K 125219 – PI: Mihály Józsi

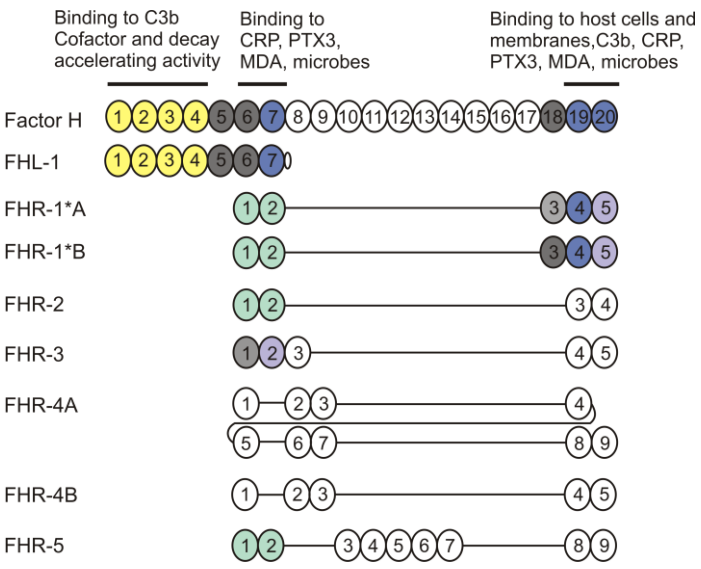
Studies on the function of human complement factor H family proteins

Final report

1. Introduction

The main aim of the project was to clarify functions of the various members of the human complement factor H (FH) protein family, which were linked to a number of inflammatory and infectious diseases. The complement system is a major humoral component of innate immunity that plays important roles in the protection against infections, in the clearance of cellular debris and in modulation of immune responses. Complement can be activated via three major pathways, the alternative, the lectin and the classical pathways. In addition, the alternative pathway amplifies complement activation initiated by any of the three pathways. Complement activation, however, is strictly regulated to prevent damage to the host. Excessive or insufficient activation or defective regulation of this system may lead to various diseases, such as atypical hemolytic uremic syndrome (aHUS), C3 glomerulopathies and age-related macular degeneration (AMD).

FH is a major inhibitor of the complement alternative pathway, but the role of the FH-related (FHR) proteins is less known and controversial. Being derived by gene duplication events and subsequent diversification, the individual domains of the FHR molecules show high amino acid sequence similarity to related domains of the complement inhibitor FH; however, they all lack the complement regulatory domains of FH. FHRs and FH bind to the same or similar ligands due to conserved binding sites, and competition between some of the FHRs and FH for ligand binding, leading to modulation of complement alternative pathway activation, was described. The project aimed at characterizing ligand interactions and functions of the FHR proteins. We studied the role of the FHRs in modulating complement activation by competing with the binding of the complement regulators FH and C4b-binding protein (C4BP), or with C1q, for certain ligands, or by recruiting complement activating molecules, such as C3b and pentraxins.



The human factor H protein family. The five human FHR proteins retained domains homologous to domains 6-9 and 18-20 of factor H (showed by vertical alignment). Colors indicate domains identical between factor H and FHRs; light shades indicate high sequence similarity but not complete identity. The domains marked green are closely related to each other but only distantly to factor H and mediate dimerization of FHR-1, FHR-2 and FHR-5. Functional sites in factor H are shown by horizontal lines. FHL-1 is a splice variant of factor H.

II. Results

Published results are briefly summarized and are indicated with the respective publication (with link); unpublished results are shown in more detail.

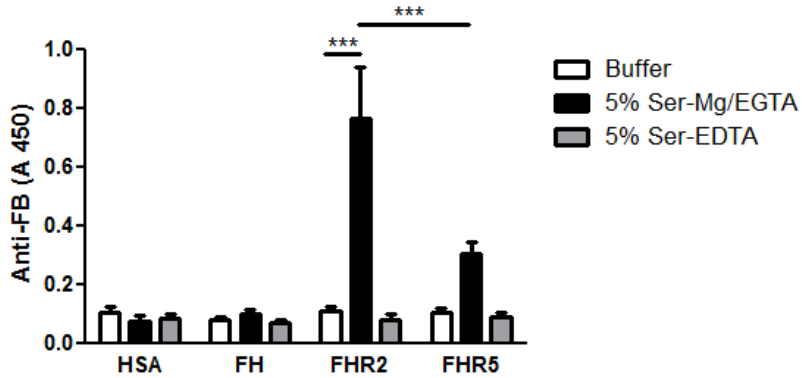
Ligands and function of FHR-2

Recent studies on some of the human FHRs strongly suggest a role for them in enhancing complement activation by competing with factor H for binding to certain ligands and surfaces. FHR-2 consists of four complement control protein domains, and was described to bind C3b, inhibit the alternative pathway C3 convertase and the terminal pathway. The aim of our study was to further characterize FHR-2 for ligand interactions and function.

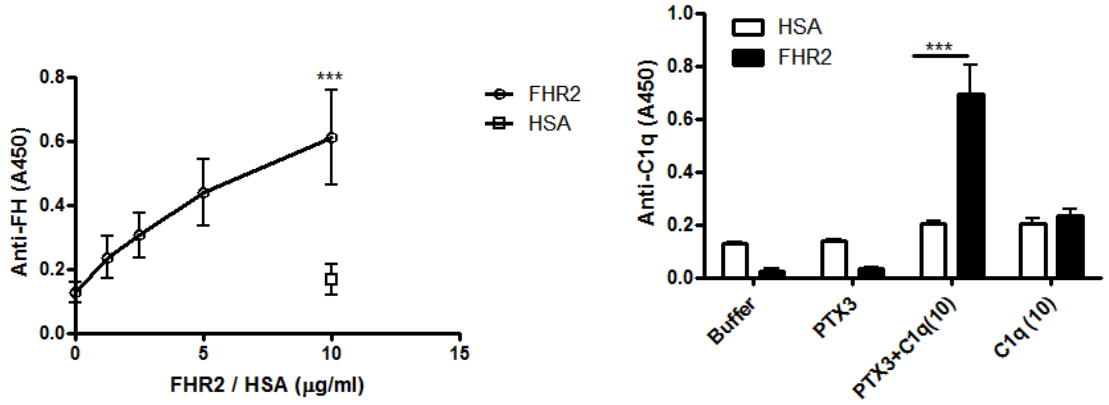
Recombinant FHR-2 was expressed in insect cells. Binding of C3 fragments, C-reactive protein (CRP) and pentraxin 3 (PTX3), assembly of the C3bBb convertase and complement activation were measured by ELISA. Hemolysis assay was performed using sheep erythrocytes; lysis was induced by adding the anti-factor H monoclonal antibody OX24.

