Crosstalk between the three complement activation pathways: The individual roles of the serine proteases

Final report

Introduction

The complement system is the most important molecular effector component of the innate immune system. It consists of about 50 protein components that can be found in the bloodstream and on the surface of cells (e.g. leukocytes, endothelial cells). The complement system is a proteolytic cascade system in which serine protease enzymes activates each-other in a strictly ordered manner. The complement system forms one of the first defense lines against the invading pathogens (e.g. bacteria, viruses) and damaged or altered host cells (e.g. apoptotic cells, cancer cells). An intact complement system is indispensable for killing the pathogen microorganisms and for maintaining the inner homeostasis of the body. Because of the cascade-like activation manner, the complement system has an enormous cell-damaging potential, which can harm self-tissues if the regulation of the system is not appropriate. Uncontrolled overactivation of the complement system can contribute to the developing and/or aggravation of serious disease conditions, such as ischemia-reperfusion injury (myocardial infraction, stroke), age-related macular degeneration, and neurodegenerative diseases (1). In order to be able to effectively treat the complement-related diseases, we need to know the exact activation mechanism of the complement system and to identify the relevant drug target molecules.

The complement system can be activated through three activation pathways: the classical, the lectin and the alternative pathway. These initiation pathways have been considered as independent linear cascades. In reality the complement system, like the other blood-borne proteolytic cascades, should rather be depicted as a hub-like network that is connected in many ways to other systems. During the OTKA project we have studied the role of the individual serine proteases in the complement activation. We have made important discoveries regarding the activation mechanism, and we have revealed hitherto hidden connections between the different activation routes.

Results

Connections between the lectin and the alternative pathway

In a previous OTKA project we showed that MASP-3, a protease considered as a member of the lectin pathway, is responsible for the activation of pro-factor D (pro-FD), the initiation protease of the alternative pathway. In the course of this project, we studied the role of MASP-1 in the alternative pathway. MASP-1 has central role in the activation of the lectin pathway. MASP-1 is the exclusive activator of MASP-2 during lectin pathway activation. Inhibition of the proteolytic activity of MASP-1 completely prevents lectin pathway activation. Recently we have discovered that inhibition of MASP-1 abolishes alternative pathway activation on certain activation surfaces. We followed the C3b deposition on different activation surfaces, using 6-times diluted normal human serum in Mg^{2+}-EGTA buffer. Under these conditions, inhibition of MASP-1 prevents C3b deposition on surfaces

covered by bacterial LPS, while it has no effect on surfaces covered by zymosan. In these experiments we used our MASP-1 specific inhibitor SGMI-1. In order to confirm these results, we also used other types of inhibitors, as well. C1-inhibitor (the physiological inhibitor of MASP-1), MASP-1 specific antibody, the N-terminal fragment of MASP-1 elicited the same phenomenon. Finally, we used MASP-1 depleted serum, and we have demonstrated beyond doubt, that MASP-1 is required for the C3b deposition on LPS-surfaces. LPS is a major component of the outer membrane of the Gram-negative bacteria. We hypothesize that in the presence of Ca^{2+}, where MBL and other pattern recognition molecules bind to their targets, the alternative pathway-promoting activity of PRM–MASP-1 complexes is even higher and may contribute significantly to the defense against Gram-negative bacteria. To check this hypothesis, we used C2-depleted serum and E. coli bacteria, and we followed the C3b deposition on the surface of the bacteria by flow cytometry. In the C2-depleted serum, only the alternative pathway C3 convertase complex (C3bBb) can be formed. Under these circumstances we could detect C3b deposition on the surface of the E. coli bacteria, which was abolished in the presence of the MASP-1 specific inhibitor. Regarding the mechanism, we checked the ability of MASP-1 to cleave the components of the alternative pathway. We found that MASP-1 can cleave C3, pro-FD and C3b-bound factor B (FB) only in very low efficiency. Taken together, it seems unlikely that MASP-1 exerts its proteolytic effect directly on the known components of the alternative pathway. It is much more likely that MASP-1 acts indirectly by influencing the regulation of the alternative pathway. These results were published in the “Cutting edge” section of “The Journal of Immunology” (2).

