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Controlling the membrane attack complex

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The membrane attack complex (MAC) is a large macromolecular immune pore that punches holes in target cells. While a potent weapon of the innate immune defense, MAC pores can also damage human cells if not properly controlled. Here we use cryoEM to understand the molecular basis for how MAC pore formation is controlled in human cells during an immune response. By solving the structure of a soluble regulated form of MAC called sMAC, we explain how blood-based chaperones scavenge and clear potentially harmful complement activation by-products. Most recently we have created a membrane model system that incorporates a synthetic GPI-anchored cellular receptor (CD59) that inhibits MAC. Using cryoEM, we show how CD59 captures and deflects pore-forming beta-hairpins of complement proteins, rerouting their membrane trajectory. Moreover, we have discovered how the membrane environment influences the role ofCD59 in complement regulation and in host-pathogen interactions. Our results open new lines of investigation into the importance of lipids in immune homeostasis that may be relevant for therapies that regulate complement.

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Synovial complement activation and imbalance after anterior cruciate ligament injury or meniscus tear as a risk factor in the development of post-traumatic osteoarthritis

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Osteoarthritis (OA) is the most common form of arthritis. Anterior cruciate ligament (ACL) injury and meniscal tear (MT) are major causal factors for developing post-traumatic osteoarthritis (PTOA), but the biological mechanism(s) underlying risk are uncertain. Our hypothesis is that the synovial complement activation would occur after ACL injury and/or MT and drive subsequent PTOA. We explored the presence of complement proteins and immune cells in discarded surgical synovial tissue (DSST) collected during arthroscopic ACL reconstructive surgery (n = 16), MT-related meniscectomy (n = 16), patients with OA (n = 3) and normal controls (n = 4). Samples were studied by Multiplexed immunohistochemistry (MIHC) to determine the presence of complement proteins, receptors, and immune cells. Examination of synovium from uninjured control tissues did not reveal the presence of complement or immune cells. However, DSST from patients undergoing ACL and MT repair demonstrated significant increases in both features. In ACL DSST, a significantly ($p < 0.05$) higher percentage of C4d+, CFH+, CFHR4+ and C5b-9+ synovial cells were present compared with MT DSST. However, no substantial differences were seen between ACL and OA DSST, demonstrating that previously observed complement activation in chronic OA can be found very early in the disease course. Increased numbers of cells expressing C3aR1 and C5aR1, and a significant increase in mast cells, were found in ACL as compared to MT synovium. Conversely, the percentage of monocytes was increased in the MT synovium. Our data demonstrate that complement is activated in the synovium following injury and is associated with marked complement activation and immune cell infiltration, with a more pronounced effect following ACL as compared to MT injury. Complement activation, associated with an increase in mast cells and macrophages after injury, is likely to contribute very early to the development of PTOA, providing opportunities for therapeutic intervention in the post-trauma period.

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Development of a C3 humanized rat as a new model for evaluating novel C3 inhibitors

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Excessive activation of complement, a crucial component of innate immunity, causes many diseases such as paroxysmal nocturnal hemoglobinuria (PNH) and age-related macular degeneration (AMD). Among the complement components, C3 is central for all complement activation pathways, thus an attractive therapeutic target. Many C3-targeted agents are under extensive development with one already approved for treating PNH and dry AMD.

However, most, if not all of these C3 inhibitors are primate C3-specific, making evaluating their treatment efficacies in vivo before a clinical trial extremely difficult and costly. We previously developed a C3 knockout (KO) rat. By supplementing purified human C3 protein into the blood of the C3 KO rats, we found that human C3 is compatible to the rat complement system. We thus developed a human C3 knockin (KI) rat by knocking in a human C3 expression construct into the rat C3 loci using CRISPR/Cas 9 technology. The resultant human C3 KI rats expressed human but not rat C3 as examined by respective ELISA, Western blot and/or RT-PCR, and possessed complement activities both in vitro and in vivo. More importantly, the complement hemolytic activity of the human C3 KI rats was inhibited by compstatin, a C3 inhibitor known to be specific only to primate C3. The successful development of human C3 KI rats provided a much-desired rodent model to evaluate novel C3 inhibitors in vivo as potential drugs.

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A nanobody-based complement inhibitor targeting complement component 2 reduces hemolysis in a complement humanized mouse model of autoimmune hemolytic anemia

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C2 is an attractive therapeutic target for many complement-mediated diseases. We developed Nab1B10, a new anti-C2 nanobody that potently and selectively inhibits both the classical and lectin pathways of complement activation. Mechanistically, Nab1B10 binds to the C2a portion of C2 and inhibits the assembly of C3 convertase C4b2a. Nab1B10 cross-reacts with monkey but not rodent C2 and inhibits classical pathway-mediated hemolysis. Using a new complement humanized mouse model of autoimmune hemolytic anemia (AIHA), we demonstrated that Nab1B10 abolished classical pathway complement activation-mediated hemolysis in vivo. We also developed C2-neutralizing bi- and tetra-valent antibodies based on Nab1B10 and found these antibodies significantly more potent than the other anti-C2 monoclonal antibody that is already in clinical trials. These data suggest that these novel C2-neutralizing nanobodies could be further developed as new therapeutics for many complement-mediated diseases, in which pathogenesis is dependent on the classical and/or lectin pathway of complement activation.

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Humanization of the mouse anti-human complement C6 monoclonal antibody as a potential therapeutic for certain complement-mediated diseases

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The assembly of tissue-damaging membrane attack complexes (MACs; C5b–9) is a major mechanism by which excessive complement activation causes diseases. We previously developed a mouse anti-human C6 monoclonal antibody (mAb) 1C9 that selectively inhibits the assembly of MACs, suggesting that it could be a potential therapeutic for treating MAC-mediated diseases. In this project, we showed that 1C9 cross-reacted with rat and guinea pig C6 and used different truncated C6 proteins to locate its binding site to the FIM1 and FIM2 domains of C6. We humanized the anti-C6 mAb by using a molecular modelling-led approach to graft the complementarity-determining regions into human variable region frameworks whilst maintaining key interactions. After biochemical and functional screening of a library of 276 humanized variants with different combinations of humanized light and heavy chains in a human IgG4, K format, the top 5 candidates were selected for further characterization. Among them, clone 3713 showed the best thermostability, lowest tendency for aggregation, and a C6 binding affinity of 340 pM. Thus, 3713 had improved binding and biophysical properties compared to a mouse-human chimeric version of the parental 1C9 mAb. This humanized 3713 mAb inhibited both human, monkey and rat complement-mediated hemolysis in vitro, and more importantly, it significantly reduced complement-mediated hemolysis in vivo in rats. These results demonstrated the successful humanization of the anti-C6 mAb 1C9 and suggest that the humanized 3713 mAb could be further developed as a new therapeutic that selectively targets MAC for certain complement-mediated pathological conditions.

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Structural evolution of a complement evasion determinant shapes Lyme borreliae host tropism

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Background: Modern infectious disease outbreaks often involve changes in host tropism, the preferential adaptation of

pathogens to specific hosts. Transmitted by ticks and maintained primarily in rodents and birds, the Lyme disease (LD)-causing bacterium Borrelia burgdorferi (Bb) is an ideal model to investigate the molecular mechanisms of host tropism. To survive in hosts and escape complement-mediated immune clearance, Bb produces the outer surface protein, CspZ, which binds to the complement inhibitor factor H (FH) to facilitate bacterial dissemination in vertebrates. Despite high sequence conservation, CspZ variants differ in human FH-binding ability. Together with the FH polymorphisms amongst vertebrate hosts, these findings suggest that minor sequence variation in this bacterial outer surface protein may confer dramatic differences in host-specific, FH-binding-mediated infectivity.

Methods: We determined the crystal structure of the CspZ-human FH complex. This complex was superimposed with our newly-obtained high-resolution structures of a CspZ variant that does not bind to FH, and the computationally modeled FH structures from mouse and quail, the rodent and bird models of LD infection. We also tested the mouse and quail FH-binding activities of those CspZ variants. Additionally, we produced CspZ variants in a cspZ-deficient Bb strains and tested the infectivity of these strains in mice and quail. Finally, we reconstructed phylogenetic relationships using 174 CspZ variants and paired the resulting phylogeny with the human, mouse, and quail FH-binding activities of those variants.

Results: Using the overlay of the CspZ and FH structures, we identified minor variation localized in the FH-binding interface yielding bird and rodent FH-specific binding activity that directly impacts infectivity. Swapping the divergent region in the FH-binding interface between rodent- and bird-associated CspZ variants alter the ability to promote rodent- and bird-specific, early-onset dissemination. phylogenetic analyses linked these loops and respective host-specific, complement-dependent phenotypes with distinct CspZ lineages, elucidating evolutionary mechanisms driving host tropism emergence.

Conclusion: Our multidisciplinary work uses CspZ and FH as a model, providing a novel molecular basis for a single, short motif that impacts the pathogen-host tropism of Lyme borreliae.

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Structure/Function Analysis of collectin-K1 and collectin-L1

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Collectins are pathogen-recognition receptors of the lectin pathway that initiate complement activation via associated MASPs. They target pathogens via C-terminal carbohydrate-recognition domains (CRD) linked via N-terminal collagen-like domains. Collectin liver-1 (CL-L1; aka collectin-10) and collectin kidney-1 (CL-K1; aka collectin-11) form heterooligomers in serum, while CL-K1 can also form homooligomers. As well as targetting pathogens, both CL-K1 and CL-L1 appear to have an as yet unknown role in development, with mutations associated with the developmental disorder, 3MC syndrome (Michels, Malpuech , Mingarelli and Carnevale). They are also known to initiate kidney damage following ischaemia when collectin-mediated complement activation occurs following binding to fucosylated structures that are upregulated on host tissues. CL-K1 interacts with high-mannose oligosaccharides and fucosylated glycans, while the targets of CL-L1 are less well characterised. In order to characterize CL-K1 and CL-L1 structure and function, structural analysis was performed using X-ray diffraction after production of recombinant forms of protein fragments in E. coli. This study will present our recent structural analysis of CL-K1 and CL-L1 with respect to oligomerisation and ligand recognition.

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Prevention of terminal pathway activation improves survival in a lethal malarial infection mouse model associated with multiple organ failure

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Background: Malaria is an important parasite-induced disease which can be fatal in humans as a consequence of the development of severe multiple organ failure (MOF) resulting in severe anemia, bleeding, encephalopathy, acute respiratory distress syndrome and renal failure. We here explored roles of the terminal pathway (TP) of the complement (C) activation system in progression of disease in a mouse model of lethal malaria caused by Plasmodium (P.) bergei infection.

Methods: To clarify roles of TP in the lethal malaria, we compared survival between C57BL/6J mice with C6 component deficiency (C6def) and wild type (WT) after infection with 10^6 P. bergei parasites. We evaluated survival, blood parameters and microscopic tissue injuries together with tissue deposition of the C activation products.

Results: After infection, C6def mice survived significantly longer than WT mice which rapidly succumbed to MOF. Tissue injuries in liver and lung were milder and C activation product deposits were less in C6def mice compared to WT mice on day 7. Blood platelet counts were markedly reduced by day 7 in WT mice but were significantly higher in C6def mice; serum IL6 levels were significantly higher in WT compared to C6def mice at day 7. In C6def mice, blood hemoglobin levels, serum albumin levels and serum cholinesterase levels were all significantly higher compared to levels in WT mice at day 7. No differences in parasite proliferation were observed between C6def and WT mice.

Conclusion: Our results show that TP activation contributes to the development of fatal MOF associated with P. bergei infection without impacting parasite proliferation. The findings suggest that inhibition of TP activation is a potential therapeutic approach to improve survival in malaria and increase the therapeutic opportunity for anti-malarial medicines.

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Differential contributions of C5b-9 and C5a/C5aR pathways to microvascular and macrovascular thrombosis in complement-mediated thrombotic microangiopathy patients

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Background: This study aimed to clarify the critical role of C5a/C5aR and C5b-9 pathways in macrovascular thrombosis (MAT) and renal microthrombosis (MIT) formations based on a complement-mediated thrombotic microangiopathy (C-TMA) cohort.

Methods: Seventy-three renal biopsy-proven C-TMA patients during 2012 to 2019 in Peking University First Hospital were collected. Amongst 73 C-TMA patients, 9 patients with pure MAT and 13 ones with pure MIT were selected for further study. Their plasma and urinary C5a and soluble C5b-9 (sC5b-9) levels were evaluated, respectively. C5a receptors, including C5aR1 and C5L2, and C5b-9 depositions in renal biopsied specimens were assessed by immumohistochemical staining. The formation of neutrophil extracellular traps (NETs) in plasma and kidney tissues were also detected. The associations of above indices and clinico-histopathological features of these C-TMA patients with MAT or MIT were further evaluated.

Results: Compared to patients with pure MAT, patients with pure MIT had lower levels of hemoglobin (P=0.008) and eGFR (P=0.049), and higher renal acute arterial scores (P=0.011). Plasma C5a and sC5b-9 levels were significantly higher in C-TMA patients with MAT than those with MIT (P=0.008, P=0.041, respectively). The mean optical density (MOD) of C5aR1 in kidney was significantly higher in MAT patients than those with MIT (P < 0.001). No significant difference was found in MOD of C5b-9 or C5L2 in kidney or urinary C5a and C5b-9 levels adjusted by urinary creatinine between the two groups. However, urinary sC5b-9 level and renal depositions of C5b-9 were both associated with renal MIT formations (P=0.009; P=0.031, respectively). Furthermore, renal citrullinated histone H3 (CitH3) and neutrophil elastase (NE) positive area ratio were both significantly higher in MAT group than those in MIT group (P=0.006; P=0.020, respectively), while plasma levels of NETs and DNase I were similar between the two groups.

Conclusion: MAT was not rare in C-TMA patients. The local C5b-9 and C5a/C5aR1 pathways might have differential contributions to MIT and MAT formations in the disease.

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Functional activity of CD35 (Complement Receptor 1; CR1) in the conversion of complement fragment iC3b to C3dg

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Regulation of the complement system is maintained in part by the presence of complement regulatory proteins, e.g. CD55/CD59, on erythrocyte surfaces. Paroxysmal Nocturnal Haemoglobinuria (PNH) is a rare haematological disorder wherein patients' erythrocytes lack these surface proteins, leaving them susceptible to complement mediated attack. Eculizumab is an anti-C5 monoclonal antibody treatment that prevents the PNH-associated intravascular haemolysis, however a subsection of those receiving treatment observe a continued low-level extravascular haemolysis (EVH), caused by phagocytosis of erythrocytes. CR1 (Complement Receptor 1) is a cofactor in the conversion of iC3b to C3dg. iC3b is the opsonin which triggers phagocytosis by engaging CR3 (Complement Receptor 3) on phagocytes. It has been hypothesised that polymorphisms in the CR1 protein, the HindIII and length polymorphisms, affect its ability to carry out the conversion, predisposing the erythrocytes of that patient subset to EVH. The aim of the study was to produce a functional assay to monitor erythrocyte CR1 conversion of iC3b to C3dg in vitro. Zymosan was used to activate complement in serum and produce iC3b. sCR1 (soluble CR1) or erythrocytes were added as a source of cofactor, and conversion of iC3b to C3dg was measured using novel ELISAs. Western blotting was carried out on CR1 proteins extracted from donor blood cells to identify the length polymorphism. An assay was successfully developed to monitor conversion of iC3b to C3dg; this was demonstrated using both soluble CR1 and erythrocytes. Unfortunately, the Western blot did not clearly demonstrate the variant of CR1 present on cells, further optimisation was required. Further study is required to investigate whether the polymorphisms present in the CR1 gene affect the efficacy with which iC3b is converted to C3dg. This has future application in PNH research and whether patients in receipt of Eculizumab treatment may possess genetic predispositions to the erythrocyte opsonisation and phagocytosis that precipitates the EVH.