We found that FHR-2 bound C3b, iC3b and C3d. When immobilized on a surface, FHR-2 supported the assembly of the C3bBb alternative pathway C3 convertase via its interaction with C3b. The FHR-2 bound convertase was active as demonstrated by the generation of C3a from C3 added to the system. This activity was confirmed by detecting C3 activation in human serum. FHR-2 also bound to PTX3 and both pentameric native CRP and monomeric CRP. FHR-2 enhanced alternative pathway activation on immobilized pentraxins when added to serum. In addition, FHR-2 bound PTX3 and mCRP could bind the initiator molecule C1q of the classical pathway. FHR-2 inhibited zymosan-induced complement activation, measured at the C9 level, and also slightly inhibited OX24-induced hemolysis of sheep erythrocytes.

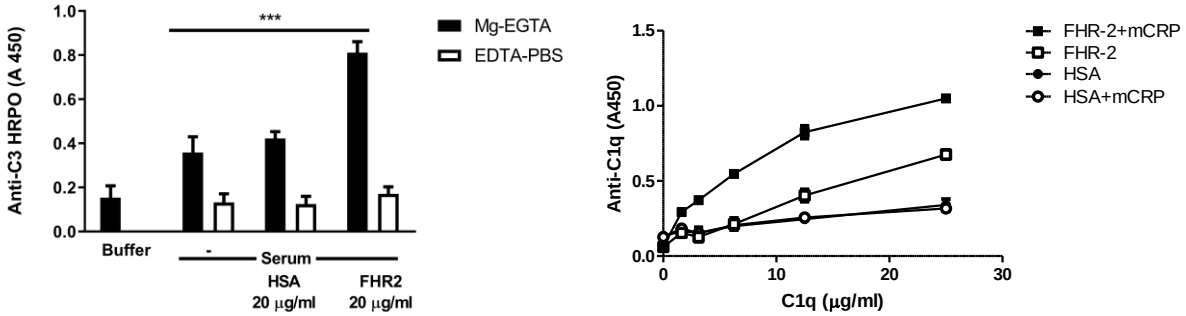
Altogether, these data identify human pentraxins as novel ligands of FHR-2 and demonstrate that, similar to human FHR-1, FHR-4 and FHR-5, FHR-2 modulates complement activity by promoting complement activation via interaction with C3b and pentraxins.



Complement activation by FHR2. *5 µg/ml recombinant FHR2, FHR5, and FH and HSA as negative controls were immobilized in microplate wells, followed by incubation with 10% normal human serum in 5 mM Mg^{2+}-EGTA buffer to allow activation of the alternative pathway only, or with 5% normal human serum in 20 mM EDTA buffer to inhibit complement activation. Deposition of FB was detected using the corresponding Ab. Data are mean absorbance values ± SD from three independent experiments. ***p<0.001, two-way ANOVA.*



Interaction of FHR2 with PTX3. *(left) 5 µg/ml recombinant human PTX3 was immobilized in TBS containing 2 mM $CaCl_2$ and 1 mM $MgCl_2$ in microplate wells. After blocking, serial dilutions of HSA and FHR2 (up to 10 µg/ml) were added and its binding was detected with polyclonal anti-FH Ab. Data are mean absorbance values ± SD from three independent experiments. ***p<0.001, two-way ANOVA. (right) C1q binds to FHR2-bound PTX3. Microplate wells were coated with 5 µg/ml recombinant human FHR2 and as control with HSA. After blocking, sequentially incubated with 5 µg/ml PTX3 and 10 µg/ml C1q. C1q binding was measured using C1q Ab. Data are mean absorbance values ± SD from three independent experiments. ***p<0.001, two-way ANOVA.*



Interaction of FHR2 with mCRP. *(left) 5 µg/ml recombinant CRP was immobilized in EDTA-PBS, followed by incubation with 20 µg/ml FHR2 or HSA in 5% normal human serum in 5 mM Mg^{2+}-EGTA buffer to allow only for alternative pathway activation or with 5% normal human serum in 20 mM EDTA buffer to inhibit complement activation. C3 fragment deposition was detected using HRP-conjugated anti-human C3 antibody. Data are mean absorbance values ± SD from three independent experiments. ***p<0.001, two-way ANOVA. (right) FHR2 was immobilized in microplate wells and incubated with or without 5 µg/ml mCRP. After washing, serial dilutions of purified C1q in the indicated concentrations were added, and C1q binding was measured with a polyclonal anti-C1q Ab. Gelatin was used as a negative control. Data are means ± SD from three experiments.*

Conference abstract:

*Marcell Cserhalmi, Domonkos Czárán, Barbara Uzonyi, **Mihály Józsi**. FHR2 binds to pentraxins and modulates complement activation. MOLECULAR IMMUNOLOGY 114 pp. 481-481. , 1 p. (2019)*

Interaction of FHR-1 and FHR-5 with the extracellular matrix (ECM), and modulation of complement activation

Components of the extracellular matrix (ECM), when exposed to body fluids may promote local complement activation and inflammation. Pathologic complement activation at the glomerular basement membrane and at the Bruch's membrane is implicated in renal and eye diseases, respectively. Binding of soluble complement inhibitors to the ECM, including factor H (FH), is important to prevent excessive complement activation. Since the FH-related (FHR) proteins FHR1 and FHR5 are also implicated in these diseases, our aim was to study whether these FHRs can also bind to ECM components and affect local FH activity and complement activation. Both FH and the FHRs showed variable binding to ECM components. We identified laminin, fibromodulin, osteoadherin and PRELP as ligands of FHR1 and FHR5, and found that FHR1 bound to these ECM components through its C-terminal complement control protein (CCP) domains 4-5, whereas FHR5 bound via its middle region, CCPs 3-7. Aggrecan, biglycan and decorin did not bind FH, FHR1 and FHR5. FHR5 also bound to immobilized C3b, a model of surface-deposited C3b, via CCPs 3-7. By contrast, soluble C3, C3(H_2O), and the C3 fragments C3b, iC3b and C3d bound to CCPs 8-9 of FHR5. Properdin, which was previously described to bind via CCPs 1-2 to FHR5, did not bind in its physiologically occurring serum forms in our assays. FHR1 and FHR5 inhibited the binding of FH to the identified ECM proteins in a dose-dependent manner, which resulted in reduced FH cofactor activity. Moreover, both FHR1 and FHR5 enhanced alternative complement pathway activation on immobilized ECM proteins when exposed to human serum, resulting in the increased deposition of C3-fragments, factor B and C5b-9. Thus, our results identify novel ECM ligands of FH family proteins and indicate that FHR1 and FHR5 are competitive inhibitors of FH on ECM and, when bound to these ligands, they may enhance local complement activation and promote inflammation under pathological conditions.