The mechanism of alternative pathway activation

Previously, we showed that MASP-3 is the exclusive activator of pro-FD in normal human serum. This is a very important connection between the lectin and the alternative pathway. In the “resting” human blood MASP-3 continuously cleaves and activates pro-FD, even before any activation signal appears. This mechanism ensures that FD circulates predominantly in the cleaved form, and when C3b appears, it is ready to cleave C3b-bound FB in the proconvertase complex. Only activated MASP-3 can cleave pro-FD. The zymogen form of MASP-3 has no proteolytic activity, and it cannot autoactivate. The main question of our research was: What is the activator of MASP-3 in the circulation? First, we checked the activation status of MASP-3 in the normal human serum. We found that in the resting blood, where no complement activation takes place, MASP-3 is present mainly (about 80%) in the activated form (3). It is interesting that MASP-1 and MASP-2 that have autoactivating ability are present in zymogen form, while MASP-3 that cannot autoactivate is present in cleaved form. Since MASP-1, MASP-2 and MASP-3 bind to the same pattern recognition molecules, it seemed logical to assume that MASP-3 is activated by MASP-1 or MASP-2. In in vitro experiments both MASP-1 and MASP-2 cleaved MASP-3 efficiently. However, when we followed the conversion of MASP-3 in human plasma the MASP-1- and MASP-2-specific inhibitors did not prevent the cleavage. In EDTA plasma the conversion of MASP-3 does not take place. It suggests that the enzyme responsible for the cleavage is Ca^{2+} dependent. We also found that the conversion can be blocked by the general proprotein convertase inhibitor dec-RVKR-cmk. Proprotein convertases are Ca^{2+} dependent enzymes that catalyzes

the maturation of the secreted proteins (hormones, receptors, etc.) along the secretory pathway or on the surface of the cells. They typically cleave after multibasic sequences. It was quite unexpected that a proprotein convertase cleaves zymogen MASP-3 in the blood. There are two proprotein convertases that have secreted form and consequently can potentially be present in the blood: PCSK6 (aka PACE4) and PC5A. We expressed these proteases in recombinant form and checked whether they are able to activate MASP-3. Both recombinant PCSK6 and PC5A efficiently activated zymogen MASP-3. We managed to detect PCSK6 in human serum in 50-150 ng/ml concentration. Because PC5A cannot be detected in the serum, we presume that PCSK6 is the only activator of MASP-3 in the circulation. We further study this question using sera obtained from PCSK6 KO mice.

This surprising discovery highlights that the proteolytic systems are interconnected in the body even to a greater extent than it was previously thought. Proprotein convertases are new enzymes in respect of innate immunity. It was already known that there are cross-talks between the blood coagulation, fibrinolytic and complement systems. The finding that a proprotein convertase is involved in the complement activation opens up new perspectives in studying the mechanism of complement activation and also in identifying new drug targets for treating complement-related diseases.

Summing up our results we can say that a proprotein convertase is the highest-level activator of the alternative pathway. In the pre-initiation phase of the alternative pathway PCSK6 activates MASP-3, which in turn cleaves pro-FD. In this way FD is present in cleaved activated form in the blood. In the initiation and amplification phases FD cleaves C3b-bound FB building up a positive feed-back loop. In this way the alternative pathway is responsible for at least the 80% of the net complement activation even if it was initiated by the classical or the lectin pathway. These results were published in the Journal of Immunology and the paper was recommended as a “Top read” by the editors (4).