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Defining the impact of factor H, factor H-related 1, and factor H-related 5 on C3b deposition on mouse mesangial cells

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The alternative pathway of complement is an integral part of the innate immune system that is constitutively active providing protection against foreign invaders in the human body. Mediators of its regulation, like Factor H, regulate amplification and prevent excessive activation, while Factor H-related 1 and Factor H-related 5 are thought to enhance the complement response. Balance between Factor H and Factor H-related proteins is therefore critical in controlling the intricate dance between clearance of pathogens and tissue inflammation and damage. As compared to controls, we have found that ratios of Factor H-related proteins to Factor H are elevated in patients with C3 glomerulopathy, an ultra-rare complement-mediated kidney disease. We therefore sought to define the impact of Factor H, Factor H-related 1, and Factor H-related 5 on mouse mesangial cells (MES13) using an in-house generated C3b deposition assay and tag-free Factor H and related proteins. We hypothesized Factor H would decrease C3b deposition on cell surfaces while Factor H-related 1 and Factor H-related 5 would have the opposite effect. Our results show that on MES13 cell surfaces: 1) the addition of Factor-H reduces or completely prevents C3b deposition; and 2) the addition of Factor H-related

5 drastically increases C3b deposition. From these results we can conclude the balance between Factor H and Factor H-related 5 may perpetuate the progression of C3 glomerulopathy. Similar work is currently ongoing with Factor H-related 1. (This work was supported in part by NIH Grant R01 DK110023 (CMN, RJS) and T32AI007485 (KL)).

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Characterization and development of CSL040, a soluble truncated fragment of complement receptor 1

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Human Complement Receptor 1 (CR1) is a potent regulator of complement both in vitro and in vivo. It functions by binding to its ligands C3b and C4b and inhibiting the classical, lectin and alternative pathways of the complement cascade via the mechanisms of decay acceleration activity and co-factor activity. We have generated and characterized CSL040, a soluble version of CR1 containing the long homologous repeat (LHR) -A, -B and -C domains. CSL040 exhibits significantly greater in vitro and in vivo inhibition of all three complement pathways compared to sCR1/TP10, the full extracellular domain of sCR1. Extensive pharmacokinetic (PK) and pharmacodynamic (PD) assessments of CSL040 in rats and cynomolgus monkeys have been performed, with a demonstrated relationship between the PK properties and asialo content of recombinant protein preparations. Our studies have uncovered striking effects on the PD parameters of CSL040, showing an extended inhibition of the alternative pathway in both rats and cynomolgus monkeys compared to the classical and lectin pathways. These non-clinical PK/PD data sets were used to simulate the PK/PD effects of CSL040 in humans at a range of doses. We have shown dose-dependent attenuation of tissue damage by CSL040 in two mouse models of disease: a warm renal ischemia-reperfusion injury model, and a model of glomerulonephritis. In addition, an in vivo toxicological assessment of CSL040 was performed at daily doses of 50, 150 and 500 mg/kg in 2-week intravenous bolus studies in both rats and cynomolgus monkeys. It was shown that CSL040 is safe and well tolerated and the NOAEL for rats and cynomolgus monkeys was determined to be 500 mg/kg. The non-clinical program demonstrated that CSL040 has a favorable safety profile and supports the clinical development of CSL040 and use in human indications where complement plays a significant role in the initiation and/or progression of tissue damage.

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A phase 2, randomized trial evaluating the safety and efficacy of Pozelimab and Cemdisiran in patients with paroxysmal nocturnal hemoglobinuria

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Background: Paroxysmal nocturnal hemoglobinuria (PNH) is an ultra-rare acquired genetic disease characterized by chronic intravascular hemolysis due to uncontrolled complement activation. Pozelimab and cemdisiran are investigational treatments that suppress terminal complement activity. The combination of pozelimab and cemdisiran is being evaluated in an ongoing phase 2 study (NCT04811716) that enrolled patients with PNH who have transitioned from pozelimab monotherapy.

Methods: Patients (n=24; all received meningococcal vaccination) were randomized (1:1) into two treatment arms; both received subcutaneous (SC) cemdisiran 200 mg every 4 weeks (Q4W) plus pozelimab 400 mg SC either Q4W (arm-1) or every 2 weeks (Q2W; arm-2). The study consists of four periods: screening (7–8 days), open-label treatment (OLTP; 28 weeks), optional open-label extension (OLEP; 52 weeks), and safety follow-up (52 weeks). Data from the completed open-label treatment period (OLTP) is presented.

Results: All patients completed the OLTP. Twenty patients (83.3%) maintained control of lactate dehydrogenase (LDH; $\leq$1.5 x upper limit of normal [ULN]) at all timepoints, with the majority maintaining values <1.0 x ULN. Most patients (75%) met the criteria for hemoglobin stabilization (did not receive a blood transfusion and had no decrease in hemoglobin $\geq$2 g/dL). Two patients had breakthrough hemolysis, associated with complement activating conditions, requiring a blood transfusion. CH50, which measured total complement hemolysis activity, remained fully suppressed in all patients at all post-baseline time-points. During the OLTP, 16 patients (66.7%; seven from arm-1 and nine from arm-2) experienced 46 treatment-emergent adverse events (TEAEs); none leading to treatment discontinuation. Three severe TEAEs occurred; one patient had anemia, another patient had two events - gastroenteritis in association with an event of breakthrough hemolysis. Three patients had one serious TEAE each (COVID-19, upper respiratory tract infection, aforementioned gastroenteritis). No serious/severe TEAEs were considered related to treatment, and all resolved. There were no meningococcal infections, thrombotic events, or deaths.

Conclusion: Pozelimab and cemdisiran was generally well tolerated in patients with PNH. Overall, 83.3% maintained adequate control of hemolysis, most maintaining normalization of LDH with 75% achieving hemoglobin stabilization during the OLTP. These findings support the ongoing development of this combination therapy.

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A phase 2, open-label study evaluating the safety and efficacy of combination pozelimab and cemdisiran therapy in patients with paroxysmal nocturnal hemoglobinuria who switch from eculizumab

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Background: Paroxysmal nocturnal hemoglobinuria (PNH) is an ultra-rare disorder, with acquired, impaired expression of complement-regulating proteins. Standard-of-care treatment includes intravenous complement C5 inhibitors. Pozelimab and cemdisiran are investigational agents administered as a subcutaneous maintenance regimen that act together to suppress terminal complement.

Methods: This single-arm, open-label, phase 2 trial (NCT04888507) consisted of: screening (up to 42 days), 32-week treatment (OLTP), optional 52-week extension, and 52-week safety follow-up periods. Adults with PNH (vaccinated for meningococcal infection) on stable eculizumab for ≥12 weeks prior to screening, transitioned to the combination over a 4-week period, and thereafter received pozelimab 400 mg and cemdisiran 200 mg subcutaneously every 4 weeks. Efficacy and safety data from the OLTP are presented.

Results: Six patients were enrolled. In the year prior to enrollment, no patient required a blood transfusion; one patient had a history of breakthrough hemolysis. Five patients completed the OLTP; one patient discontinued treatment after 29 days due to a mild, non-serious treatment-emergent adverse event (TEAE) of headache. At baseline, lactate dehydrogenase (LDH) was well controlled in all patients. During the OLTP, no patient had LDH >1.5 x the upper limit of normal (ULN), or had a breakthrough hemolysis event, including two patients previously treated with 1200 mg and 1500 mg eculizumab every 2 weeks. All but two LDH values remained normal (≤1.0 x ULN) at all timepoints evaluated. During the OLTP, no patient required a blood transfusion, and all patients maintained hemoglobin stabilization (no blood transfusion and no decrease in hemoglobin ≥2 g/dL). CH50, a measure of terminal complement activity, remained fully suppressed at 0 kIU/L. One participant with long-standing, intermittent post-menopausal bleeding experienced a serious TEAE of endometrial hyperplasia requiring hospitalization for pre-emptive hysterectomy/ovariectomy (not deemed related to study treatment by the investigator/sponsor). There were no meningococcal infections, TEAEs due to potential large drug-target-drug immune complexes, thrombotic events or TEAEs leading to death.

Conclusion: In patients with PNH transitioning from eculizumab, the combination of pozelimab and cemdisiran was generally well tolerated and provided sustained control of intravascular hemolysis without breakthrough hemolysis. Findings support the ongoing development of combination therapy.

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Patient-reported outcomes from a phase 2, randomised trial evaluating the safety and efficacy of pozelimab and cemdisiran in patients with paroxysmal nocturnal haemoglobinuria

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Background: Paroxysmal nocturnal haemoglobinuria (PNH) is an ultra-rare acquired genetic disease characterised by uncontrolled complement activation. The combination of pozelimab and cemdisiran, investigational therapeutics that suppress complement component C5, is being evaluated in an ongoing phase 2 study in patients with PNH who transitioned from pozelimab monotherapy (NCT04811716). Here, we present patient-reported outcomes for the open-label treatment period (OLTP).

Methods: Twenty-four patients were randomised (1:1) to open-label subcutaneous cemdisiran 200 mg every 4 weeks (Q4W) plus subcutaneous pozelimab 400 mg Q4W (arm 1) or every 2 weeks (arm 2). Results of the Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-Fatigue) scale (range 0-52), and the European Organization for Research and Treatment of Cancer: Quality-of-Life Questionnaire physical function and global health status (GHS)/QoL assessments (range 0-100) from the completed OLTP are reported. Higher scores indicate a better level of functioning, GHS/QoL, or less fatigue.

Results: Mean (standard deviation [SD]) pre-treatment scores (prior to pozelimab monotherapy in the previous phase 2 trial [NCT03946748]) for FACIT-Fatigue were 32.3 (15.2). For the current trial, mean (SD) baseline FACIT-Fatigue scores were 45.4 (5.6) for arm 1 and 45.6 (3.6) for arm 2 (representative of the effect of prior pozelimab monotherapy); over Weeks 2-28, mean scores were 40.3-45.2 for arm 1 and 36.5-42.9 for arm 2. For physical functioning, mean (SD) pre-treatment scores were 70.9 (22.5); baseline mean (SD) physical functioning scores were 93.3 (8.8) for arm 1 and 94.2 (9.0) for arm 2; over Weeks 2-28, mean scores were 90.0-95.0 for arm 1 and 82.0-90.9 for arm 2. For GHS/QoL, mean (SD) pre-treatment scores were 60.6 (22.4); baseline mean (SD) GHS/QoL scores were 77.8 [14.4] for arm 1 and 80.2 [20.9] for arm 2; over Weeks 2-28, mean GHS/QoL scores were 69.2-77.3 for arm 1 and 66.7-75.8 for arm 2.

Conclusion: Improvements from pre-treatment GHS/QoL, physical functioning, and fatigue scores were maintained by combination treatment through Week 28. This evidence suggests that pozelimab and cemdisiran combination therapy, particularly the Q4W regimen, improves and maintains improvements in patient fatigue, physical functioning, and QoL.

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The C5aR2 pathway as a potential novel checkpoint for B cell activation and differentiation

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Complement C5a has been shown to be critically involved in many autoimmune diseases through activation of its cognate receptors, C5aR1 and C5aR2, expressed on effector cells. While the role of C5aR1 in autoimmune disease is well understood, the function of C5aR2 remains elusive. Initially thought to be a decoy receptor that negatively regulates the functions of C5aR1, C5aR2 has recently been shown to exert both pro- and anti-inflammatory properties independent of C5aR1. Recent experiments by our group using C5aR2 reporter mice showed that B cells express C5aR2 but not C5aR1 in the steady state. We also observed an altered maturation of B cells in C5ar2−/− mice. These findings prompted us to further investigate the role of C5aR2 in B cells in the context of early autoimmunity. First, using flow cytometry to assess the role of C5aR2 for B cells under homeostatic conditions, we found that naive splenic B cells from C5ar2−/− mice showed a drastically increased expression of the decay accelerating factor (DAF, CD55) compared to B cells from wild-type and C5ar1−/− mice. Since the regulation of DAF expression has been shown to be critical for a functional germinal centre response, we hypothesised that the increased DAF expression in B cells of C5ar2−/− mice might affect their differentiation. To investigate this, we used an in vitro culture system in which we co-cultured splenic B cells with 40LB fibroblast feeder cells that exogenously express CD40–ligand (CD40L) and B cell activating factor (BAFF), which promote B cell proliferation and survival and mimic the germinal center response. Flow cytometric analysis of the co-cultured B cells revealed attenuated activation, differentiation and class switching of B cells from C5ar2−/− mice compared to the wild-type. These results also suggest a potential role for C5aR2 in autoimmunity, which we will investigate in future experiments. Taken together, our findings highlight a potential role of C5aR2 as a novel checkpoint for B cell development, possibly by regulating DAF expression during B cell maturation.

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Long-term efficacy and safety of pozelimab monotherapy in patients with paroxysmal nocturnal hemoglobinuria

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Background: Paroxysmal nocturnal hemoglobinuria (PNH) is a rare, acquired disease characterized by chronic intravascular hemolysis. Pozelimab is an investigational monoclonal antibody against complement component C5. Final results from a phase 2 study (NCT03946748) and subsequent open-label extension (OLE; NCT04162470), evaluating long-term pozelimab monotherapy in patients with PNH are presented.

Methods: During the phase 2 study, patients received an intravenous loading dose of pozelimab 30 mg/kg, then weekly subcutaneous pozelimab 800 mg. All 24 patients completed phase 2 and entered the OLE, where patients received weekly subcutaneous pozelimab 800 mg up to Week 104.