Publication:

Papp A, Papp K, Uzonyi B, Cserhalmi M, Csincsi ÁI, Szabó Z, Bánlaki Z, Ermert D, Prohászka Z, Erdei A, Ferreira VP, Blom AM, ***Józsi M****. Complement Factor H-Related Proteins FHR1 and FHR5 Interact With Extracellular Matrix Ligands, Reduce Factor H Regulatory Activity and Enhance Complement Activation. Front Immunol. 2022 Mar 22;13:845953. doi: 10.3389/fimmu.2022.845953.*

Interaction of FHR-1 and FHR-5 with DNA and dead cells

Complement plays an essential role in the opsonophagocytic clearance of apoptotic/necrotic cells. Dysregulation of this process may lead to inflammatory and autoimmune diseases. FH, a major soluble complement inhibitor, binds to dead cells and inhibits excessive complement activation on their surface, preventing lysis and the release of intracellular material, including DNA. The factor H-related proteins (FHRs) share common ligands with FH, due to their homology with this complement regulator, but they lack the domains that mediate the complement inhibitory activity of FH. Because their roles in complement regulation is controversial and incompletely understood, we studied the interaction of FHR-1 and FHR-5

with DNA and dead cells and investigated whether they influence the regulatory role of FH and the complement activation on DNA and dead cells. FH, FHR-1 and FHR-5 bound to both plasmid DNA and human genomic DNA, where both FHR proteins inhibited FH-DNA interaction. The FH cofactor activity was inhibited by FHR-1 and FHR-5 due to the reduced binding of FH to DNA in the presence of the FHRs. Both FHRs caused increased complement activation on DNA. FHR-1 and FHR-5 bound to late apoptotic and necrotic cells, and recruited monomeric C-reactive protein and pentraxin 3, and vice versa. Interactions of the FHRs with pentraxins resulted in enhanced activation of both the classical and the alternative complement pathways on dead cells when exposed to human serum. Altogether, our results demonstrate that FHR-1 and FHR-5 are competitive inhibitors of FH on DNA; moreover, FHR-pentraxin interactions promote opsonization of dead cells.

Publication:

Kárpáti É, Papp A, Schneider AE, Hajnal D, Cserhalmi M, Csincsi ÁI, Uzonyi B, ***Józsi M****. Interaction of the Factor H Family Proteins FHR-1 and FHR-5 With DNA and Dead Cells: Implications for the Regulation of Complement Activation and Opsonization. Front Immunol. 2020;11:1297. doi: 10.3389/fimmu.2020.01297.*

FHR-5 in kidney disease

FHR-5 was originally isolated from renal immune deposits and has been linked to various kidney diseases since. Due to the homology to FH, the main complement regulator of the alternative pathway, it may also be implicated in the pathomechanism of kidney diseases where FH and alternative pathway dysregulation play a role. In collaboration with the group of Prof. Zoltán Prohászka and Dr. Dorottya Csuka at Semmelweis University, we reported the first observational study on *CFHR5* variations along with serum FHR-5 levels in immune complex-mediated membranoproliferative glomerulonephritis (IC-MPGN) and C3 glomerulopathy (C3G) patients together with the clinical, genetic, complement, and follow-up data. A total of 120 patients with a histologically proven diagnosis of IC-MPGN/C3G were enrolled in the study. FHR-5 serum levels were measured in ELISA, the *CFHR5* gene was analyzed by Sanger sequencing, and selected variants were studied as recombinant proteins in ELISA and surface plasmon resonance (SPR). Eight exonic *CFHR5* variations in 14 patients (12.6%) were observed. Serum FHR-5 levels were lower in patients compared to controls. Low serum FHR-5 concentration at presentation associated with better renal survival during the follow-up period; furthermore, it showed clear association with signs of complement overactivation and clinically meaningful clusters. Our observations raise the possibility that the FHR-5 protein plays a finetuning role in the pathogenesis of IC-MPGN/C3G.

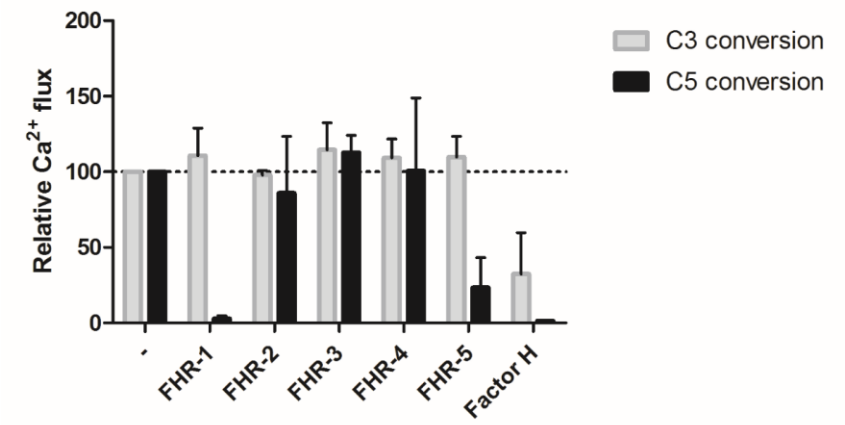
Publication:

Garam N, Cserhalmi M, Prohászka Z, Szilágyi Á, Veszeli N, Szabó E, Uzonyi B, Iliás A, Aigner C, Schmidt A, Gaggl M, Sunder-Plassmann G, Bajcsi D, Brunner J, Dumfarth A, Cejka D, Flaschberger S, Flögelova H, Haris Á, Hartmann Á, Heilos A, Mueller T, Rusai K, Arbeiter K, Hofer J, Jakab D, Sinkó M, Szigeti E, Bereczki C, Janko V, Kelen K, Reusz GS,

Szabó AJ, Klenk N, Kóbor K, Kojc N, Knechtelsdorfer M, Laganovic M, Lungu AC, Meglic A, Rus R, Kersnik Levart T, Macioniene E, Miglinas M, Pawłowska A, Stompór T, Podracka L, Rudnicki M, Mayer G, Rysava R, Reiterova J, Saraga M, Seeman T, Zieg J, Sládková E, Stajic N, Szabó T, Capitanescu A, Stancu S, Tisljar M, Galesic K, Tislér A, Vainumäe I, Windpessl M, Zaoral T, Zlatanova G, ***Józsi M****,* Csuka D.* FHR-5 Serum Levels and CFHR5 Genetic Variations in Patients With Immune Complex-Mediated Membranoproliferative Glomerulonephritis and C3-Glomerulopathy. Front Immunol. 2021 Sep 10;12:720183. doi: 10.3389/fimmu.2021.720183. *shared senior authorship*