Developing inhibitors against factor D

FD is a key enzyme in the alternative pathway. It cleaves FB in the proconvertase complex (C3bB). FD is synthetized in the adipose tissue as a zymogen and is cleaved by MASP-3 in the circulation. FD is a challenging target for inhibitor development since it has only very weak activity against synthetic substrates. Although MASP-3 cleaves the activation peptide in pro-FD, cleaved FD has a distorted conformation that renders it rather inactive. FD adopts its fully active conformation through contacting C3bB by a substrate induced mechanism. In the first round we used a Kunitz-type inhibitory domain from the tissue factor pathway inhibitor (TFPI) for the in vitro evolution process via phage display. We randomized the P3, P1’-P3’, and P5’ position in the inhibitor loop. At the P1 position, only Lys and Arg were allowed. After the selection we got two consensus sequences (P3-P5’) are ACRVAMTL and ACRVAMTP, termed variants L20 and P20, respectively. These inhibitors were specific since they did not bind to other complement proteases (e.g. MASP-1, MASP-2, MASP-3, C1r, C1s). These inhibitors were recombinantly expressed and purified to homogeneity. We tested the inhibitory capacity of the variants on FD using small synthetic substrate (Z-Lys-SBzl). These inhibitors proved to be rather weak FD inhibitors; their equilibrium inhibitory constant values being in the micromolar range. In another experiment we checked the inhibitory

capacity of ecotin on the enzymatic activity of FD. Ecotin is a serine protease inhibitor isolated from E. coli. Ecotin proved to be a weak inhibitor of FD. The wild type ecotin has methionine at the P1 position, which is suboptimal for FD; since FD as a trypsin-like protease cleaves after basic amino acids (Lys, Arg). We mutated the P1 Met of ecotin to Arg (MetP1Arg) and tested it in the FD- synthetic substrate reaction. To our great satisfaction, it turned out to be a nanomolar inhibitor of fD. MetP1Arg ecotin is a strong enough fD inhibitor to serve as a template for producing crystals of fD in complex with this protein-based, substrate-like, high-affinity inhibitor. To further increase the specificity of the ecotin-based inhibitors towards FD, we plan to conduct in vitro selection experiments by phage display.

Ecotin is a potent inhibitor of the lectin pathway

Ecitin is a pan-specific inhibitor that inhibits a large set of serine proteases having different specificities (e.g. chymotrypsin, trypsin, elastase fXa, fXIIa, plasma kallikrein) with Ki values in the 10^{-9}-10^{-13} M range. Orthologs of ecotin are present in opportunistic pathogen microbes such as Pseudomonas aeruginosa and Yersinia pestis. The lectin pathway of the complement system is an evolutionary ancient defense mechanism against bacterial infections. We tested the effects ecotins from E. coli, P. aeruginosa and Y. pestis on the lectin pathway activity in normal human serum. We found that ecotin from all three tested species are potent lectin pathway inhibitors. We also showed that ecotin knock-out E. coli cells are significantly more susceptible to complement attack, than normal cells. These experiments proved that ecotin is an important factor for the surviving of the pathogens in the human body. Our results identify ecotin as a promising anti-microbial drug target, and offer novel therapeutic options. These results were published in the prestigious Plos Pathogens journal (5).

Dissecting the inhibitory mechanism of Ecotin towards the three human MASP enzymes

Ecotin is a homodimer serine protease inhibitor. It belongs to the diverse, polyphyletic group of reversible canonical inhibitors that interact with their target protease in a substrate-like manner through a canonical conformation binding loop. In the ecotin homodimer the monomers are held together by their C-terminal tails. The canonical loop of ecotin serves as a primary binding site, but uniquely, ecotin has a secondary binding site as well. One ecotin homodimer can simultaneously bind two proteases, both being held by one primary binding site from one monomer and a secondary binding site from the other monomer. Using protein engineering, enzyme kinetics, analytical size exclusion chromatography, X-ray crystallography and serum complement assays, we showed that the secondary binding site of ecotin is essential for its MASP-inhibiting activities and is indispensable for its lectin pathway inhibitory capacity. Relative contributions of the two protease binding sites of ecotin are MASP-specific suggesting that parallel evolution of the primary and secondary binding sites was necessary to provide potent inhibition of the three important complement protease targets. We submitted a manuscript containing these results to the Journal of Biological Chemistry and it was published on the 25th of April, 2022 (6).