Results: Overall, 87.5% of patients were Asian; 54.2% were male. In phase 2, 18/24 (75.0%) patients achieved adequate control of intravascular hemolysis (defined as LDH $\leq$1.5 x upper limit of normal; 95% confidence interval [CI]: 57.7-92.3%) at every scheduled timepoint between Weeks 4-26 inclusive. During the OLE, 22/23 (95.7%) patients achieved control of intravascular hemolysis at all timepoints through Week 26 (95% CI: 87.3-100.0%); with 15/16 (93.8%; 95% CI: 81.9-100.0%) at Week 78. In phase 2, 21/24 (87.5%; 95% CI: 74.3-100.0%) patients achieved transfusion avoidance (no red blood cell transfusion). During the OLE, 22/23 (95.7%) patients achieved transfusion avoidance through the initial 26 weeks, and 15/16 (93.8%) through to Week 78. No patients experienced a breakthrough hemolysis event in either trial. Importantly, one patient with a C5 variant resistant to eculizumab/ravulizumab had adequate control of intravascular hemolysis. All patients showed a rapid decrease in total complement hemolytic activity to near zero, maintained to Week 104. In phase 2, 21 (87.5%) patients experienced 72 treatment-emergent adverse events (TEAEs); two (8.3%) patients experienced severe TEAEs; and 10 (41.7%) patients experienced TEAEs considered related to pozelimab. During the OLE, 15 (62.5%) patients experienced 41 TEAEs; two (8.3%) patients experienced serious TEAEs; two (8.3%) patients experienced severe TEAEs; two (8.3%) patients experienced TEAEs considered related to pozelimab; and no deaths or discontinuations due to TEAEs occurred.

Conclusion: Patients with PNH receiving pozelimab (up to 130 weeks) experienced improvement in intravascular hemolysis control, with no breakthrough hemolysis, and achieved transfusion avoidance. Pozelimab was also generally well tolerated.

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Study of complement modulation by reactive oxygen species: revision of existing *in vitro* data

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Background: Controversial reports describe reactive oxygen species effects on complement in vitro. Some model features seem overly artificial. We aimed to revise existing data in different models.

Methods: Model 1. H_2O_2 (10, 20 mM) was incubated with 10% normal human serum at +4°C or +37°C in the presence or absence of FeEDTA for 10 min, and serum lytic action towards rabbit erythrocytes (Er^{rab}) was assessed. Model 2. In fluid-phase design, H_2O_2 or HOCl at concentrations 0.125-16 mM was incubated with 80% serum at room temperature for 60 min, and C3a and C5a were measured by ELISA. If significant, effects were assessed in EDTA-treated serum. After incubation, all samples were diluted with MgEGTA/GVB for hemolytic assay. Model 3. HOCl was added to 5% serum in the presence of Er^{rab} in MgEGTA/GVB at +37°C for 30 min. The levels of hemolysis and C3a and C5a were measured.

Results: Model 1. Incubation with H_2O_2 under different conditions did not affect serum hemolytic activity. Model 2. HOCl, unlike H_2O_2, dose-dependently augmented the level of C3a and especially C5a accumulation. The effect was abolished in EDTA-treated serum, suggesting dependence on Mg^{2+} ions. Incubation with HOCl, but not H_2O_2, significantly reduced hemolytic activity. It was the case even in EDTA-treated serum. Therefore, loss of hemolytic activity cannot be explained solely by complement depletion. Model 3. Unexpectedly, HOCl in serum with Er^{rab} led to pronounced inhibition of hemolysis, C3a and C5a accumulation. 8-16 mM HOCl was toxic for Errab in heated serum.

Conclusion: H_2O_2 and $HO^{\cdot}$ radical seem not to affect complement activation under conditions close to physiological. HOCl triggers fluid-phase C3 and C5 conversion but surprisingly inhibits membrane-associated complement activation. We hypothesize that the both processes are due to attack of C3 thioester bond. Probably, HOCl promotes C3 conformation favorable for FB binding. C3(HOCl)Bb may cleave both C3 and C5. HOCl attacks C3b preventing its covalent binding to membranes and inhibits amplification loop.

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The C5a/C5aR1 axis controls the induction of Germinal Center B cells, their IgM/IgG1 production and plasma cell differentiation

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Background: Previously, we observed a reduced switch from antigen-specific IgM to IgG autoantibodies in C5ar1-/- mice in a murine model of autoimmune skin disease, associated with strong upregulation of C5aR1 in Germinal Center B cells (GCB). Here, we used an in-vitro model of GCB induction to define the pathways downstream of the C5a/C5ar1 axis controlling the switch from IgM to IgG production in induced GCBs (iGCB).

Methods: We co-cultured 3T3 cells overexpressing CD40-ligand and B-cell-activating factor (BAFF) (40LB cells) (Haniuda and Kitamura 2019) with naïve B cells from either C57BL/6 WT or C5ar1-/- mice in the presence of IL-4 for 4 or 6 days. At both time points, we determined the expression of the GCB markers GL7 and CD95 (Fas), IL-4-receptor, IgG1 and IgM production as well as CD138+ plasma cell (PC) differentiation. Further, we assessed expression of C5 and C5aR1 as well as C5a generation in 40LB, naïve B cells and iGCBs.

Results: We observed strong intracellular expression of C5, C5a and C5aR1 in 40LB cells. A small fraction of naïve B cells from WT or C5ar1-/- mice expressed C5 but not C5a or C5aR1. After 4d of co-culture CD95 but not GL7 expression was reduced in iGCBs from C5ar1-/- as compared to WT mice. In iGCBs from WT mice, 65% expressed C5 and 50 % C5a at d4, which was significantly lower in iGCBs from C5ar1-/- mice. Strikingly, IL-4 receptor expression was downregulated in iGCBs from C5ar1-/- mice at d4, which was associated with a reduced IgM to IgG1 switch at d4 and d6 as compared with iGCBs from WT mice. Finally, we found induction of PCs from iGCBS at d6, the frequency of which was significantly lower in cells from C5ar1-/- as compared to those from WT mice.

Conclusion: Our findings suggest a model in which autocrine (in iGCBs) and/or paracrine (in 40LB cells) production of C5 and generation of C5a activates C5aR1 in iGCBs to drive their differentiation and expression of IL-4 receptor, which is crucial for the switch from IgM to IgG1 antibody production. Later, C5a/C5aR1 axis activation controls the differentiation of PCs from iGCBs.

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A phase 2/3 study evaluating the efficacy and safety of pozelimab in patients with CD55 deficiency with hyperactivation of complement, angiopathic thrombosis, and protein-losing enteropathy (CHAPLE disease)

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Background: CD55 deficiency with hyperactivation of complement, angiopathic thrombosis, and protein-losing enteropathy (CHAPLE) disease is an ultra-rare autosomal recessive disorder caused by loss-of-function variants of the CD55 gene, leading to overactivation of the terminal complement system.1 Clinical and laboratory features of CHAPLE disease include hypoalbuminemia and edema; hypogammaglobulinemia; and gastrointestinal symptoms such as abdominal pain, loss of appetite, vomiting, and diarrhea. Patients also present with micronutrient deficiency, anemia, and growth retardation.[1] Currently, there is no approved treatment for CHAPLE disease. We assessed the efficacy and safety of pozelimab, an investigational anti-C5 antibody, in patients with CHAPLE disease.

Methods: This is an interim report of an open-label, single-arm, historically-controlled study in patients with CHAPLE disease (ClinicalTrials.gov, NCT04209634). Patients received intravenous pozelimab 30 mg/kg followed by subcutaneous, weight-based dosing once weekly. The primary endpoint was the proportion of patients who achieved normalization of serum albumin and demonstrated improvement or no worsening in clinical outcomes (abdominal pain, bowel movement frequency, facial edema, and peripheral edema) at week 24. Secondary/exploratory efficacy endpoints and safety are also reported.

Results: Ten patients were enrolled in the study, and had ≥48 weeks of efficacy measurements as of the cut-off date for this analysis. All 10 patients (100%) experienced serum albumin normalization and improvement/no worsening in clinical outcomes. Over the 48-week treatment period, most patients experienced remarkable catch-up growth. Following treatment with pozelimab, patients had reduced all-cause hospitalization days; the mean number of hospitalization days across all 10 patients decreased from 26.8 days in the 48 weeks prior to treatment to 0 days by week 48. Four patients (40%) started the treatment period on corticosteroids and all were withdrawn as of the cut-off date for this analysis. Complete inhibition of complement activity (CH50) was achieved. Seven patients (70%) experienced adverse events; none were severe, and only one patient experienced adverse events that were considered related to study drug.

Conclusion: Pozelimab inhibits complement overactivation and resolves the clinical and pathophysiological manifestations of CHAPLE disease.

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Sustained complement C1s inhibition with sutimlimab in patients with cold agglutinin disease results in continued efficacy during part B of the randomized placebo-controlled phase 3 CADENZA study (NCT03347422)

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Background: In part A of CADENZA (NCT03347422), treatment with sutimlimab (SUT), a complement pathway (CP) inhibitor, rapidly improved hemoglobin (Hb) levels, hemolytic markers, and quality of life (QoL) in patients (pts) with Cold Agglutinin Disease (CAD). Here, we report long-term efficacy, safety, and durability of response evaluated in Part B CADENZA (>1 year).

Methods: In part B, ex-SUT pts continued their bi-weekly dosing, and ex-placebo (PBO) pts started SUT on Days 0, 7, and bi-weekly thereafter. Efficacy endpoints up to Week (Wk) 79 were collected as change from baseline (BL) in Hb, hemolytic and pharmacodynamic markers, Functional Assessment of Chronic Illness Therapy (FACIT)-Fatigue. Safety was evaluated as treatment-emergent adverse events (TEAEs) and serious TEAEs (TESAEs).

Results: Among 39 pts entering part B, 32/39 (82.1%) completed the study. At Wk 79, the mean (SE) Hb levels (g/dL) were sustained in the SUT group (11.86 [0.54]) and rose rapidly and comparably with SUT initiation in the ex-placebo (PBO) group (from 9.43 [0.40] to 11.76 [0.58]). Total bilirubin normalization was sustained in SUT group; similarly in ex-PBO group upon SUT initiation. At Wk 87, improvements in mean (SE) FACIT-Fatigue scores were sustained in the SUT group (44.31 [2.19]); mean (SE) score increased to comparable levels in the ex-PBO group (41.40 [2.71]). Hb, bilirubin, and FACIT-Fatigue improvements correlated with C4 normalization and near-complete CP activity inhibition. Mean absolute reticulocyte count reduction and increase in haptoglobin levels with SUT in part A were maintained in part B and ex-PBO group levels were comparable. In part B, 9 pts (23.1%) received $\geq$1 transfusion. In part B, 36 pts (92.3%) reported TEAEs, and 7 (17.9%) reported 11 TESAEs (1 hypertension event assessed as SUT related). Non-serious thromboembolic events were seen in 2 pts (transient ischemic attack; deep vein thrombosis; neither related to SUT). No meningococcal infections, serious hypersensitivity events, anaphylaxis, or systemic lupus erythematosus were reported. One patient (ex-PBO) with history of tobacco use developed squamous cell carcinoma of the lung (TESAE) and died.

Conclusions: Long-term SUT maintained mean Hb levels >11 g/dL, normalized bilirubin, and improved FACIT-Fatigue scores with a favorable safety profile.

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Elucidating the molecular mechanisms mediating the tumor suppressive actions of complement inhibitor CSMD1 in gliomas

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Background: Glioma which is the most malignant form of brain tumors originates in the central nervous system. Tumor suppressor role of complement inhibitor CSMD1 is still not clear although complement activation promotes more aggressive phenotype in glioma and CSMD1 is highly expressed in brain tissue. To that end, we investigated the role of CSMD1 in glioma using in vivo and in vitro approaches.

Materials and methods: A total of 1507 patients with glioma from three different databases including TCGA, CGGA and GEO were analyzed to determine the prognostic role of CSMD1. CSMD1 was introduced into glioma cell lines and its effect on aggressiveness was assessed through performing functional studies. We next evaluated the effect of CSMD1 overexpression in glioma cells on activation of CD4+ and CD8+ T cells. Additionally, we used glioma mouse model and cryosections of patients with glioma to validate our findings.

Results: Our database analysis revealed that CSMD1 downregulation in glioma cohorts was associated with decreased overall survival (OS) and disease-free survival as well as higher activity of neuroinflammation signaling pathway and tumor grade. While ectopic expression of CSMD1 inhibited proliferation, migration,

invasion, tumorsphere formation, colony formation, ALDH activity (a measure of stemness), and chemoresistance, it induced apoptosis. CSMD1 also interrupted the TNF-induced activation of P65 and STAT3 pathways and inhibited AKT signaling pathway. In addition, overexpression of CSMD1 boosted the activation of CD4+ T cells in co-culture experiments. Staining of both mouse and human glioma samples demonstrated that CSMD1 is downregulated in a grade-dependent manner.

Conclusion: Our study indicates that CSMD1 expression in gliomas is related to favorable prognosis and response to chemotherapy. Consequently, CSMD1 may be used as new molecular predictor of both prognosis and response to therapy.

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C1q binding and complement activation by the IgM Fc-core domain

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Background: Immunoglobulins type-M (IgM) are the first antibody isoforms that are produced by vertebrates in response to a range of antigens from viruses to tumor cells, making them promising therapeutic targets. In serum, IgMs are found in two oligomeric forms, pentamers (5 promoters joined by J chain) and hexamers (6 promoters without J chain). These assemblies make a rigid inner core formed by the constant fragment (Fc), while the antigen-binding regions (Fab) are positioned at the extremities and adopt a very flexible conformation. The antigen-bound IgMs are known to strongly activate the complement system, in particular the classical pathway, by binding to it's first recognition molecule, C1q. Despite their essential role, the mechanism of the complement activation by the different oligomeric forms of IgMs are only starting to be elucidated in fine details through newly developed methods in protein engineering, biophysics and structural biology (Chouquet et al., 2021; Sharp et al., 2019).

Methods: Fab-truncated forms of IgMs containing only the Fc-core were produced in HEK293F cells. The purified samples were characterised by coupling size exclusion chromatography with mass photometry and negative-stain transmission electron microscopy to finely elucidate their oligomeric distributions. The binding of Fc samples to C1q and their potency in complement activation were subsequently measured using Bio-Layer Interferometry (BLI) and in-house ELISA-like assay, respectively.

Results and conclusion: The oligomeric distribution in the Fc samples was either heterogeneous or homogeneous depending on the presence of the J chain, similar to recombinant full-length IgMs studied before (Chouquet et al., 2021). Unexpectedly, both forms exhibited the ability to bind to C1q although with lower affinity than full IgMs and to activate the classical pathway in BLI and ELISA functional assays. Notably, the level of complement activation was correlated to the oligomer ratios in the different samples, confirming that Fc-core hexamers have a stronger potential to trigger complement than pentamers, as observed in full IgMs. In conclusion, the findings from BLI and in vitro complement activation studies challenge the idea that, in the absence of antigen and Fab domains, the IgM-Fc (either pentameric or hexameric) is incapable of activating the complement by C1q binding.