FHRs and complement convertase enzymes

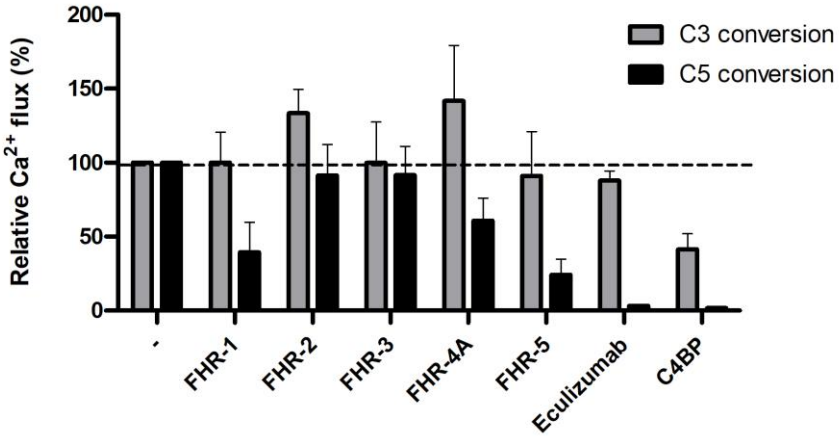
Convertase enzymes play a critical role in complement activation and produce the key mediators of complement. C3 convertases cleave C3 to generate the anaphylatoxin C3a and label target cells with C3b, which promotes phagocytosis. C5 convertases cleave C5 into the chemoattractant C5a and C5b, the initiator of the terminal pathway leading to formation of the membrane attack complex and cell lysis. The human factor H (FH) protein family includes the highly similar proteins FH, FHL-1 and five FH-related proteins. In contrast to the complement- and convertase inhibiting role of FH and FHL-1, the FHRs were described as positive modulators of complement activation through competitive inhibition of FH. Because the possible alternative pathway (AP) and classical pathway (CP) C3/C5 convertase inhibition by the FHR proteins is currently a controversial issue, our aim was to characterize all FHRs to determine their effect on C3 and C5 conversion. In collaboration with Prof. Suzan Rooijakkers (Amsterdam), we used a C3aR and C5aR U937 reporter cell systems. These model systems use CP/AP C3/C5 convertases formed on biotinylated C3b-coated streptavidin beads and quantify C3 and C5 conversion via functional analysis of released C3a/C5a. We found that FHR-1 and FHR-5, and to a lesser extent FHR-3 and FHR-4, inhibited the CP C5 convertase, while leaving C3 conversion unaffected. The AP C5 convertase was inhibited by FHR-1, FHR-2 and FHR-5; here the C3 conversion was also unaffected. In addition, we found that the most C-terminal domains of FHR-5 mediate its AP C5 convertase inhibiting effect. Altogether, these data demonstrate that FHRs can selectively inhibit C5 conversion, which may help to better understand the mechanisms of the complement related diseases.



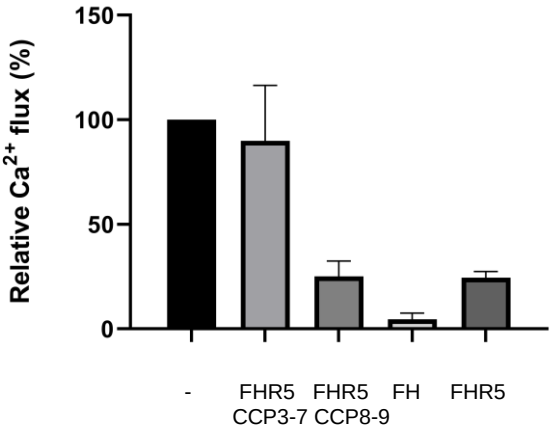
C3 and C5 conversion in the alternative pathway convertase model in the presence of FHRs. *C3b-biotin was incubated with streptavidin-coated magnetic beads, followed by*

incubation with FB. After washing, we added C5 and FD; the FHR proteins and FH were added in 100 nM concentration. Conversion of C3 and C5 were measured by calcium mobilization in U937-C3aR and U937-C5aR cells, respectively. Data represent means ± SD from three independent experiments.

At higher concentrations (500 nM), in addition to FHR-1 and FHR-5, FHR-2 reduced the amount of generated C5a in the alternative pathway C5 convertase model. To complete these analyses, we also evaluated the potential effect of the FHRs on the classical pathway convertases. In the classical pathway, FHR-1 and FHR-5 strongly inhibited C5 conversion:



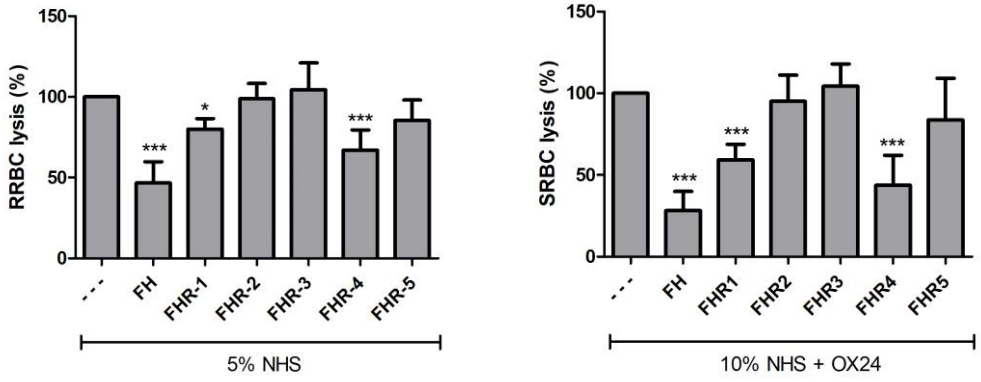
Conversion of C3 and C5 in the classical pathway convertase model in the presence of FHRs. *DNP-biotin was incubated with streptavidin-coated magnetic beads, incubated with anti-DNP IgG, followed by CP components, then C3 and C5. The FHR proteins and FH were added in 100 nM concentration. Conversion of C3 and C5 were measured by calcium mobilization in U937-C3aR and U937-C5aR cells, respectively. Data represent means ± SD from three independent experiments. The anti-C5 mAb eculizumab and the classical pathway inhibitor C4BP were used as controls.*



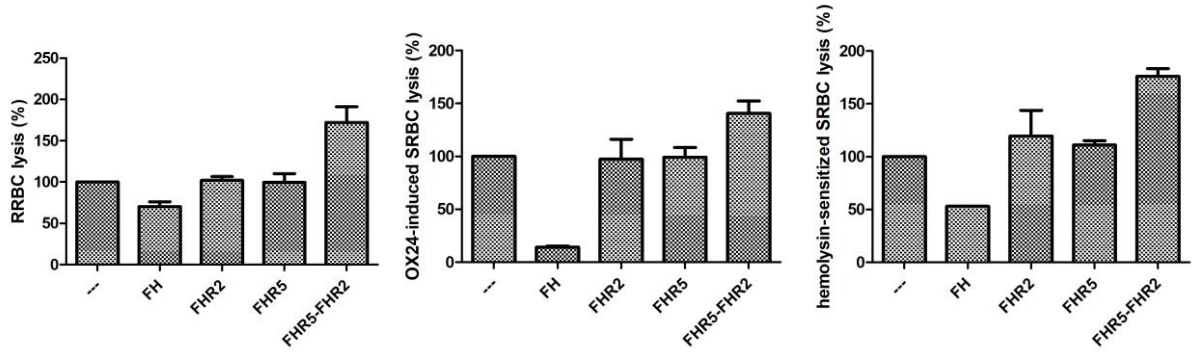
Conversion of C5 in the alternative pathway convertase model *in the presence of FHR-5 fragments comprising CCPs 3-7 or 8-9, and FH and FHR-5 as positive controls, measured by calcium mobilization in U937-C5aR cells, respectively. Conversion is shown as a percentage relative to the control without inhibitor. Data of three independent experiments, presented as mean ± SD.*