New generation of MASP-2 inhibitors developed by directed protein evolution have great therapeutic potential

Uncontrolled activation of the lectin pathway can contribute to the development of serious disease conditions. It has previously proven, that MASP-2 is a potential drug target to treat ischemia-reperfusion injury (IRI) associated with myocardial infarct, stroke, and several other clinical conditions. We have previously developed the first two generations of MASP-2 inhibitors by directed evolution of two unrelated canonical serine proteinase inhibitors. These inhibitors were selective LP inhibitors, but their nonhuman origin rendered them suboptimal lead molecules for drug development. During this OTKA project we developed third-generation MASP-2 inhibitors based on a human inhibitor scaffold. We subjected the second Kunitz domain of human tissue factor pathway inhibitor 1 (TFPI1 D2) to directed evolution using phage display to yield inhibitors against human and rat MASP-2. These novel TFPI1-based MASP-2 inhibitor (TFMI-2) variants are potent and selective LP inhibitors in both human and rat serum. Directed evolution of the first Kunitz domain of TFPI1 had already yielded the potent kallikrein inhibitor, Kalbitor® (ecallantide), which is an FDA-approved drug to treat acute attacks of hereditary angioedema. Like hereditary angioedema, acute IRI is also related to the uncontrolled activation of a specific plasma serine proteinase. Therefore, TFMI-2 variants are promising lead molecules for drug development against IRI. We published these results in the Journal of Biological Chemistry (7).

We revealed the binding mechanism of our first generation SFMI type lectin pathway inhibitors

Our first generation MASP-1 and MASP-2 inhibitors were based on the smallest natural trypsin inhibitor peptide, the 14-amino acid Sunflower Trypsin Inhibitor (SFTI), which is totally inactive against the MASP enzymes. SFTI was taken through a phage-display based directed evolution resulting in the very first lectin pathway selective complement inhibitors. From these SFTI-based MASP inhibitors, the SFMIs, SFMI-1 was a strong MASP-1 and weaker MASP-2 inhibitor, while SFMI2 was a selective MASP-2 inhibitor. During this OTKA project we finally managed to crystallize and solve the structure of the MASP-1/SFMI1 complex. Together with previous X-ray structures of free MASP-1 and MASP-2, our recent structural NMR studies on the SFMIs, and our recent MD simulation on all combinations of the SFMI1/2 and MASP-1/2 complexes, we deciphered how directed evolution enabled these peptides with efficient MASP-inhibitory potency. We found that the single most important step in directed evolution was to disintegrate the original stable hairpin structure of SFTI by disrupting its wild-type hydrophobic intramolecular cluster of three residues. SFTI has a rigid structure and a lock and key binding mode to the open substrate-binding cleft of trypsin. The MASP enzymes have large gatekeeper surface loops that largely block their substrate-binding cleft. This is the major reason why SFTI does not inhibit these enzymes. By disrupting the original intramolecular interaction network of residues, directed evolution yielded SFMIs having great structural plasticity. Instead of a lock and key mechanism, these peptides bind to the MASP enzymes by an induced fit mechanism, in which the enzyme structure remains practically unchanged, while the inhibitor adopts a stable structure only in the complex. We also managed to replace the original disulfide of SFMI2 with thioether linkers, which can

potentially increase biological stabilities of these peptides. We published these results in the prestigious ACS Chemical Biology journal in the 4th of April, 2022 (8).

Domestic and international scientific collaborations

During this OTKA project we collaborated with Hungarian and foreign research groups in revealing the mechanism of complement activation and revealing the role of complement in diseases.

The complement system is evolutionary and functionally closely related to the blood coagulation and fibrinolysis. In an international collaboration with Verena Schroeder's group from the University of Bern we study the interaction between these proteolytic cascades. So far, we published one paper in PLoS One (9), one paper in Molecular Immunology (10), and one paper has recently been accepted for publication in Frontiers in Immunology.

We have a fruitful collaboration with László Cervenak's group from Semmelweis University. We study the effect of MASP-1 on endothelial cells. In the frame of this OTKA project we published one paper in Frontiers of Immunology (11).

We also studied the C1-Inhibitor/Plasma serine protease complexes in healthy humans and in hereditary angioedema patients, in collaboration with Henriette Farkas's group in Semmelweis University. We published one paper in Frontiers of Immunology (12).