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Humoral innate immunity and acute phase proteins in COVID-19

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Innate immunity includes a cellular and a humoral arm. Acute and chronic inflammation, including Covid-19, are associated with profound systemic changes, the so called acute phase reaction, with augmented production of a number of proteins (e.g. C reactive protein, fibrinogen, PTX3 etc.) which serve as invaluable clinical diagnostic tools. A number of acute phase reactants have antibody-like functions and are components of the humoral arm of innate immunity. They are produced by the liver and by tissue immune cells such as macrophages. The general significance of the acute phase response is amplification of innate immunity and promotion and regulation of tissue repair.

We studied the involvement of selected humoral innate immunity and acute-phase proteins as diagnostic and prognostic biomarkers, as well as their potential as therapeutic tools and targets in COVID-19. In COVID-19, the long pentraxin PTX3 emerged as independent strong prognostic indicator of short-term mortality, as well as biomarker of COVID-19-associated secondary infections. Mannose-Binding Lectin (MBL) bound trimeric Spike protein, including that of variants of concern (VoC), in a glycan-dependent way and inhibited SARS-CoV-2 in in vitro and in vivo models of SARS-CoV-2 infection. Moreover, upon binding to Spike, MBL activated the lectin pathway of complement activation.

Thus, in addition to a role as biomarkers of severity, selected humoral fluid phase pattern recognition molecules can play an important role in resistance to, and pathogenesis of, COVID-19, a finding with translational implications.

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C5 cleavage inhibition combined with C3 convertase targeting protects from disease development in a preclinical model of autoimmune skin disease

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Background: Epidermolysis Bullosa Acquisita (EBA) is an autoimmune skin blistering disease characterized by strong production of autoantibodies (AAbs) against type VII collagen (COL7) at the dermal epidermal junction (DEJ). Local complement activa-

tion drives C5a generation associated with neutrophil recruitment and activation resulting in skin lesions and inflammation. The clinical effector phase of EBA can be mimicked by repeated injection of rabbit COL7-specific IgG into mice. Here we tested the impact of prophylactic or therapeutic C5a or combined C5/C3 targeting on disease development and skin inflammation in this model.

Methods: C57BL/6 mice were immunized s.c. with purified rabbit anti-mouse-COL7 IgG. An anti-mouse C5a/C5adesArg IgG1 mAb (M031), a fusion protein between an anti-mouse C5 IgG1 mAb and FH-SCR1-5 (M014), or an IgG1 isotype control (n = 12/group) were injected (i.p.) prophylactically on days −1, 2, 5 and 8 or therapeutically on days 5 and 8. Then, formation of skin lesions was evaluated every other day. DEJ separation was assessed in cryosections from day 12 and stained with H&E. Tissue-bound IgGs AAbs, C3/C3b deposition as well as MPO^{+} or $Ly6G^{+}$ neutrophil infiltration was identified by direct immunofluorescence.

Results: Isotype-treated mice developed first skin lesions at day 4 peaking at day 12. Prophylactic treatment with either M031 or M014 markedly reduced the development of skin lesions. Also, dermal/epidermal separation as well as neutrophil infiltration on day 12 were significantly lower upon M031 or M014 treatment. Surprisingly, C5/C3 inhibition by M014 but not C5a inhibition by M031 markedly reduced the development of skin lesions and the dermal/epidermal separation after therapeutic treatment as well as the infiltration with MPO^{hi} neutrophils. IgG and C3/C3b deposition did not differ in the three treatment groups.

Conclusion: Prophylactic C5/C3 and C5a targeting is equally effective in reducing the development of skin lesions and tissue inflammation in a preclinical model of EBA. In contrast, only combined C5/C3 but not C5a inhibition protects from the development of skin lesions and skin inflammation after therapeutic administration at day 5 when first skin lesions become evident. Our findings point toward an important role of C5a as well as other complement effectors during disease development in EBA.

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Anti-tumor effect of resident macrophages bearing complement component C1q in targeted therapy with CD19- Chimeric antigen receptor (CAR)-T cell

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Background: CAR genetically engineered T cells can activate an immune response to a cancer-specific antigen. Excellent results from treatment in hematological malignancies were not replicated in solid tumors, possibly due to a hostile tumor microenvironment (TME). Here we describe the effect of CAR T on bystander resident macrophages.

Methods: HeLa-CD19 cells were stably transduced with pLenti-PGK-V5-Luc-Neo. For CAR preparation, fresh mononuclear cells were introduced to CD28+CD3+ beads and transfected with third generation a CD19-CAR plasmid. SCID-Bg mice were injected IP with human HeLa-CD19 or HeLa-CD19-luciferase cells, and 10 × 106 CD19-CAR-T cells or mock T cells.

RNA from the barcoded cells from each sample was subsequently reverse-transcribed and sequencing libraries were constructed. Single-cell RNA-seq analyses were performed using Seurat 4.3.0. Data was normalized with sctransform, and principal component analysis followed by Uniform Manifold. Approximation and Projection (UMAP) was performed. Results were confirmed by Flow-Cytometry.

Results: Mice survived 30 ± 5 days (range 27–37). CAR-T cell therapy significantly ameliorated survival to 55 ± 11 days (range 34–76, $p < 0.05$ vs MOCK). We performed on day 23 in silico sorting and further sub-clustering and annotation by a peritoneal macrophage reference and marker genes. We identified the normal resident peritoneal macrophages in normal strain SCID-Bg mouse, which include large peritoneal macrophages (LPMs) and small peritoneal macrophages (SPM). Upon cancer progression there was a major shift from the normal macrophages to tumor-associated macrophages (TAMs) which have no or low expression of the common LPMs and SPM markers.

Upon tumor shrinkage during CAR-T treatment, we again observed a change in cancer-related peritoneal macrophages. The scRNA-seq analysis showed that there was a shift back towards the original macrophages' subtypes, but with some modulations (termed modified peritoneal macrophages (MPM)). One subtype was characterized by high expression of the complement genes C1qa and C1qb.

Conclusion: During IP tumor progression, resident LPMs macrophages depletion correlated with tumor growth. CAR-T cell therapy reduced this depletion. Complement component C1q that may appear in both TAMs and resident macrophages was more pronounced in LPMs and less in TAMs and mostly pronounced in MPM.C1q that appeared following shrinkage of the tumor and disappearance of TAMs.

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Deciphering complement system-dependent cellular pathways in human rheumatoid arthritis synovial tissues using large single-cell computational omics

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Background: The complement system is a major component of innate immunity and plays a vital role in experimental models of autoimmune pathogenesis. In patients with rheumatoid arthritis (RA), local activations of the complement pathways in the synovium will interact with macrophages and other synovial mesenchymal cells. However, it remains to be determined how specific complement components in the RA synovium modulate

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cell type functions to strengthen phagocytosis and inflammation resolution.

Methods: We have classified the spectrum of RA biopsies into six inflammatory phenotypes named CTAPs (Cell Type Abundance Phenotypes) using a comprehensive synovial single-cell multimodal cell atlas (>314,000 cells, 70 RA patients) generated from NIH-funded AMP RA/SLE consortium. In this study, we linked complement activation pathways with cell-type heterogeneity and tested significant associations with different CTAPs using computational models. Next, we characterized myeloid cell differentiation using single-cell trajectory analysis and aligned specific complement pathways to myeloid functional states. Furthermore, our cell type interactome analysis reveals complement-dependent receptor-ligand pairs that likely contribute to tissue pathology.

Results: We generated an Autoimmune Complement Cellular Graph comprehensively characterizing interactions between complement components and cell-type co-varying patterns in RA synovium. We found that complement components present unexpected distinct transcriptomic expressions across cell types. Specifically, expressions of complement C1QA-C and C3AR1 are correlated with myeloid phagocytic trajectory, while complement FCN1 and CFP are correlated with myeloid pro-inflammatory trajectory, which suggests different complement pathways interact with myeloid states in RA synovium. Intriguingly, we observed a phagocytic MERTK+ tissue macrophage cluster, overexpanded in a stratified RA synovial phenotype that lacks lymphocytes, display two complement pathways, namely the phagocytic C1QA-C factors and the inflammatory receptor C3AR1, while sublining fibroblasts highly expressed complement C3. This suggests the interactions between MERTK+ macrophages and fibroblasts that activate complement pathways in the synovium.

Conclusion: Through systematically aligning complement pathways with tissue cellular heterogeneity, we generated a graph describing how complement components can modulate functional cell states in stratified RA patients. This approach of analyzing high-resolution single-cell data in human tissues brings insights into new complement pathway-based cellular targets for patients with RA, and provides a roadmap for other complex pathways in tissues.

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Phase 1 clinical data for single-dose subcutaneous injection of RLYB116, a C5 blocking Affibody® molecule linked to an Albumod® albumin binding domain

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Background: RLYB116 comprises an Affibody molecule with high affinity for blocking C5 linked to an albumin binding domain for half-life extension and is in Phase 1 clinical development. The Affibody platform may provide opportunity for a small molecular sized protein with high specificity for target to be administered infrequently with low volume subcutaneous injection.

Methods: A Phase 1 single-blind, placebo-controlled single and multiple ascending dose study was initiated with objectives including evaluation of the safety and tolerability as well as pharmacokinetic and pharmacodynamic properties of RLYB116 in healthy participants. The single dose phase of the study included doses of 2, 10, 30, 100, and 300 mg administered by subcutaneous injection in sequential cohorts of 8 participants each. Each cohort included 6 assigned to RLYB116 and 2 to placebo. Serum concentrations of RLYB116 were measured utilizing LC-MS/MS and the pharmacodynamic measure of free C5 serum concentration was assessed utilizing a Gyrolab® immunoassay.

Results: At the 100 mg dose, a mean Cmax concentration of 1.33 μM at 24 h following dose administration was measured. At the 300 mg dose, a mean Cmax concentration of 3.68 μM at 36 h following dose administration was measured. The estimated half-life for RLYB116 at the 300 mg dose was 338 h. Free C5 concentrations measured for each participant were reduced by greater than 99% at 24 h after the 100 mg dose and at 12, 24, and 72 h after the 300 mg dose of RLYB116. Reported adverse events were mild to moderate in severity and there were no serious adverse events or discontinuations from the study due to adverse events.

Conclusion: RLYB116 is a low molecular weight protein containing an Affibody molecule with C5 binding specificity linked to an albumin binding domain for half-life extension. Phase 1 data has demonstrated that single-dose administration of 100 and 300 mg resulted in maximum exposures greater than 1 μM and 3 μM, respectively, and substantial reductions in free C5 concentrations. Single dose administration of RLYB116 was characterized by no severe or serious adverse events. Further investigation of RLYB116 with weekly multiple-dose administration in healthy participants is planned.

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Intracellular complement in atypical hemolytic-uremic syndrome

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Atypical Hemolytic Uremic Syndrome (aHUS) is a rare kidney disease associated with pathogenic variants of the central complement component C3 and its regulators, including Factor H (CFH). Overactivation of the complement alternative pathway is a key driver of the pathogenesis of aHUS. Recently an intracellular role of these proteins has been discovered in different cell types. Particularly, a contribution of CFH to maintaining of the endothelial cell monolayer integrity and activation status has been reported. Still, the specific intracellular mechanisms by which these complement components affect endothelial cells (EC) and whether they contribute to the pathophysiology of aHUS have not been elucidated yet. To better understand the role of intracellular C3, CFB and CFH in endothelial cells in human EC, we used a model of microvascular EC (dermal microvascular endothelial cells, HMEC-1). We detected, via western blot and ELISA, the presence of C3, CFB, and CFH in the lysate, supernatant and in subcellular fractions. Additionally, we studied the effect of silencing of CFH, CFB and C3. By imaging, wound healing assay and CFSE cell proliferation test, we observed that the knockdown cells showed differences in mortality, migration, proliferation, and morphology. We plan to continue our analyses on glomerular endothelial cells (GEnC) to explore the specific contribution of these proteins in the context of aHUS. Kidney microvascular EC are a primary site of complement activation in aHUS, and understanding the role of intracellular CFH, CFB and C3 in these cells could provide important insights into the pathogenesis of the disease. Investigating the expression,

localization, and function of intracellular complement in EC may provide insights into the broader role of intracellular complement regulation in aHUS and other complement-mediated diseases.

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Evaluation of minimal Factor H therapy administered to kidneys during ex vivo normothermic perfusion as a treatment to improve ischaemia reperfusion injury

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Background: Ischaemia reperfusion injury is an inevitable consequence of transplant with the complement system, in particular the alternative pathway being one of the key drivers of damage. The main regulator of the alternative pathway is factor H. We hypothesised that homodimeric mini-factor H (HDM-FH; PMID:29588430) would protect kidneys when administered during normothermic machine perfusion (NMP) with whole blood which mirrors the clinical setting of transplantation.

Methods: A model of porcine whole blood NMP with extended warm and cold ischemic times was optimised to induce complement activation and ischaemic injury in kidneys so that the full efficacy of HDM-FH could be assessed. Utilising this model, both kidneys were retrieved from female white landrace pigs. One kidney from each pair was randomised to receive 5 mg of HDM-FH (~8 μg/mL). Kidneys were perfused at 37 °C with autologous blood for 6 h. HDM-FH binding within kidneys was confirmed using immunofluorescence. HDM-FH levels in perfusate and urine were measured using ELISA. Complement activation was measured by quantifying Bb deposition in tissue and C5a levels in urine. Fibrosis, inflammatory cytokines, and apoptosis were measured as indicators of downstream ischaemic injury. 'Cold binding' of HDM-FH was assessed by flushing kidneys with 4 °C saline containing HDM-FH.

Results: 25 minutes warm ischaemic time followed by 16 h cold ischaemic time led to an increase in complement activation and markers of ischaemic injury. ~4 mgs of HDM-FH bound from perfusate during perfusion at 37 °C, with <10% lost in urine suggesting saturation was achieved. HDM-FH localised to glomeruli with deposition increasing during the perfusion. Complement activation was reduced in kidneys receiving HDM-FH as demonstrated by reduced Bb deposition in tissue and reduced C5a levels in urine. Fibrinogen deposition, inflammatory cytokine and apoptosis levels were reduced. HDM-FH inhibits complement activity in serum in a dose-dependent manner. HDM-FH also binds at 4 °C, experiments are ongoing using this approach.

Conclusion: Infusion with HDM-FH during simulated kidney transplant conditions reduced complement activation and downstream ischaemic injury to the organ. Therefore, organ perfusion with HDM-FH is highly likely to help prolong graft survival after transplant and this will be assessed in future studies.