In order to test if such inhibitory activities can be measured also in serum, we performed various hemolysis assays, which effectively measure the terminal membrane attack complex (C5b-9) formation and consequent cell lysis. First, we used rabbit red blood cells (RRBC) that are “complement activators”, because they lack cell surface sialic acids and cannot bind factor H; thus complement activation on their surface can proceed unhindered. When incubated in 5% normal human serum (NHS), ca. 50% of the RRBC lysed compared with total lysis in distilled water. In these assays, 500 nM FH/FHRs were used as potential convertase modulators. FH had ~50% inhibitory effect on RRBC lysis, as expected (“positive control”). Among the FHRs, FHR1 had slight but significant inhibitory effect and FHR4 had even stronger inhibitory effect. We also used sheep erythrocytes (SRBC) that act as “complement non-activators”, since they bind FH through cell surface sialic acids. We induced suboptimal (ca. 40%) lysis of SRBC by blocking the FH regulatory domains using the mAb OX24 (binding in CCP5); in these assays, in addition to FH, 500 nM FHR-1 and FHR-4 significantly inhibited SRBC lysis, but FHR-2 and FHR-5 had no effect. In the classical pathway assay using hemolysin-sensitized SRBC none of the FHRs had inhibitory effect (not shown).

We also expressed a disease-associated mutant FHR5-FHR2 hybrid protein (Xiao X. et al., Mol. Immunol. 2016; 77:89-96.), which essentially has the CCPs 1-2 of FHR5 (mediating dimerization) fused with the whole FHR2 protein, and analyzed its effect in the above hemolysis assays. In all tests, this hybrid protein strongly enhanced complement-mediated red cell lysis in human serum, explaining its pathogenic, complement-activating effect in C3G.



Alternative pathway mediated lysis of target cells. Left panel: *RRBC lysis induced by human complement (5% NHS as complement source) without or with 500 nM FH/FHR added.* ***Right panel:*** *SRBC lysis induced by the FH function blocking mAb OX24 in 10% NHS. Lysis was measured by hemoglobin release and is shown as a percentage relative to maximal osmotic lysis caused by distilled water (MQ). Data of three independent experiments, presented as mean ± SD.*



Analysis of the C3G-associated FHR5-FHR2 hybrid protein. Left panel: *RRBC lysis induced by human complement (5% NHS as complement source) without or with 500 nM FH/FHR added.* ***Middle panel:*** *SRBC lysis induced by the FH function blocking mAb OX24 in 10% NHS without or with 500 nM FH/FHR added.* ***Right panel:*** *Hemolysin-sensitized SRBC lysis mediated by classical pathway in 1% NHS without or with 200 nM FH/FHR added. Lysis was measured by hemoglobin release and is shown as a percentage relative to maximal osmotic lysis caused by distilled water (MQ). Data of three independent experiments, presented as mean ± SD.*

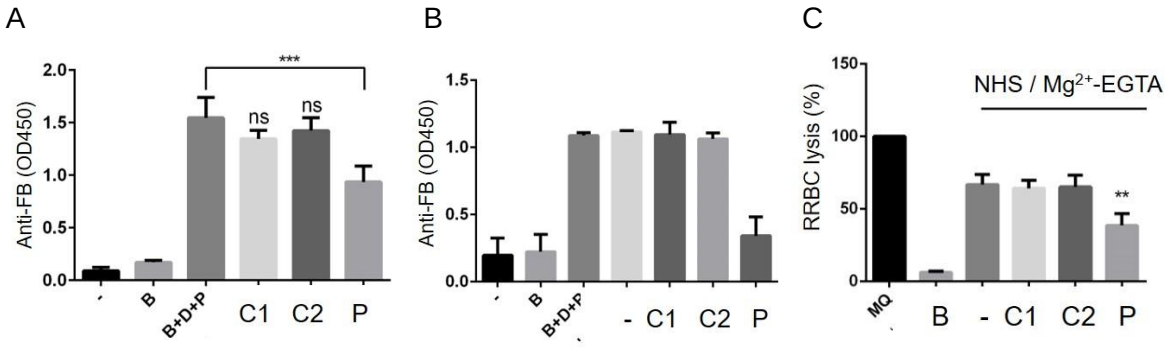
These results demonstrate that while C5 convertase inhibition can be demonstrated for some of the FHRs (particularly FHR-1 and FHR-5) in purified convertase models, in more complex systems such as cell surfaces and serum, the effects are less pronounced or may require higher FHR concentrations. In hemolysis assays using serum, i.e. when FHRs are in a complex fluid with other serum factors, FHR-1 and FHR-4 had inhibitory effect, whereas the slight effect of FHR5 was not significant statistically. The disease-associated mutant FHR5::FHR2 protein likely has higher competitive activity (against FH), thus causing enhanced hemolysis.

Conference abstract:

Marcell Cserhalmi, Alexandra Papp, Anna E. van Beek, Eva Struijf, Maartje Ruyken, Ilse Jongerius, Taco W. Kuijpers, Suzan H.M. Rooijakkers, ***Mihály Józsi****. Regulation of complement C3 and C5 convertases by factor H family proteins. MOLECULAR IMMUNOLOGY 114 pp. 481-482. , 2 p. (2019)*

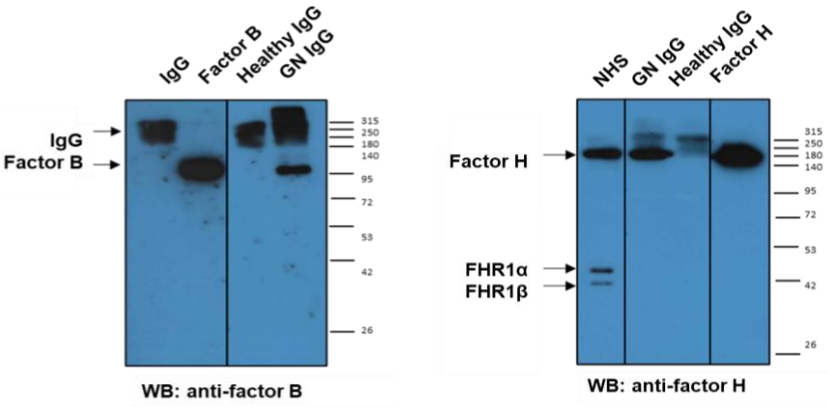
Autoantibodies to FH in IC-MPGN

In collaboration with the group of Prof. Zoltán Prohászka and Dr. Dorottya Csuka (Semmelweis University), we analyzed an interesting case of immune-complex mediated membranoproliferative glomerulonephritis (IC-MPGN), where in the patient's serum autoantibodies against both FB and FH could be detected. In functional assays, the net effect of the autoantibodies (when applied as purified IgG fraction) was inhibition of solid phase C3 convertase assembly, acceleration of convertase decay and inhibition of rabbit red blood cell (RRBC) lysis.