In collaboration with Michael Holers' group form University of Colorado, Aurora, CO we study the role of the lectin pathway in the rheumatoid arthritis. During this OTKA project we published one paper in Frontiers of Immunology (13).

Previously we showed that heparin significantly inhibits the lectin pathway very likely through the potentiation of the serpin-protease reactions. In the frame of this OTKA project we further studied the interactions between MASP-2 and heparins and heparan sulfates in collaboration with Jacob van den Born, University of Groningen, Netherlands. We published our results in Frontiers of Immunology (14).

In a broad international collaboration formed by the leading role of Andreas Kistler at University of Zurich we contributed to revealing a major role of the lectin pathway in the pathogenic mechanism of primary membranous nephropathy. Our results demonstrated a mechanism by which aberrantly glycosylated IgG4 activates the lectin pathway and induces podocyte injury in primary membranous nephropathy. The manuscript has been published in the prestigious Journal of Clinical Investigation (15).

Steffen Thiel at University of Aarhus, Denmark is a long-term collaborator of our group. Recently, we have discovered a new physiological inhibitor of the lectin pathway: Inter-α-inhibitor heavy chain 4 (ITIH4). We showed that ITIH4 inhibits the MASPs by sterically preventing larger protein substrates from accessing their active sites. We published our results in Science Advances (16).

The lectin pathway can contribute to the elimination of different pathogens. In collaboration with John P. Dalton's group from National University of Ireland, Galway we studied how Fasciola hepatica evades the attack of the complement system. We found that the serpins

produced by the pathogen inhibit the MASPs. We published our results in Plos Pathogens (17).

List of publications

1.) Dobó J, Kocsis A, Gál P. (2018) Be on Target: Strategies of Targeting Alternative and Lectin Pathway Components in Complement-Mediated Diseases. *Front Immunol.* **9,** 1851. doi: 10.3389/fimmu.2018.01851 IF: 4.716

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3.) Oroszlán G, Dani R, Szilágyi A, Závodszky P, Thiel S, Gál P, Dobó J. (2017) Extensive Basal Level Activation of Complement Mannose-Binding Lectin-Associated Serine Protease-3: Kinetic Modeling of Lectin Pathway Activation Provides Possible Mechanism. *Front Immunol.* **8,** 1821. IF: 5.511

4.) Oroszlán G, Dani R, Végh BM, Varga D, Ács AV, Pál G, Závodszky P, Farkas H, Gál P, Dobó J. (2021) Proprotein Convertase Is the Highest-Level Activator of the Alternative Complement Pathway in the Blood. *J Immunol.* **206,** 2198-2205. IF: 4.886

5.) Nagy ZA, Szakács D, Boros E, Héja D, Vígh E, Sándor N, Józsi M, Oroszlán G, Dobó J, Gál P, Pál G. (2019) Ecotin, a microbial inhibitor of serine proteases, blocks multiple complement dependent and independent microbicidal activities of human serum. *PLoS Pathog.* **15**:e1008232. IF: 6.463

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Directed Evolution-Driven Increase of Structural Plasticity Is a Prerequisite for Binding the Complement Lectin Pathway Blocking MASP-Inhibitor Peptides.

ACS Chem Biol. 2022 Apr 4. doi: 10.1021/acschembio.2c00114. IF: 5.100

9.) Jenny L, Dobó J, Gál P, Pál G, Lam WA, Schroeder V. (2018) MASP-1 of the complement system enhances clot formation in a microvascular whole blood flow model. *PLoS One.* **13**:e0191292. IF: 2.766

10.) Jenny L, Noser D, Larsen JB, Dobó J, Gál P, Pál G, Schroeder V. (2019) MASP-1 of the complement system alters fibrinolytic behaviour of blood clots. *Mol Immunol.* **114,** 1-9. IF: 3.064

11.) Debreczeni ML, Németh Z, Kajdácsi E, Schwaner E, Makó V, Masszi A, Doleschall Z, Rigó J, Walter FR, Deli MA, Pál G, Dobó J, Gál P, Cervenak L. (2019) MASP-1 Increases Endothelial Permeability. *Front Immunol.* **10,** 991. IF: 4.716

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