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Humoral complementomics – exploration of non-invasive complement biomarkers as predictors of renal cancer progression

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Despite the advancements made in cancer therapy, the prognosis of many patients with solid tumours remains dismal. As such, there is a need for the development of reliable non-invasive biomarkers that can accurately predict both patient survival and therapy response. Here we propose a Humoral Complementomics approach which involves the application of validated assays to comprehensively evaluate complement proteins, their activation fragments, and autoantibodies in the context of the most common renal cancer: clear cell renal cell carcinoma (ccRCC). The proposed approach involves the integration of two ELISA-based methods to detect C1s-C1INH complexes (Hycult) and C4d fragments (SVAR), along with the MicroVue Complement Multiplex (Quidel) for global complement analysis. In parallel, a Luminex particle-based antibody assay is performed for the detection of autoantibodies. In two different ccRCC cohorts, patient plasma presented higher levels of C2, C5, FD and properdin when compared to healthy controls, reflecting a global inflammatory phenotype that correlated with calprotectin levels in plasma and did not associate with patient prognosis. Conversely, autoantibodies against the reduced form of FH (a tumour neo-epitope reported in lung cancer) were associated with a favourable outcome. Elevated plasma levels of C4d and C1s/C1Inh were observed in patient groups, indicating initiation of the classical pathway. Additionally, Ba and Bb were detected in the same groups, featuring alternative pathway activation. Boostrapped Lasso regularized Cox regression analysis revealed that the most predictive complement activation biomarkers were C4d and Bb which correlated with poor prognosis. In conclusion, we propose an unbiased Humoral Complementomics approach applicable to any pathological plasma samples and disease context. Its implementation for ccRCC revealed that elevated C4d and Bb in plasma are promising prognostic biomarkers, correlating with shorter progression-free survival.

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How FHR-1 deficiency affects the infection with the malaria pathogen *Plasmodium falciparum*

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The obligate intracellular parasite Plasmodium falciparum causes malaria tropica, the most severe form of malaria, which is particularly acute in the WHO African region. With more than 247 million cases and 619,000 deaths each year, malaria is still one of the deadliest infectious diseases in the world. Malaria infection is characterized by multiple symptoms, like fever, head- and body aches, and diarrhea. Severe complications further include anemia, shock, cerebral malaria, multiple organ failure, and ultimately death. During evolution, the parasite has developed various mechanisms to evade attack by human complement, such as binding of proteins of the Factor H (FH) family. In previous studies, we have shown that the Factor H-Related Protein 1 (FHR-1) competes with FH for binding sites and thereby prevents FH-mediated complement evasion (Reiss et al., 2018). Furthermore, FHR-1, when binding to malaria-lysed erythrocytes, activates neutrophils and thus contributes to inflammation. Remarkably, up to one third of the African population harbors a chromosomal deletion of the FHR-1 gene. To investigate a potential link between FHR-1 deficiencies and malaria severity, we investigated two cohorts of malaria tropica-infected African patients. We here show that patients with FHR-1 deficiencies are more likely to suffer from a mild malaria with less signs of anemia and lower levels of inflammation markers compared to FHR-1 expressing individuals. Our data underline the potent role of FHR-1 in inflammation, which likely enhances the immune response in Malaria infections and the risk of severe Malaria anemia.

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Assessing the feasibility of genetically modified porcine red blood cells as an alternative to human red blood cells for transfusion

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Background: The shortage of blood products and declining blood donation rates present significant challenges for medical societies worldwide. One potential solution is the use of porcine red blood cells (pRBCs) from genetically modified pigs as an alternative to human red blood cells (hRBCs). However, adverse immunological reactions, including hemolysis, remain a significant obstacle to their use. This study aimed to evaluate the performance of diverse genetically modified pRBCs in human serum regarding antibody reactivities, complement activation, and human serum-mediated hemolysis.

Methods: Human complement-competent serum, complement 7 (C7)-deficient serum, and hRBCs of all ABO blood types were commercially acquired. Additionally, leftover clinical samples from health checkups were used for further evaluation. The pRBCs were collected from wild-type (WT) and three genetically modified pigs: α1,3-galactosyltransferase-, cytidine monophosphate-N-acetylneuraminic acid hydroxylase-, and β1,4 N-acetylgalactosaminyltransferase-knockout (TKO), additional isoglobotrihexosylceramide synthase-knockout (QKO), and α 1,3-galactosyltransferase- and β1,4 N-acetylgalactosaminyltransferase 2-knockout and human CD55- and human CD39-knockin (DKO/hCD55KI/hCD39KI). Flow cytometry was used to measure the extent of C3 deposition on the RBCs post-incubation in C7-deficient serum diluted in Ca++-enriched or Ca++-depleted buffer. IgM/IgG antibody binding to the RBCs and the extent of hemolysis of the RBCs post-incubation in each ABO-type human serum was also evaluated.

Results: All three genetic modifications significantly improved the compatibility of pRBCs with human serum compared to WT pRBCs. However, the extent of IgM/IgG binding to pRBCs varied. Total complement activation on TKO or QKO pRBCs was weaker than that on DKO/hCD55KI/hCD39KI pRBCs, while alternative pathway complement activation did not differ between them. DKO/hCD55KI/hCD39KI pRBCs hemolysis was significantly higher than that of TKO or QKO pRBCs, comparable to type O hRBCs.

Conclusion: Eliminating porcine carbohydrate antigens in TKO or QKO pigs resulted in a significant enhancement of pRBCs compatibility with human serum compared to ABO-incompatible hRBCs and WT pRBCs. The improved compatibility was comparable to that of type O hRBCs. These findings provide valuable insights for developing pRBCs as a potential alternative to hRBCs. (Supported by the ICMTC under grant No. 22-CM-EC-18).

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APPARENT: A multicenter, randomized, double-blind, placebo-controlled Phase 3 study to assess the efficacy and safety of iptacopan in idiopathic (primary) immune complex-mediated membranoproliferative glomerulonephritis

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Background: Immune complex-mediated membranoproliferative glomerulonephritis (IC-MPGN) is a fast-progressing kidney disease that may be idiopathic (primary) or secondary to chronic infection, autoimmune disorders, or monoclonal gammopathies. Idiopathic IC-MPGN is rare and has a comparable clinical course to complement 3 glomerulopathy (C3G), which is also characterized by membranoproliferative histology. Dysregulation of the alternative complement pathway is implicated in the pathophysiology of both glomerular diseases. Currently, there are no approved targeted treatments for IC-MPGN. Iptacopan (LNP023) is an oral, first-in-class, highly potent proximal complement inhibitor that specifically binds to factor B and inhibits the alternative pathway (AP).

Methods: This randomized, double-blind, placebo-controlled, pivotal Phase 3 study (APPARENT; NCT05755386) is the first to evaluate the efficacy and safety of iptacopan in patients with idiopathic IC-MPGN. Approximately 68 patients (including a minimum of 10 adolescents) aged 12–60 years with biopsy-confirmed IC-MPGN, proteinuria $\geq$1 g/g, and estimated glomerular filtration rate (eGFR) $\geq$30 mL/min/1.73 m^2 will be randomized. All patients will have received maximally tolerated angiotensin-converting enzyme inhibitor/angiotensin receptor blocker and vaccination against encapsulated bacteria. Patients with any organ transplant, progressive crescentic glomerulonephritis, or kidney biopsy with >50% interstitial fibrosis/tubular atrophy will be excluded. Patients will be randomized 1:1 to receive either iptacopan 200 mg twice daily (bid) or placebo for 6 months (double-blind period), followed by open-label treatment with iptacopan 200 mg bid for all patients for 6 months. At the end of the study, patients will have the option to transition to an open-label extension study.

The primary objective is to evaluate the efficacy of iptacopan versus placebo on proteinuria reduction as measured by urine protein–creatinine ratio (24-h urine) at 6 months. Key secondary endpoints will assess kidney function measured by eGFR, patients who achieve a proteinuria–eGFR composite renal endpoint, and patient-reported fatigue. The safety objectives are to evaluate the safety and tolerability of iptacopan in all patients and perform cardiovascular surveillance in adolescent patients (blood pressure, heart rate, cardiac function and biomarkers).

Results: The study is expected to start in Q2 2023.

Conclusion: This study will provide evidence towards the efficacy and safety of iptacopan in idiopathic IC-MPGN.

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Evaluation of iptacopan in atypical hemolytic uremic syndrome: Design and rationale of the Phase 3 open-label multicenter APPELHUS study

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Introduction: Atypical hemolytic uremic syndrome (aHUS) is a rare, progressive, and life-threatening form of thrombotic microangiopathy (TMA) caused by the dysregulation of the alternative complement pathway (AP). Complement inhibition via oral administration is an attractive therapeutic target in aHUS as current approved therapies require intravenous or subcutaneous administration. Furthermore, not all such intravenous therapies are available in many countries. Iptacopan (LNP023) is an oral, first-in-class, highly potent, selective inhibitor of factor B, a key regulator of the AP. In Phase 2 studies in IgA nephropathy, paroxysmal nocturnal hemoglobinuria, and C3 glomerulopathy, iptacopan inhibited the AP, showed clinically relevant benefits, and was well tolerated. Moreover, iptacopan showed clinically meaningful results in the Phase 3 APPLY PNH study. Thus, Iptacopan has the potential to become an effective and safe treatment for aHUS, with the convenience of oral administration.

Methods: APPELHUS (NCT04889430) is a multicenter, single-arm, open-label, Phase 3 study evaluating the efficacy and safety of iptacopan 200 mg twice daily in adult patients with aHUS (N = 50) naïve to complement inhibitor therapy. Eligible patients must have evidence of TMA (platelet count $<150 \times 10^9$/L, LDH $\geq 1.5 \times$ ULN, hemoglobin $\leq$LLN, serum creatinine $\geq$ULN). Primary endpoint is the proportion of patients achieving complete TMA response without the use of plasma exchange/plasma infusion or anti-C5 antibody during 26 weeks of treatment. This treatment period is followed by an extension treatment period of 26 weeks of treatment with iptacopan. Upon completion, eligible patients will be offered post-trial access to iptacopan.

Results: The study is currently recruiting in 32 sites worldwide.

Conclusion: APPELHUS will determine if iptacopan is safe and efficacious in patients with aHUS.

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Bi-specific antibodies targeting C1q and a tumor antigen activate the complement system independent of C1q-Fc interactions

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Complement dependent cytotoxicity (CDC) is an effector function employed by many therapeutic antibodies to kill their target cells. Classical pathway complement activation is initiated when C1q binds to a cluster of IgG molecules bound to a cell surface. However, not all antibodies are able to effectively activate complement, because of constraints like antigen density, clustering, and orientation. To overcome these limitations we have developed bispecific antibodies (bsAb), binding both C1q and cellular targets, to activate complement optimally.

Human IgG1 bsAb were produced by Fab-arm exchange, combining several different C1q-binding arms with arms binding CD20 (Rituximab), CD37 (clone 37-3) and HER2 (Trastuzumab). Antigen binding, complement activation and CDC were evaluated by flowcytometry on Ramos, Raji and AU565 cancer cell lines.

On all cellular targets tested, the bsAb provided more complement C3 deposition and CDC than the corresponding monovalent antibody without C1q-binding Fab arm. The wildtype bivalent Trastuzumab antibody do not yield CDC, importantly however anti-C1q bsAb based on Trastuzumab could kill their HER2-positive target cells. For anti-CD37, the low level CDC by the bivalent antibody was enhanced by anti-C1q bsAb, while high CDC by Rituximab was matched by the corresponding anti-C1q bsAb. Experiments with inactive Fc-domain mutants and Fab2 fragments of bsAb indicate that complement activation by these bsAb does not require the Fc-domain. Comparing different C1q-targeting arms in the bsAb revealed that, although they all strongly increase C1q binding, only some also enhance complement activation and CDC, reflecting the different modes of C1q binding.

From the presented data, we conclude that (1) complement activation by therapeutic bispecific antibodies through Fab-C1q binding is feasible and (2) that it can improve complement activation over traditional Fc-mediated activation, with important implications especially for low abundant antigens.

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Expression of complement regulators on red blood cell progenitors during *in vitro* and *in vivo* erythropoiesis

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Background: The complement system is tightly regulated to prevent damage to host cells. Human cells can express four membrane-bound complement regulators: CD35, CD46, CD55 and CD59, with varying expression patterns between cell types. Erythrocytes do not express CD46, while their progenitor hematopoietic stem cells do express CD46. Here, we aim to track complement regulators during erythropoiesis, determine how expression is controlled and assess complement sensitivity during differentiation. This will provide insights into protection of erythrocytes against complement in different developmental stages and better understanding pathologies such as autoimmune hemolytic anemia (AIHA). Ultimately, this could aid in the development of erythrocytes for transfusion that are better protected against complement-mediated lysis.

Methods: Peripheral blood mononuclear cells (PBMCs) from healthy donors were used to induce differentiation from hematopoietic stem and progenitor cells to erythrocytes, using a 3-phase in vitro culture system. Cells were incubated with AIHA patient serum or recombinant antibodies to induce complement activation and assess sensitivity. During differentiation cells were stained for differentiation markers, complement regulators and complement activation using FACS on multiple days and compared to gene expression profiles. Bone marrow of healthy donors was analysed similarly to compare in vitro to in vivo erythropoiesis. In addition, presence of soluble CD46 in culture supernatant was determined by ELISA.

Results: In vitro erythropoiesis was confirmed by enucleation, reduced CD71 and increased CD235a expression during the differentiation phase. Expression of CD55 and CD59 was similar in vitro and in vivo and constant throughout differentiation. CD35 was completely absent on in vitro cultured reticulocytes, while it was highly expressed on in vivo reticulocytes. CD46 expression disappeared completely between day 2 and 7 of in vitro differentiation. Preliminary results suggest that complement sensitivity was highest at day 1-7 of differentiation.

Conclusion: During in vitro erythropoiesis, CD46 expression was lost between day 2 and 7, before enucleation. This reflected the loss of CD46 as observed during in vivo erythropoiesis. In addition, in vitro generated reticulocytes lack CD35, normally expressed on donor reticulocytes. Future research will further investigate how this altered expression of complement regulators relates to the complement sensitivity of the cells during differentiation.

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The mycobacterial strain-dependent impact of the complement system in tuberculosis

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Tuberculosis (TB) is the most prevalent bacterial infectious disease in humans and is caused by bacteria of the Mycobacterium tuberculosis complex (MTBC), which are composed of three different human-adapted pathogens Mycobacterium tuberculosis (Mtb), Mycobacterium africanum, and Mycobacterium canettii. Besides the genetic diversity of MTBC clinical isolates, host factors also significantly influence the pathogenesis of TB. However, the relevance of the complement system which is activated by the classical, the lectin or the alternative pathway has yet to be adequately investigated. Mannose-binding lectin (MBL) recognizes specific cell surface carbohydrates such as mannosylated lipoarabinomannan, one of the main cell wall components of slow-growing mycobacteria. MBL can act as an opsonin or activate the lectin pathway (LP) of the complement cascade and thereby modulate the innate and adaptive immune response. Although MBL may be considered the main factor activating the LP upon recognition of mycobacteria, ficolins may also act as carbohydrate-binding molecules. Nevertheless, most findings are based on the lab-adpated Mtb strain H37Rv, Mycobacterium bovis Calmette-Guérin, and non-virulent mycobacteria.