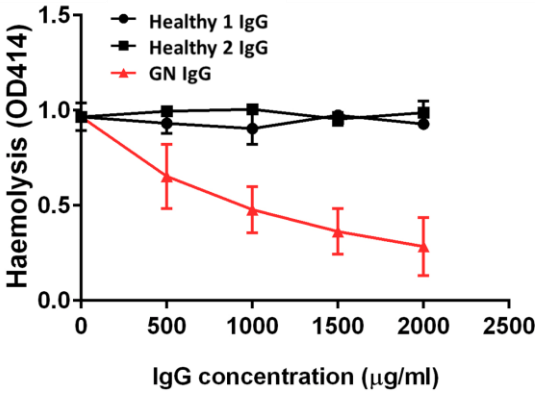
Effect of purified autoantibodies on the C3bBb convertase and RRBC lysis. (A) *The C3bBb convertase was built up in microplate wells in the presence of purified IgG of healthy controls (C1, C2) and patient (P).* ***(B)*** *Decay of the convertase in the presence of the IgG fractions. Convertases were detected with anti-FB.* ***(C)*** *Lysis of RRBC in NHS in the presence of the purified IgGs; B, buffer only.*

It was not possible to separate these autoantibodies from each other, e.g. on antigen columns, due to the limited amount of serum; however, complexes were removed to eliminate the potential effect of complexed factor H in the complement assays, which could influence the assembly and decay of the convertase.

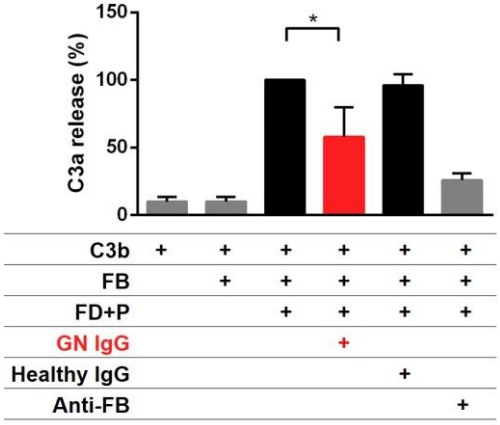


Detection of in vivo formed immune complexes. *To investigate the presence of* in vivo *formed autoantibody-FH and autoantibody-FB complexes in the plasma, Western blots were performed. 10 µg IgG isolated on Protein G column was separated on 10% SDS-PAGE and after blotting, the presence of FB or FH was detected with polyclonal anti-FB and anti-FH antibodies. Representative blots of three experiments are shown. These complexes were removed from the sample before further investigation of the autoantibodies.*

After removal of the FH/FB-autoantibody complexes, no effect on the convertase assembly and decays was observed. However, the patient’s IgG dose-dependently inhibited the alternative pathway mediated hemolysis of rabbit red blood cells. This is likely due to the inhibition of the C3 convertase activity, as shown by the inhibition of C3 conversion *in vitro*.



Autoantibodies inhibited the hemolysis of rabbit red blood cells. *To investigate the effect of patient's autoantibodies on the alternative pathway, RRBCs were mixed with pooled normal human serum and serial dilutions of IgG purified from the patient or healthy controls. Samples were incubated at 37°C for 30 min and, after centrifugation, optical density of the supernatants was measured at 414 nm. Data are means ±SD of two independent experiments.*



Effects of the autoantibodies on the solid phase C3 convertase. *Solid phase C3 convertase of the alternative pathway was built up in microplate wells. C3b was immobilized and after washing FB, factor D (FD) and properdin (P) were added for 1 hour at 37°C in Ni^{2+}-containing buffer. Convertase formation was detected with polyclonal anti-FB antibody. To investigate the effect of the autoantibodies on the activity of the formed convertase, C3 cleavage was measured by detecting C3a from the supernatant with C3a EIA (Quidel). Patient's IgG inhibited the activity of the convertase. Data are mean ±SD of three independent experiments. * $p<0.05$*

These data demonstrate the difficulty of analyzing and interpreting results of autoantibody positive patients, e.g. to conclude on the pathogenic role of the antibodies and what 'autoantibody positivity' really means in the context of the disease. However, the results are valuable because such convertase blocking antibodies may be useful as inhibitors of complement activation. While cloning of such autoantibodies may not be easy, identifying similar antibodies such as monoclonal antibodies with comparable effects could be a way to go.

Autoantibodies in rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disorder affecting the joints. Many patients carry anti-citrullinated protein autoantibodies (ACPA). Overactivation of the complement system seems to be part of the pathogenesis of RA, and autoantibodies against the pathway initiators C1q and MBL, and the regulator of the complement alternative pathway, factor H (FH), were previously reported. Our aim was to analyze the presence and role of autoantibodies against complement proteins in a Hungarian RA cohort.

To this end, serum samples of 97 ACPA-positive RA patients and 117 healthy controls were analyzed for autoantibodies against FH, factor B (FB), C3b, C3-convertase (C3bBbP), C1q, MBL and factor I. In this cohort, we did not detect any patient with FH autoantibodies but detected C1q autoantibodies in four patients, MBL autoantibodies in two patients and FB autoantibodies in five patients. Since the latter autoantibodies were previously reported in patients with kidney diseases but not in RA, we set out to further characterize such FB autoantibodies. The isotypes of the analyzed autoantibodies were IgG2, IgG3, IgGκ, IgGλ and their binding site was localized in the Bb part of FB. We detected in vivo formed FB–autoanti-FB complexes by Western blot. The effect of the autoantibodies on the formation, activity and FH-mediated decay of the C3 convertase in solid phase convertase assays was determined. In order to investigate the effect of the autoantibodies on complement functions, hemolysis assays and fluid phase complement activation assays were performed. The autoantibodies partially inhibited the complement-mediated hemolysis of rabbit red blood cells, inhibited the activity of the solid phase C3-convertase and C3 and C5b-9 deposition on complement activating surfaces.

