into pJet (Thermo Fisher Scientific). MFHR1, FHR-1, FHR-5, FH_{1-4} FHR-1_{1-2} and mini-FH fragments were cloned via *AgeI* and *KpnI restriction sites* into pFastbac-gp67-10His, expressed in *Spodoptera frugiperda* (*Sf9*) cells following manufacturer's instructions (Bac-to-Bac® System, Invitrogen™LifeTechnologies™, Darmstadt, Germany).

C3b binding ELISA

For C3b binding ELISA, 10µg/ml C3b in phosphate buffered saline (PBS) (Gibco) was coated on 96-well MaxiSorb plates (Nunc, Roskilde, Denmark) overnight at 4°C. Following washing and blocking with PBS/2% bovine serum albumin (BSA, Thermo Scientific) in PBS for 2 hours at 37°C, increasing amounts of MFHR1 or hFH were added and incubated for 1 hour at 37°C. After washing, detection of C3b bound MFHR1 and hFH was performed using anti-hFH antibody and anti-goat-HRP.

Cell binding and cell-based cofactor assay

HUVEC cells were cultivated after manufacturer's instructions (Promo-Cell, Heidelberg, Germany). After cultivation under serum-starved conditions for 24 hours, cells were blocked with 1% BSA in 0.5X PBS and were incubated with 2µM MFHR1 or hFH for 1 hour at 37°C. Binding was analyzed from cell lysates with DPBS containing 1% Triton X-100 and Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany) for 1 hour at 4°C. Cellular extracts were separated on 8% SDS-PAGE and were transferred for immunoblotting using standard procedures. Bound MFHR1 or hFH were detected using anti-FH antibodies or anti-Actin antibodies as control (Santa Cruz, Heidelberg, Germany) followed by HRP-conjugated secondary Ab. For cofactor activity, cells incubated with MFHR1 or hFH were incubated with

200 ng C3b and 50 ng FI in 50 µl for 1 hour at 37˚C. Supernatants were analyzed for C3b cleavage by western blot.

Binding of MFHR1 to FHR-1, -2, -5

Purified MFHR1 as well as a dimerization mutant MFHR1-mut was labeled with biotin according to standard techniques. In the MFHR1-mut Protein the dimerization motif of MFHR1 was mutated from YYSCEY to SYYCEE. Biotin labeled MFHR1 or MFHR1mut was added to immobilized FHR-1, or FHR-2. Following incubation and washing bound MFHR1 was detected with avidin. In addition gelatin served as a control. Production and purification were performed as described above.