By using clinical isolates of MTBC, we found in the present study that collectins of the LP recognize mycobacteria and that high binding of C1q additionally indicates the participation of the classical pathway (CP). Interestingly, we observed a differential binding of MBL and complement component (C)1q to various MTBC clinical isolates. Based on these results we further investigated the activation of the complement cascade and found that MBL or C1q binding leads to the deposition of the complement components C4b, C3b and the terminal complement complex (TCC) on the surface of mycobacteria in a strain-dependent manner. Furthermore, first results also imply a relevance of the alternative pathway for complement deposition. Finally, to understand the impact of complement opsonization on macrophage infection we observed in first experiments a significant decrease in the uptake of mycobacteria when using complement-depleted heat-inactivated autologous serum. Together, our results so far suggest a previously underappreciated role of the complement system during infection with clinical isolates of the MTBC.

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Plasma factor D cross-sectionally associates with low-grade inflammation, endothelial dysfunction and cardiovascular disease, but not with intima-media thickness or ankle-brachial index: The Maastricht Study

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Background and aim: The complement system, particularly the alternative complement pathway, may contribute to vascular damage and development of cardiovascular disease (CVD). We investigated the association of factor D, the rate-limiting protease in alternative pathway activation, with adverse cardiovascular outcomes.

Methods: In 2947 population-based participants (50.6% men, 59.9±8.2 years, 26.5% type 2 diabetes [T2D], oversampled) we measured low-grade inflammation (LGI, composite score, in SD), endothelial dysfunction (ED, composite score, in SD), carotid intima-media thickness (cIMT, in μm), ankle-brachial index (ABI, ratio [no unit]), prevalent CVD and plasma concentrations of factor D (in SD). Associations between factor D (main independent) and the cardiovascular outcomes were estimated using multiple linear and logistic regression, adjusting for demographic, lifestyle, and dietary factors.

Results: Factor D (per SD) significantly associated with LGI (0.17 SD [0.14; 0.21]), ED (0.16 SD [0.12; 0.19]) and CVD (OR 1.15 [1.04; 1.27]) but not significantly with cIMT (-6.62 μm [-13.51; 0.27]) or ABI (-0.003 [-0.007; 0.001]). Interaction analyses show that factor D more strongly associated with ED in those without diabetes (0.24 SD [0.18; 0.29] than in participants with T2D (0.10 SD [0.03; 0.16]), $P_{interaction}$ <0.05. In contrast, factor D inversely associated with cIMT in non-diabetes (-13.37 μm [-21.84; -4.90]), but not in participants with T2D (4.49 [-7.91; 16.89]), $P_{interaction}$ <0.05.

Conclusion: Plasma factor D is independently associated with LGI, ED, and prevalent CVD but not with ABI or cIMT. Hence, greater plasma factor D concentration in CVD may potentially induce complement activation which, in turn, might contribute to further disease progression via a process that may involve inflammation and endothelial dysfunction but be directly related to neither atherosclerosis nor arterial injury. The observation that, in participants without diabetes, factor D associated with worse ED but smaller cIMT warrants further investigation.

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The binding and kinetics of normal versus pathogenic C3 convertase autoantibodies

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Background: We have identified an antibody reactive to the C3 convertase (labelled C3CAb) in a high proportion of normal test subjects. Despite the presence of this antibody, none of the test subjects displays excessive complement activity as is typical in the presence of pathogenic anti-convertase autoantibodies called C3 Nephritic Factors (Nefs). To begin characterizing C3CAbs and their differences from C3Nefs, we investigated the effect of temperature on the antibody-antigen interaction.

Methods: Utilizing surface plasmon resonance, C3 convertase was formed at various temperatures ranging from 22-40C in the presence of Mg++. At each temperature, three samples were injected: FB with FD alone, FB with FD and C3Nef+ IgG, or FB with FD and C3CAb+ IgG. Binding was recorded, normalizing decay kinetics between each sample.

Results: Both C3Nefs and C3CAbs bind to reagent convertase regardless of temperature. For example, at 37C the reagent proteins produced a response of 59.6 RUs while the C3Nef sample had 154.4 RUs and the C3CAb sample had 147.9 RUs. At 25C, RUs were 59.5, 162.3, and 132.9 respectively. However, the ability to influence convertase kinetics was temperature dependent. At 37C, the baseline convertase half-life was 92.5s, the C3Nef half-life was 377s, and the C3CAb half-life was 102.5s. At 40C, times were 64, 284.5, and 66 seconds, and at 28C, they were 181, 696, and 267 seconds, respectively. Thus, while the C3Nef sample always maintained a longer half-life, the C3CAb had minimal impact on convertase decay at physiologic temperatures. At cooler temperatures, C3CAb stabilization was more pronounced, although never to the extent of the C3Nef.

Discussion: Our results show that C3CAb and C3Nef bind C3 convertase across a range of temperatures. However, unlike C3Nef, C3CAb do not increase complement activity at physiological temperatures. The results from our study suggest this difference is due, in part, to the reduced impact C3CAb have on convertase stabilization.

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Protective role of complement factor H against the development of preeclampsia

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Complement system plays a crucial role in the successful establishment of pregnancy and parturition. Its dysregulation can lead to complications such as preeclampsia, spontaneous abortion, and preterm birth. Complement regulators at the maternal-foetal interface are likely to prevent inappropriate complement activation to protect the foetus. Factor H (FH) is an important negative humoral regulator of the alternative pathway. Thus, the expression and localization of FH in human placenta (tissue and cells) and serum was investigated in normal pregnancy (NP) and Preeclampsia (PE). The association of FH with placental syncytiotrophoblast derived extracellular vesicles (STBEVs) was also examined. The RT-qPCR revealed that gene expression levels of FH followed the order of decidual stromal cells (DSC) > decidual endothelial cells (DEC) > extravillous trophoblast (EVT) isolated from 1st trimester NP. However, the human umbilical vein endothelial cells (HUVEC) showed the highest FH expression. In the 1st and 3rd trimester NP tissue, two-fold increase of FH gene expression was observed compared to HUVEC. FH expression was lower in PE compared to NP tissue and serum. FH mRNA expression in DECs and DSCs were downregulated following their treatment with serum of PE mothers for 24 hrs as compared to NP serum. When DECs were treated for 24 hrs with syncytotrophoblast microvesicles (STBMs) isolated from the placental tissue of NP and PE mothers, STBMs from PE downregulated FH expression. IHC revealed higher FH staining in NP placenta than the PE that was localised in the foetal villi and intervillous space. When DSC, DEC and EVT cells isolated from 1st trimester NP placenta were treated with pro-inflammatory IL-1β and TNF-α, FH expression was significantly upregulated. Our results suggest a protective role of FH in pregnancy; and its downregulation possibly may cause adverse pregnancy conditions such as PE.

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Activation of complement in a mouse model of severe COVID-19

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Background: Research has demonstrated complement activation is a hallmark of severe COVID-19. While the association between COVID-19 and complement has been studied extensively in humans, it has yet to be investigated in an animal model. Here using a mouse model of severe COVID-19 lung disease, we find evidence of complement activation and describe its possible role in lung injury.

Methods: To characterize the complement response to SARS-CoV-2 infection, 6-8 week old BALB/c mice were inoculated intranasally with $5x10^3$ PFU of mouse-adapted SARS-CoV-2 virus (SARS-CoV-2_{MA30}), which reliably recapitulates key phenotypic features of severe COVID-19 seen in humans[1]. We next utilized $C3^{-/-}$ mice on a C57BL/6J background to evaluate the response to infection in the absence of complement activity. Mice were euthanized, with organs and serum collected for analysis of complement response and evaluation of disease phenotype.

Results: BALB/c mice develop exuberant complement activation in response to SARS-CoV-2, with increases in serum C3, C4, and CH50 activity levels by 2 days post-infection (d.p.i.). Immunofluorescence (IF) staining demonstrates pronounced C3 deposition in the lungs by 4 d.p.i. Colocalization by IF staining suggests direct interaction with type I alveolar, ciliated, and endothelial cells, with immunoblot and IF for C3b/iC3b/C3c confirming presence of C3 fragments consistent with activation. In C57BL/6J

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mice, the complement response to SARS-CoV-2_{MA30} was diminished compared to BALB/c mice. Surprisingly, in $C3^{-/-}$ mice the absence of C3 resulted in no significant difference in weight loss or mortality. Indeed, C3 appears to have a protective role early in disease course. In its absence, mice have increased viral burden in lung tissue, delayed viral clearance, increased lung injury, and increased early cytokine markers of systemic inflammation.

Conclusion: We have successfully produced a reliable mouse model of severe COVID-19. Similar to humans, mice infected with SARS-CoV-2_{MA30} develop significant complement response with pulmonary activation. Despite strain differences in the degree of complement activity, our results indicate that complement activation in the lung plays an important role in the immune response to this deadly virus.

Reference

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Human factor H and properdin modulate SARS-CoV-2 infection in a complement-independent manner

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Background: The complement alternative pathway (AP) is associated with undesirable inflammation during severe SARS-CoV-2 infection. Dysregulated serum levels of AP regulatory proteins, such as low levels of factor H and elevated levels of properdin, have been reported in cases of severe COVID-19 infection. This study aimed to determine the role of locally produced factor H and Properdin by fibroblasts and neutrophils, respectively, in SARS-CoV-2 infection, independent of complement activation.

Methods: Interactions of Factor H and Properdin with the SARS-CoV-2 spike protein were examined via ELISA. Cell binding and luciferase reporter assays were used to evaluate the potential effects of factor H and Properdin on SARS-CoV-2 cell entry and binding. The immune modulatory roles of factor H and Properdin in the SARS-CoV-2 inflammatory response were assessed using RT-qPCR.

Results: ELISA revealed that the SARS-CoV-2 spike protein, and the receptor binding domain (RBD), bound Properdin via thrombospondin type I repeats 4 and 5 (TSR4+5) and factor H. Additionally, treatment of SARS-CoV-2 lentiviral pseudoparticles with either properdin or recombinant TSR 4+5 (rTSR 4+5) increased viral binding and entry in A549 cells expressing human ACE2 and TMPRSS2. The higher infectivity observed was reversed by adding a Properdin-specific antibody, confirming that the increased infectivity was Properdin mediated. However, factor H treatment reduced SARS-CoV-2 lentiviral pseudotype cell entry and binding. Properdin and rTSR4+5 treatment was also found to promote NF-kB activation A549-h ACE2 + TMPRSS2 cells challenged SARS-CoV-2 spike protein, whilst FH treatment reduced NF-kB activation. The mRNA levels of IL-1β, IL-8, IL-6, TNF-α, IFN-α, NF-kB and RANTES were also shown to be elevated in A549-hACE2 +TMPRSS2 cells challenged with SARS-CoV-2 alphaviral pseudoparticles treated Properdin or rTSR4+5, while factor H treatment downregulated the mRNA expression of pro-inflammatory cytokines compared to their respective control.

Conclusions: These findings suggest that factor H and Properdin differentially modulate SARS-CoV-2 infection and associated inflammatory events. These results reveal that complement regulatory proteins, which act as a first point of interaction during SARS-CoV-2 infection, can modulate infection independent of complement activation.

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Increased infiltration of $CD4^+$ T cell in the complement deficient lymphedema model

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Lymphedema is an intractable disease that can be caused by injury to lymphatic vessels, such as by surgical treatments for cancer. It can lead to impaired joint mobility in the extremities and reduced patient quality of life. Chronic inflammation due to infiltration of various immune cells (such as $CD4^+$ T lymphocytes, macrophages, and neutrophils) in a lymphedema region is thought to lead to local fibrosis. However, the detailed molecular pathogenesis of lymphedema remains unclear. Elucidation of the immunological mechanisms involved in progression of lymphedema is important for the development of effective therapies. The complement system is an innate immune system, and the activated complements eliminate not only pathogens, but also altered host cells, such as apoptotic and necrotic cells. Complement-targeted therapies have recently been clinically applied to various diseases caused by complement overactivation. Determination of whether complement activation is involved in the development of lymphedema is therefore important.

In the present study, we used mouse tail lymphedema models to show increased expression of C3, and that the classical or lectin pathway is activated in the local region. Complement activation is thus suggested to be involved in lymphedema progression. We compared the C3 knockout (KO) mouse lymphedema model with the wild-type. There was no difference in the degree of edema at three weeks postoperatively, but there was a significant increase of $TUNEL^+$ necrotic cells and $CD4^+$ T cells in the C3 KO mice. Infiltration of macrophages and granulocytes was not significantly elevated in C3 KO or C5 KO mice compared with those of wild-type mice. Impaired opsonization and decreased migration of macrophages and granulocytes due to C3 deficiency should therefore induce the accumulation of dead cells and may lead to increased infiltration of $CD4^+$ T cells. The present study demonstrates the need for vigilance for exacerbation of lymphedema when surgical

treatments may have potential to have injured lymphatic vessels in patients undergoing these complement-targeted therapies, or in patients with complement deficiency.

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Functional assessment of the alternative complement pathway in the first line treatment of B cell Non-Hodgkin Lymphoma

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Background: Non-Hodgkin's lymphoma is considered as the first hematological cancer in terms of health and human costs. Since no study has addressed yet the alternative complement (ACP) activation and its regulation by using fictional assays, we sought to investigate at which extent ACP functional activity can be relevant in B cell Non-Hodgkin Lymphoma (BCNHL).

Methods: A longitudinal kinetic follow up of 28 BCNHL patients was performed by monitoring ACP and complement factor H (FH) functional activities over the entire first-line treatment period.

Results: Indeed, continuous impairment in ACP function (AH50 non responders=74.38 ± 9.99 %NHP; AH50 responders = 84.38 ± 11.01 %NHP; p=0.001) and time-dependent enhancement of FH activity (FH non responders = 91.84 ± 5.4%NHP; FH responders = 86.62 ± 5.08%NHP, p=10-3) were found in patients who failed to the first line treatment. Furthermore, significant enhancement of AH50 along the cumulative doses of first line therapy (R2=0.19; p=10-3) was observed responder patients.

Conclusion: Our study confirms that impairment in ACP functional activity yielded poorer clinical outcomes in BCNHL.

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Association between autoantibodies and complement activation in systemic lupus erythematosus

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Background: Systemic lupus erythematosus (SLE) is an immune complex deposition disease; therefore, complement plays an essential role in its pathogenesis. Since SLE is characterized by the production of a variety of autoantibodies, we sought to identify the most relevant autoantibodies for complement activation.

Methods: Seventy SLE patients were enrolled for autoantibody profiling (anti-soluble nuclear antigen, anti-double-stranded DNA, histone autoantibodies, anti-nucleosome; anti-C1q, anti-cardiolipin, anti-β2GPI and circulating immune complexes (CIC)) and complement assessment (total complement activity (CH50) and serum C3, C4, C1q and C2).