In summary, in ACPA-positive RA patients we identified FB autoantibodies. The characterized FB autoantibodies did not enhance complement activation, rather, they had inhibitory effect on complement. These results support the involvement of the complement system in the pathomechanism of RA and raise the possibility that protective autoantibodies may be generated in some patients against the alternative pathway C3 convertase. However, further analyses are needed to assess the exact role of such autoantibodies.

Publication accepted in Frontiers in Immunology:

Alexandra T. Matola, Angéla Fülöp, Bernadette Rojkovich, György Nagy, Gabriella Sármay, ***Mihály Józsi****,* Barbara Uzonyi.* Autoantibodies against complement factor B in rheumatoid arthritis. * shared last authorship*

Modulation of classical complement pathway activation by FHRs

The main soluble regulatory proteins of the classical/lectin pathways, C4b-binding protein (C4BP), and that of the alternative pathway (AP), factor H (FH), do not only act in solution on C4b or C3b but also interact with ligands such as C-reactive protein (CRP) and extracellular matrix (ECM) proteins, and regulate complement on cellular and non-cellular surfaces. Next to FH, in humans there are five structurally related proteins, the FH-related (FHR) proteins, whose function is poorly understood. Accumulating evidence support a role for FHRs as competitors of FH for binding to certain ligands and enhancers of AP activation. Due to their homology to FH, FHRs were mainly studied for their effect on the AP. Since the

FHRs have overlapping ligand binding profile with both FH and C4BP, we studied the potential effect of FHR-1, FHR-2 and FHR-5 on the binding and regulatory activity of C4BP and on classical pathway (CP) activation.

Both FHR-1 and FHR-5 inhibited C4BP binding to immobilized CRP and the ECM protein osteoadherin in ELISA. This translated to reduced surface cofactor activity of C4BP on CRP in the presence of the FHRs as shown by Western blot analysis of C4b cleavage. Unexpectedly, FHR-5 rather inhibited CP activation in a dose-dependent manner, as shown by decreased C3- and C4-fragment deposition in ELISA on CRP and osteoadherin exposed to 1% human serum, whereas FHR-1 did not influence CP activation in this assay. The effect of FHR-5 was due to inhibition of C1q binding to both CRP and osteoadherin. Despite binding to immobilized CRP and osteoadherin, FHR-2 did not modulate the binding of C4BP and C1q, and did not affect CP activation.

In conclusion, our results provide further insight into the functions of the FHR proteins and reveal a novel role of FHR-5 in modulating the activity of the complement classical pathway.

Conference abstract:

Hammad Hani Hashim, Alexandra Papp, Ádám I. Csincsi, Barbara Uzonyi, Anna M. Blom, ***Mihály Józsi****. Modulation of the classical complement pathway by factor H-related proteins. Molecular Immunology 2022;150:204-205.*

Inactivation of factor H family proteins by fungal proteases

Because complement evasion is a major strategy of microbes to avoid elimination by the innate immune system, we collaborated with Prof. Attila Gácser's group (University of Szeged) to study the potential of fungal proteases to inactivate human complement components. We found that *Candida parapsilosis* Sapp1p and Sapp2p proteases, in addition to the opsonins C3b and C4b, could cleave FH and FHR-5, but not FHL-1 and FHR-1.

Candida parapsilosis is an emerging non-albicans *Candida* species that largely affects low-birth-weight infants and immunocompromised patients. Fungal pathogenesis is promoted by the dynamic expression of diverse virulence factors, with secreted proteolytic enzymes being linked to the establishment and progression of disease. Although secreted aspartyl proteases (Sap) are critical for *Candida albicans* pathogenicity, their role in *C. parapsilosis* is poorly elucidated. In this study, we aimed to examine the contribution of *C. parapsilosis* SAPP genes SAPP1, SAPP2, and SAPP3 to the virulence of the species. Our results indicate that SAPP1 and SAPP2, but not SAPP3, influence adhesion, host cell damage, phagosome-lysosome maturation, phagocytosis, killing capacity, and cytokine secretion by human peripheral blood-derived macrophages. Purified Sapp1p and Sapp2p were also shown to efficiently cleave host complement component 3b (C3b) and C4b proteins and complement regulator factor H. Additionally, Sapp2p was able to cleave factor H-related protein 5 (FHR-5). Altogether, these data demonstrate the diverse, significant contributions that SAPP1 and SAPP2 make to the establishment and progression of disease by *C. parapsilosis* through enabling the attachment of the yeast cells to mammalian cells and modulating macrophage

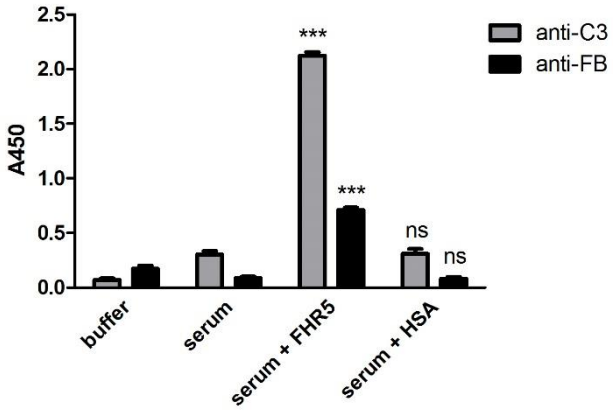
biology and disruption of the complement cascade.

Publication:

*Singh DK, Németh T, Papp A, Tóth R, Lukácsi S, Heidingsfeld O, Dostal J, Vágvölgyi C, Bajtay Z, **Józsi M**, Gácser A. Functional Characterization of Secreted Aspartyl Proteases in Candida parapsilosis. mSphere. 2019;4(4):e00484-19. doi: 10.1128/mSphere.00484-19.*

Interaction of FHRs with microbial FH-binding proteins

Pathogenic microorganisms have developed several means to evade the host immune system. A commonly used microbial strategy is the binding of complement inhibitors, such FH, from body fluids to escape from complement-mediated destruction. In addition to FH, binding of FHRs to microbes has also been described. FHR1 and FHR5 belong to the human FH protein family and are structurally related to FH. Recent data suggest that these FHRs, in contrast to FH, are positive regulators of complement activation. FHR1 contains five and FHR5 nine complement control protein domains (CCPs). FH binds to various ligands and surfaces mainly via CCP6-7 and CCP19-20. The latter domains show strong sequence similarity to homologous domains in FHR1/FHR5. Our aim was to study whether FHR1 and FHR5 bind to selected bacterial proteins and influence the activity of FH and complement activation. The microbial proteins leptospiral LcpA, Lig-A and Lig-B, borrelial OspE and streptococcal GBS-enolase were recombinantly expressed. Binding studies, competition assays, cofactor- and complement activation assays were performed by ELISA and Western blotting. FHR1 and FHR5 bound to these bacterial receptors both from serum and as recombinant proteins. FHR1 and FHR5, bound via their C-termini, dose-dependently competed with FH on OspE, caused reduced FH cofactor activity, and also enhanced alternative pathway activation and C3 deposition on OspE when exposed to serum. Similarly, FHR5 bound via CCP3-7 to GBS-enolase and the tested leptospiral proteins, competed with FH and increased alternative pathway activation in human serum, measured by enhanced deposition of C3-fragments and Bb in wells coated with bacterial proteins. Thus, by competing with FH on microbial proteins, FHR1 and FHR5 reduce the complement inhibitory activity of FH and may also directly promote alternative pathway activation. Altogether, our results suggest that FHR1 and FHR5 may enhance the opsonization of microbes.