Results: Our work shows that the presence of anti-SSA autoantibodies was associated with the consumption of C3 (p=0.000) and that the anti-dsDNA FidisTM antibodies correlated with the decrease in CH50 (r=0.579; p=0.001) while anti-dsDNA ELISA antibodies correlated with anti C1q antibodies (r=0.593; p=0.001). Anti-histone positivity was associated with higher levels of C1q (p= 0.016). Also, high levels of CIC are associated with the decrease in CH50 (p=0.017) and correlated with anti C1q levels (r=0.574; p=0.001).

Conclusion: We found that anti dsDNA, anti SSA and anti C1q antibodies are the most associated with complement consumption in SLE.

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Complement biomarkers reflect the pathological status of neuromyelitis optica spectrum disorders

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Background: Complement is involved in the pathogenesis of neuroimmune disease, but the detailed pathological roles of the complement pathway remain incompletely understood. Recently, eculizumab, a humanized anti-C5 monoclonal antibody, has been clinically applied against neuroimmune diseases such as myasthenia gravis and neuromyelitis optica spectrum disorders (NMOSD). Clinical application of eculizumab is also being investigated for another neuroimmune disease, Guillain-Barré syndrome (GBS). However, the effectiveness of eculizumab for NMOSD is extremely high in many cases, and there are some cases with no or limited efficacy of eculizumab for myasthenia gravis and GBS. Development of effective biomarkers that reflect complement activation in these diseases is therefore important.

Methods: We retrospectively analyzed serum levels of complement factors in 21 patients with NMOSD and 25 patients with GBS to identify biomarkers that could predict disease status.

Results: Ba, an activation marker of the alternative complement pathway, was elevated in the acute phases of both NMOSD and GBS, but sC5b-9, an activation marker generated by the terminal complement pathway, was elevated in NMOSD but not in GBS. Complement factor H (CFH), a complement regulatory factor, was decreased in the acute phase as well as the remission phase of NMOSD, but not in any phases of GBS.

Conclusion: In this study, we suggest that complement biomarkers, such as Ba, sC5b-9 and CFH in peripheral blood, have potential utility in understanding the pathological status of NMOSD.

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Opsonization and efferocytosis of dying cells are modulated by the presence of malondialdehyde epitopes

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Background: Increased oxidative stress leads to the formation of lipid peroxidation-derived structures that form oxidation-specific epitopes (OSEs) and act as damage-associated molecular patterns (DAMPs). Malondialdehyde (MDA)-adducts are prototypical examples of such OSEs and can be found on dying cells, oxidized lipoproteins, and a subset of extracellular vesicles. We and others have shown that MDA-epitopes are recognized by scavenger receptors, natural IgMs, factor H (FH), and FH-related proteins (FHR1, FHR3, FHR5). Once bound to MDA-epitopes, these proteins determine consequent innate immune responses. To elucidate the role of MDA-epitopes in the removal of dying cells and homeostasis maintenance, we investigated the mechanisms of their appearance and their roles in opsonization and efferocytosis.

Methods & results: Using flow cytometry, we found that the presence of MDA-epitopes on dying cells correlates with loss of cellular membrane integrity, as measured by 7-Aminoactinomycin D positivity. MDA-epitopes were detected on the surfaces of dying cells generated by extrinsic or intrinsic apoptotic-, ferroptotic-, and necroptotic cellular death pathways. Furthermore, we demonstrated by ELISA that initiators of the classical and alternative complement pathways, C1q and C3b, also bind to MDA-epitopes. However, the extent of complement activation on MDA-decorated surfaces was controlled by the MDA-dependent recruitment of the complement regulators FH and C4b-binding protein (C4BP). Using flow cytometry, we further found that the opsonization of dying cells by IgMs, C1q, C4BP, C3b, and C5b-C9 complexes depends partially on the presence of MDA-epitopes. Finally, we observed that the efferocytosis rate was determined by the density of MDA-epitopes and the repertoire of opsonins.

Conclusion: Our data suggest that MDA-epitopes on dying cells with impaired membrane integrity guide opsonization by complement components and thereby modulate their recognition and clearance by phagocytic cells.

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DNA-templated antibody complexes provide insights into the geometric requirements of human complement activation

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The classical complement cascade can be activated via antigen-bound antibodies (IgG or IgM) in response to pathogens during infection or to autoantigens in autoimmune diseases. Whereas IgM circulates as a pre-formed pentamer or hexamer, IgG exists as monomers which have the ability to oligomerize via non-covalent Fc-interactions upon surface binding. Furthermore, it is known that at least two IgGs are required for C1 binding, with recent studies having shown that IgG1 mutants with enhanced hexameric oligomerization potential are able to activate the complement system more efficiently. However, structural data reveal that it is not necessary to bind all 6 C1q arms to initiate the complement cascade, but that already binding of 4 or 5 C1q arms is sufficient. These observations reveal a symmetry mismatch between C1 and the activating hexameric Abs complex, which has not been adequately explained.

Here we use DNA nanotechnology to produce specific nanostructures in order to template nanometre precise chemically conjugated antigens for antibody binding. We can bind these self-assembled DNA platforms to cell-mimetic lipid membranes, and assess complement activation in a controlled environment. This system enabled us to determine the effect of valency on complement activation, without the requirement of mutated antibodies. We investigated this using biophysical assays together with 3D cryo-electron tomography (cryoET). Our data revealed that pre-formed antibody complexes activated complement to a greater extent than the same number of antibodies left un-patterned on the surface of liposomal cell mimetics. Furthermore, biophysical analysis showed that, for similar C1 binding, increased valency caused an increased activation of the protease domains of C1 as measured via C4 cleavage and MAC pore formation.

Together, these data provide insights into how nanopatterning antigen-antibody complexes influences activation of the C1 complex, and suggests routes to modulate complement activation by antibody engineering. Furthermore, this is the first time DNA nanotechnology has been used to study the activation of the complement system.

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Pattern recognition plays a role in the initiation of the alternative complement pathway

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Pattern recognition is essential to restrict complement activation to unwanted surfaces. Contrary to the classical and the lectin pathways triggered by specific macromolecular interactions, the alternative pathway (AP) constantly opsonizes surfaces at low level.

Imbalance in complement regulation can drive various pathologies, such as age-related macular degeneration, the leading cause of blindness in elderly. Genome-wide association studies strongly suggest that the AP plays a pivotal role in the pathogenesis of this disease, but the underlying molecular mechanism still remains obscure.

To gain insight into pathological AP activation, we set out to study the initiation of this process on various eukaryotic cell surfaces. Human serum is effective at lysing unsensitized rabbit erythrocytes under conditions permissive only for the AP. As a first step, we used this well-established model system to gain insight into the activation of this proteolytic cascade. Surprisingly, we found striking differences among rabbit red cells in their capacity to become opsonized by C3b when exposed to normal human serum. This initial heterogeneity remains detectable also at subsequent stages in the cascade also at the level of MAC formation. The number of C3d-positive rabbit erythrocytes largely increased when cells were incubated with FH-deficient serum. Nevertheless, the heterogeneity in opsonization still persisted under these conditions. The same discriminative feature was detected when the above experiments were repeated by using sheep erythrocytes and human cell lines lacking complement protection. We also asked if purified components of the AP mixed in physiological concentration are sufficient to discriminate among potential target membranes. Notably, this mix was not sufficient to catalyze the deposition of C3b.

These results suggest that the AP exhibits a certain surface preference even at a very early stage of fluid-phase activation. C3b-acceptor molecules may become more available on deteriorated cells and if so, these scavengers can divert an emerging AP activation and preserve cells with greater fitness. Similarly, cells have different abilities to recruit complement regulators that can confer complement resistance and ultimately cell survival. A better understanding of the AP selectivity and regulation will pave the way for therapies that protect worn membranes and surfaces present in old eyes.

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Association between novel lipoprotein particles and age-related macular degeneration

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Background: Previous studies have shown an association between extra-large high-density lipoprotein (XL-HDL) levels in blood and age-related macular degeneration (AMD). In addition, there is increasing evidence to support a relationship between lipoproteins and the complement system, the major pathway in AMD pathogenesis. Recently, a novel lipoprotein fraction with a size between XL-HDL and low-density lipoprotein 4 (LDL IV), termed the midzone, was identified by ion mobility assay and found to be correlated with cardiovascular disease. We tested whether this midzone fraction is also associated with complement activation and AMD.

Methods: Plasma samples from 197 advanced AMD (60% women, mean (SD) age 74.93 yrs (9.52 yrs)) with 200 controls (59% women, mean (SD) age 73.59 yrs (9.64 yrs)) and 100 intermediate AMD patients (60% women, mean (SD) age 75.85 yrs (9.63 yrs)) with 102 controls (61% women, mean (SD) age 75.97 yrs (8.47 yrs)) were selected from our biobank. The lipoprotein spectrum was determined using an ion mobility assay. Complement activation levels determined as the C3d/C3 ratio were available for all samples, measured by rocket immunoelectrophoresis and radial immunodiffusion respectively. We performed linear regression analysis to assess associations in R using the PheWAS package.

Results: A significant association was observed between the midzone concentration and complement activation (in advanced AMD samples: p = 4.6 * 10-5, R^2 = 0.13; in intermediate AMD samples: p = 1.9 * 10-4, R^2 0.14). Concentrations of the HDL-midzone boundary, a subfraction of the midzone, were also significantly increased in advanced AMD (median nmol/L (Q1-Q3): AMD 368.1 (316.3-427.5), controls 355.8 (307.1-401.8), p = 0.0328; corrected for age). No significant difference was found between intermediate AMD and controls (p = 0.959).

Conclusions: Novel midzone lipoprotein particles appear to play a role in complement activation and in AMD. Although in need of confirmation, our findings may explain the underlying biology in the association between HDL and AMD.

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Acquired ficolin-3 deficiency in patients with Systemic Lupus Erythematosus

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Background: Ficolin-3 is the main initiator of the lectin pathway in humans. Case reports of ficolin-3 deficient patients have suggested that ficolin-3 deficiency may be enriched in patients with Systemic Lupus Erythematosus (SLE), a systemic autoimmune disease where complement plays an important role. Therefore, this study aimed to investigate the activity levels of ficolin-3 and to identify potential ficolin-3 deficient individuals in two Swedish SLE cohorts.

Methods: Serum or plasma samples from SLE patients (n=810) and matched controls (n=566) were collected from the Karolinska Institute (KI) and Umeå University Hospital. The ficolin-3 activity levels were measured by an in-house developed functional ELISA with a pooled normal human serum sample as a reference. Serial samples were analyzed for ficolin-3 deficient patients when available. Sequencing data were analyzed for FCN3 frame-shift mutation +1637delC (rs532781899) and other potential loss-of-function (LoF) variants.

Results: This screening revealed that the level of ficolin-3 activity varies largely in patients with SLE. The activity levels also show that SLE patients seem to generally have elevated ficolin-3 activity compared to the control group ($p<0.0001$). Out of 810 patients with SLE, four patients were determined to be ficolin-3 deficient. For two of these patients, the ficolin-3 activity was at normal levels at the time of diagnosis and thereafter depleted over time, indicating an acquired deficiency. For deficient patients, no or very low ficolin-3 protein levels and no lectin pathway-dependent complement activation could be detected. Autoantibodies against ficolin-3 were not detectable. No patients were homozygous for the +1637delC frameshift mutation, whereas in total 10 patients were determined to be heterozygous carriers. These heterozygous patients displayed lower levels of ficolin-3 activity but did not include the deficient patients. Additional possible LoF variants were analyzed but none were enriched in either patients or controls.

Conclusions: Contrary to the classical pathway of the complement system we show that genetic ficolin-3 deficiency is not a risk factor for SLE. Instead, acquired ficolin-3 deficiency was observed in a subgroup of SLE patients, possibly due to a potent activation of the lectin pathway that depleted ficolin-3 plasma levels in these individuals.

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Heme-induced complement activation in invasive aspergillosis

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Background: Invasive aspergillosis is among the most common fungal infections in immunocompromised patients. With the increasing number of susceptible patients, the development of host-centric interventions is of paramount importance. Pulmonary hemorrhage is a characteristic clinical feature of invasive aspergillosis, and is associated with worse lung injury and a more lethal infection. However, the effects of free heme on the host during invasive aspergillosis are poorly understood. We hypothesized that a mechanism by which free heme leads to worse pulmonary injury is via activating the terminal complement pathway, driving inflammation and tissue injury.

Methods: C57BL/6 mice were neutrophil depleted and then infected with A. fumigatus. To characterize the dynamics of complement activation in the alveolar space, we measured levels of C3a and C5a in the bronchoalveolar lavage fluid during the infection. To test the effects of heme on the host, mice were administered exogenous hemin after infection, and C3a and C5a levels were measured by ELISA, and compared to mice challenged with vehicle control. As a secondary model of heme accumulation, we infected hemopexin -/- mice and measured complement levels. Lastly, we antagonized C5aR1 and measured fungal burden and lung injury on C57BL/6 compared to vehicle control.

Results: Complement is activated in the alveolar space of Aspergillus-infected mice with C5a levels peaking before C3a levels, and exogenous administration of hemin leads to higher levels of anaphylatoxins C3a and C5a in the alveolar space. Hemopexin-deficient mice had higher levels of alveolar C3a, and administration of exogenous hemopexin to hemopexin -/- mice led to a decrease of C3a in the alveolar space– suggesting that heme scavenging attenuates complement activation. Due to the early peak of alveolar C5a levels, and the putative inflammatory and potentially harmful role of C5aR1 signaling (as shown in other models of acute lung injury) we antagonized C5aR1, which led to a decrease in the lung fungal burden of infected female mice, but not in male mice.

Conclusion: Complement is induced in the alveolar space during invasive aspergillosis in mice. This study indicates that inhibiting C5a signaling results in better microbial clearance during invasive pulmonary aspergillosis in female mice.

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Loss of the complement regulator CD55 alters marginal zone B cell homeostasis

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Background: Complement and B cells are implicated in the pathophysiology of rheumatoid arthritis, an autoimmune disease characterized by synovial inflammation. Loss of complement regulator CD55 leads to delayed onset of inflammatory arthritis in the collagen-induced mouse model of arthritis (CIA).1 Marginal zone

(MZ) B cells, an innate-like B cell population, serve as first responders to blood-borne pathogens by rapidly differentiating into antibody-producing plasmablasts, and are important initiators of CIA.2 Whether and how CD55 regulates MZ B cells in CIA pathogenesis is unknown.

Methods: We examined naïve and sorted MZ B cell populations in 3-5 week-old and 10-12 week-old CD55-/- and compared to wildtype (WT) C57BL/6 mice using flow cytometry, immunofluorescence, bromodeoxyuridine (BrdU) incorporation, and bulk RNA-seq. Differential gene expression analysis was performed, followed by Gene Sequence Enrichment Analysis (GSEA).