FHR5 enhances alternative pathway activation on OspE of *Borrelia burgdorferi*. 5 µg/ml OspE was immobilized and incubated with 5% serum in the presence or absence of 10 µg/ml recombinant FHR5, in buffer containing Mg^{2+}/EGTA. Deposition of C3 fragments and FB were detected. Data are means ± SD from three independent experiments. ***p <0.0001, one way ANOVA.

Conference abstract:

Papp, Alexandra; Brandus, Bianca; Cserhalmi, Marcell; Uzonyi, Barbara; Ermert, David; Barbosa, Angela Silva; Blom, Anna M.; Isaac, Lourdes; Meri, Seppo; ***Jozsi, Mihaly.*** *FHR1 and FHR5 compete with factor H for the binding to bacterial proteins and enhance complement activation. MOLECULAR IMMUNOLOGY 102 pp. 196-196. , 1 p. (2018)*

Non-canonical / cellular functions of FH family proteins

Besides being a key effector arm of innate immunity, a plethora of non-canonical functions of complement has recently been emerging. FH, the main regulator of the alternative pathway of complement activation, has been reported to bind to various immune cells and regulate their functions, beyond its role in modulating complement activation. In this study we investigated the effect of FH, its alternative splice product FH-like protein 1 (FHL-1), the FH-related proteins FHR-1 and FHR-5, and the recently developed artificial complement inhibitor mini-FH, on two key innate immune cells, monocytes and neutrophilic granulocytes. We found that, similar to FH, the other factor H family proteins FHL-1, FHR-1 and FHR-5, as well as the recombinant mini-FH, are able to bind to both monocytes and neutrophils. As a functional outcome, immobilized FH and FHR-1 inhibited PMA-induced NET formation, but increased the adherence and IL-8 production of neutrophils. FHL-1 increased only the adherence of the cells, while FHR-5 was ineffective in altering these functions. The adherence of monocytes was increased on FH, recombinant mini-FH and FHL-1 covered surfaces and, except for FHL-1, the same molecules also enhanced secretion of the inflammatory cytokines IL-1β and TNFα. When monocytes were stimulated with LPS in the presence of immobilized FH family proteins, FH, FHL-1 and mini-FH enhanced whereas FHR-1 and FHR-5 decreased the secretion of TNFα; FHL-1 and mini-FH also enhanced IL-10 release compared to the effect of LPS alone. Our results reveal heterogeneous effects of FH and FH family members on monocytes and neutrophils, altering key features involved in pathogen killing, and also demonstrate that FH-based complement inhibitors, such as mini-FH, may have effects beyond their function of inhibiting complement activation. Thus, our data provide new insight into the non-canonical functions of FH, FHL-1, FHR-1 and FHR-5 that might be exploited during protection against infections and in vaccine development.

Publication:

Kárpáti É, Kremlitzka M, Sándor N, Hajnal D, Schneider AE, ***Józsi M****. Complement Factor H Family Proteins Modulate Monocyte and Neutrophil Granulocyte Functions. Front Immunol. 2021;12:660852. doi: 10.3389/fimmu.2021.660852.*

Review articles:

In addition to the above research results, during the project period we covered various aspects of the topic in review articles as listed below.

Matola AT, ***Józsi M****, Uzonyi B. Overview on the role of complement-specific autoantibodies in diseases. Mol Immunol. 2022 Nov;151:52-60. doi: 10.1016/j.molimm.2022.08.011.*

*Cserhalmi M, Papp A, Brandus B, Uzonyi B, **Józsi M**. Regulation of regulators: Role of the complement factor H-related proteins. Semin Immunol. 2019 Oct;45:101341. doi: 10.1016/j.smim.2019.101341.*

*Smith RJH, Appel GB, Blom AM, Cook HT, D'Agati VD, Fakhouri F, Fremeaux-Bacchi V, **Józsi M**, Kavanagh D, Lambris JD, Noris M, Pickering MC, Remuzzi G, de Córdoba SR, Sethi S, Van der Vlag J, Zipfel PF, Nester CM. C3 glomerulopathy - understanding a rare complement-driven renal disease. Nat Rev Nephrol. 2019 Mar;15(3):129-143. doi: 10.1038/s41581-018-0107-2.*

*Sánchez-Corral P, Pouw RB, López-Trascasa M, **Józsi M**. Self-Damage Caused by Dysregulation of the Complement Alternative Pathway: Relevance of the Factor H Protein Family. Front Immunol. 2018 Jul 12;9:1607. doi: 10.3389/fimmu.2018.01607.*

Under review:

*Noémi Sándor, Andrea E. Schneider, Alexandra T. Matola, Veronika H. Barbai, Dániel Bencze, Hammad Hani Hashim, Alexandra Papp, Dorottya Kövesdi, Barbara Uzonyi, **Mihály Józsi.** The human Factor H protein family – an update*

Editorial:

***Mihály Józsi**, Paul Nigel Barlow and Seppo Meri. Editorial: Function and Dysfunction of Complement Factor H. Front Immunol. 2022 Jan 13;12: 831044. doi: 10.3389/fimmu.2021.831044*

Four MSc theses were written:

(1) Domonkos Czárán, on the characterization of FHR-2 ligands and function (2018).

(2) Alexandra Matola, on the characterization of FHR-3 ligands and function (2018).

(3) Bianca Brandus, regarding the competition between FHRs and factor H for bacterial ligands; she also won 1st prize with this work at the national students' competition conference (OTDK) in 2019.

(4) Hammad Hani Hashim, regarding the classical pathway modulatory effect of FHR-5; he also won 2nd prize at the OTDK in 2021.

Part of the following PhD theses were prepared from this project:

(1) Marcell Cserhalmi, Investigation of the complement inhibitory molecule factor H and the pathological role of the FHR molecules, 2019 (Cserhalmi Marcell védése (doktori.hu)).

(2) Éva Kárpáti, Interactions of the factor H molecular family with DNA and cells: functional consequences of interactions, 2022 (Kárpáti Éva védése (doktori.hu)).

(3) Alexandra Papp, The role of the human factor H-related proteins FHR1 and FHR5 in the complement system, 2022 (Papp Alexandra Éva védése (doktori.hu)).

The results have been presented at several national and international conferences.