Results: We found that loss of CD55 decreased the number of MZ B cells in 10-12 week-old, but not 3-5 week-old mice. The precursors to MZ B cells were unchanged at both endpoints, indicating that loss of MZ B cells was not due to failure of MZ B cell differentiation. En vivo BrdU incorporation showed no difference between CD55-/- and WT, indicating MZ B cell differences are not due to loss of proliferative capacity. Differential gene expression analysis showed decreased expression of genes involved in preventing apoptosis in MZ B cells of CD55-/- compared to WT mice (HSP90b1, STIP1, AHSA1, and FKBP4). GSEA showed decreased expression of cell cycle/cell survival pathways in CD55-/- cells relative to WT. Flow cytometry revealed that CD55-/- MZ B cells had corresponding increases in activated caspase-3 and caspase-7, as well as increases in cellular reactive oxygen species relative to WT cells.

Conclusion: Loss of CD55 led to increased reactive oxygen species and activation of caspases in MZ B cell populations, which may prevent the normal expansion of the MZ B cell subset over time. These studies suggest a role for the complement regulator CD55 in MZ B cell homeostasis and survival. Future experiments are aimed at defining whether loss of CD55 impacts MZ B cell survival through complement-dependent or independent mechanisms.

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Tumor-derived complement is required for tumor growth in syngeneic murine STK11-mutant lung adenocarcinoma

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Background: Loss of function STK11 mutations occur in 15-20% of non-small cell lung cancer (NSCLC) and are associated with poor survival and resistance to immune checkpoint inhibitors (ICI). We observed in human STK11-mutant NSCLC upregulation of C3, Factor D, Factor H, and CD55. We previously observed that in the tumor microenvironment (TME), neutrophils (PMN) acquire complement-dependent T cell inhibitory function. Less is known about the roles of tumor-derived complement in tumor progression. We hypothesized that tumor-derived complement will promote growth of STK11-mutant tumors in vivo.

Methods: CMT167 (syngeneic NSCLC) tumor cells with Stk11 deletion with and without C3 deletion were generated by CRISPR-Cas9. To evaluate the role of tumor-derived versus systemic complement in tumor progression, we compared growth of subcutaneous (s.c.) administered Stk11KOC3WT and Stk11C3KO tumor in WT versus C3-/- mice. To test the interaction of tumor-derived C3 and T cell immunity in regulating tumor growth, we compared tumor growth in WT versus nude mice. Finally, we evaluated whether PMN depletion with anti-Ly6G or Cxcr2 inhibition would overcome resistance of Stk11KO tumors to anti-PD-1 treatment.

Results: We observed high infiltration of PMN and low CD8 T cell in Stk11KO tumor in vivo. Systemic C3 deficiency resulted in modest delay of growth of Stk11WT tumors, but had no effect on growth of Stk11KO tumors. Deletion of C3 in CMT167-Stk11KO (CMT167 Stk11/C3KO) resulted in dramatic inhibition of tumor growth in immune competent mice, but had no effect in nude mice. While anti-PD1 treatment alone had modest to no effect in the growth of Stk11KO tumors, the addition of anti-Ly6G or Cxcr2 inhibitor resulted in improved control of tumor growth.

Conclusions: Tumor-derived C3 drives Stk11KO tumor growth in mice. Growth of Stk11KO tumor was strikingly dependent on tumor-derived C3 in immunocompetent mice but dispensable in nude mice. These results support a role for tumor-derived C3 suppressing T-cell immunity, potentially indirectly through recruitment of PMN or inducing PMN suppressor function. Our results also provide rationale for targeting tumor-derived complement and inhibiting Cxcr2 to enhance ICI efficacy as novel therapeutic approaches in STK11-mutant NSCLC.

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Strategic targeting of complement to regulate neuroinflammation and synapse pruning in Alzheimer's disease

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Alzheimer's disease (AD), resulting in progressive cognitive decline, is the most prevalent form of dementia in the elderly. Neuroinflammation is now recognized as a contributor to the progression of late onset AD, and complement activation is associated with pathological hallmarks of AD. In animal models of AD, early components of the classical complement pathway are involved in aberrant synapse elimination. In previous studies, genetic ablation of C5aR1 or pharmacologic antagonism of C5aR1, resulted in less activation of microglia and astrocytes, preservation of neuronal complexity, reduction of cognitive loss and suppression of synapse engulfment by microglia, all implicating a role for C5a-C5aR1 signaling in disease progression. Gene expression data from adult microglia isolated from brain demonstrated that a genetic lack of C5aR1 prevented the polarization of microglia to a more inflammatory state while enhancing expression of genes involved in phagocytosis and lysosomal degradative enzymes and limiting synaptic pruning, all disease mitigating functions. Single cell (microglia)- and single nucleus-RNA seq now reveal differential expansion of specific cell clusters of astrocytes, as well as microglia, and predict pathways of cell communication involving oligodendrocyte precursor cells providing insight into the cellular and molecular mechanisms underlying the neuroprotection achieved by inhibiting C5a-C5aR1 signaling in adult mice. Specifically, some microglia and astrocyte clusters induced in the AD mouse models were C5aR1-independent, but others were almost completely suppressed by C5aR1 antagonism. Additional clusters were reduced in the AD mice, but restored by inhibition of C5aR1. Functional pathways that were suppressed by inhibition of C5aR1 included inflamma-

tory, neurotoxic or synapse pruning, while those that were rescued by C5aR1 inhibition were neuroprotective or included regulation of inflammation. Importantly, these studies suggest that the specific inhibition of C5a-C5aR1 signaling is a promising strategy for suppressing loss of function in AD and other neurogenerative disorders in which neuroinflammation and synapse loss are evident.

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C5a receptor 1 controls antigen and TLR-driven T cell proliferation and differentiation by splenic conventional type 2 dendritic cells

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Background: C5a regulates the differentiation and function of dendritic cell (DC) subsets from bone marrow-derived precursors through C5aR1 activation. Here, we determined the impact of the C5a/C5aR1 axis on FMS-like tyrosine kinase 3 ligand (Flt3L)-induced in vivo generation and function of splenic conventional DC (cDC) subsets.

Methods: Flt3L expressing B16 melanoma cells were subcutaneously injected into wildtype and C5ar1$^{-/-}$ mice. After 10 days spleens were harvested. cDC1 and cDC2 subsets were FACS-sorted, incubated with LPS-free ovalbumin (3 or 10 ug/ml) ± TLR ligands (LPS, PAM3) and co-cultured for 72h with ovalbumin-specific TCR transgenic OT-II CFSE-labelled CD4 T cells. Then we determined the generation of effector memory (TEM) and effector T cells (TEFF) and their differentiation towards Th1 (IFN-y) and Th17 (IL-17A) effector lineages.

Results: Flt3L induced mobilization of cDC1 and cDC2 cells in the spleen was independent of C5aR1 expression. Ovalbumin stimulation ex vivo resulted in strong TEFF and less TEM generation by cDC2, which was markedly reduced in cDC2 from C5ar1$^{-/-}$ mice at low ovalbumin concentration. Surprisingly, TEFF and TEM generation was absent using cDC1 cells. Additional LPS/PAM3 stimulation of cDC2 cells from either wildtype or C5ar1$^{-/-}$ mice increased the dominant TEFF cell differentiation. In contrast, TEM and TEFF induction was similar using cDC1 cells from wildtype or C5aR1-deficient mice. Strikingly, we found a strong and dominant IFN-y production upon wildtype cDC2 cell stimulation with OVA ± TLR ligands and OT-II cell co-culture, which was significantly reduced using C5ar1$^{-/-}$ cDC2 cells.

Conclusion: Our findings suggest that cDC2 but not cDC1 cells are critical for antigen-driven T cell proliferation and TEFF differentiation in the absence of TLR ligands. Further, C5aR1 activation seems to be crucial for this effect at low but not at high doses of antigen. Also, while C5aR1 activation did not affect TEFF or TEM cell differentiation induced by cDC1 or cDC2 following OVA ± TLR ligand stimulation, it controlled the dominant Th1 induction mediated by cDC2 cells. Thus, we identified a novel role for C5aR1 in antigen-driven TEFF differentiation by splenic cDC2 cells and subsequent differentiation into Th1 cells in response to pattern recognition receptor activation.

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Acinetobacter baumannii clinical isolates evade complement-mediated lysis by inhibiting the complement cascade and improperly depositing MAC

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Background: Acinetobacter baumannii is an opportunistic human pathogen causing life-threatening infections in immunocompromised patients. The complement system plays a significant role in protecting against bacterial invasion, yet knowledge about the complement activation during the infection is limited. Previously, we showed that Acinetobacter spp. clinical isolates (n=50) were recognized by the complement system, however, they survived in human serum. Being a gram-negative bacterium A. baumannii should be directly killed by MAC, hence we studied a MAC deposition and potential MAC-evasion mechanisms that A. baumannii has.

Methods: Bacteria were incubated with normal human serum (NHS), antibacterial peptide Nisin A and SYTOX Green to assess complement-mediated bacterial cell damage. Western blot was used to evaluate the cleavage of C5 protein and deposition of C5b. An ELISA was used to detect C5a and soluble MAC in the solution. A MAC deposition was measured after incubation with either NHS or purified C9 and mutant C9 TMH-1 protein, followed by the detection with an anti-C5b-9 antibody. A trypsin-shaving method was used to assess proper MAC insertion into the outer membrane.

Results: We observed a MAC deposition on most tested strains, which, however, were not killed in serum. Flow cytometry analyses showed polymerization of wildtype C9 but not mutant C9 TMH-1, thus MAC was properly formed. No significant MAC-mediated killing was detected, even after the addition of Nisin A, which should pass through MAC-created pores in the outer membrane and damage the inner membrane. Treatment of a deposited MAC with trypsin showed a significant reduction of MAC, indicating that the complex was not embedded in the membrane. Additionally, we observed a few A. baumannii isolates that did not deposit MAC. Several experimental methods revealed inhibition of the complement cascade by these bacteria at the level of C5 protein.

Conclusion: Our results indicated two possible mechanisms of MAC evasion by A. baumannii. Firstly, bacteria may inhibit the complement system at the level of C5 activation, thus staying protected from MAC deposition and lysis. Secondly, bacteria may deposit MAC, but the complex is not correctly inserted into the membrane and therefore does not induce the lysis of bacteria.

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Elucidation of novel exosite interactions in the activation and control of the classical pathway of complement

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The complement cascade is initially controlled by the conversion of mostly inert zymogen enzymes to fully active enzymes through limited proteolysis. These cleavage events lead to several conformational changes in the proteases that ultimately form the basis for the recognition of substrates. The proteases of the classical and lectin pathway are regulated directly by the serine protease inhibitor (SERPIN), C1 esterase inhibitor (C1-INH). The SERPIN mechanism relies on the substrate recognition of a reactive center loop that upon reacting results in a covalent SERPIN-protease complex, effectively eliminating protease activity. SERPINs are often specific to functionally distinct proteolytic cascades, but C1-INH is considered promiscuous due to its control of 11 blood proteases of the coagulation, fibrinolytic, and complement systems. Recent characterization of classical pathway specific protease inhibitors BBK32 and ElpB/Q, from the bacterial pathogen Borrelia burgdorferi, have uncovered non-active site protein-protein interaction surfaces on complement proteases C1r and C1s that are crucial to their inhibitory properties. These surfaces, termed exosites, function as key determinants for protease specificity and can be altered by conformational changes in the transition from zymogen to enzyme. Due to the increased protease recognition range, we hypothesized that areas outside of the reactive center loop of C1-INH may also have a role in the formation of the Michaelis-Menten complex preceding the covalent SERPIN reaction. To test this hypothesis, we implemented protein structure prediction techniques to model the Michaelis-Menten complex of C1-INH with each of its 11 target proteases. This approach yielded complexes of each C1-INH/protease pair and surface contact analysis revealed that C1-INH exosites contributed 21 to 62% of the protein-protein interaction surfaces. Candidate C1-INH/protease interaction surfaces were selected outside of the reactive center loop to investigate the potential role of exosites in C1-INH specificity. Using site directed mutagenesis coupled to surface plasmon resonance binding assays and assays of C1-INH function our data suggest a novel role for exosite-mediated interactions in contributing to the protease specificity of C1-INH. Broadly this work reveals a potential role for exosites in inhibitory SERPINs like C1-INH and may provide a tractable platform for rational design of complement protease specific inhibitors.

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Dengue virus NS1-epitope specific monoclonal antibodies inhibit NS1-mediated complement evasion

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Background: The non-structural protein 1 (NS1) of dengue virus (DENV) plays a crucial role in evading the immune system. NS1 can bind to various complement proteins, such as C1s, C4, C4b binding protein (C4BP), and mannose binding lectin (MBL), to protect the virus and infected cells from complement attack. We aimed to investigate whether monoclonal antibodies (mAbs) targeting specific NS1 epitopes could inhibit NS1-mediated complement evasion.

Methods: Mouse mAbs recognizing NS1 epitopes in the wing domain (aa 81-122 and aa 41-111) and the β-ladder (aa 274-352 and aa 158-235) were humanized and transformed into IgG1 chimeric mAbs. These chimeric mAbs were incubated with NS1 before incubating with coated complement proteins on ELISA plates. The inhibitory effect of each chimeric mAb on NS1 binding to complement proteins was represented as a percentage of inhibition.

Results: Two mAbs, 4D4 and 2G6, which target the wing domain and C-terminal tip of the β-ladder of NS1, respectively, provided stronger inhibitory effects on NS1 binding to complement proteins than the other two mAbs. Interestingly, 2G6 exhibited a high inhibitory effect on NS1 binding to proC1s, C4, and C4BP, but a lower effect on NS1-MBL binding.

Conclusion: Our findings suggest that NS1 epitopes in the wing domain and C-terminal tip of the β-ladder are crucial for NS1 binding to complement proteins. Notably, mAb clone 4D4 showed strong inhibition of NS1 binding to all complement proteins, making it a potential inhibitor of NS1-mediated complement evasion.

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αβ T cells and complement meet again: TCR crosstalk with surface complement regulators

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The complement systeḿs role during the innate immune response is well known. There is proof of its involvement also in adaptative immunity. For instance, the B cell receptor (BCR) and complement receptors such as CR2 (CD21) crosstalk when engaged by C3-opsonized antigen by reducing the BCR activation threshold. We are exploring similar interactions in T cells.

Here we studied HTVL-1-immortalized αβ $CD4^+$ T cell lines, either wild-type or with different defects in TCR invariant chains ($CD247^{+/-}$, $CD247^{-/-}$, $CD3D^{\Delta Ex2/\Delta Ex2}$) derived from patients with such mutations, which caused increasing TCR signaling impairment. Cells were stimulated with Dynabeads® T-Activator CD3/CD28 for 24h and surface expression induction of CR (CR1, CR2, CR3, CR4, C5aR1, C3aR), Creg (CD46, CD55, CD59) and CD69 (positive control) was analyzed by flow cytometry, comparing with PMA+ionomycin as a TCR-independent stimulus.

The basal CR/Creg phenotype was similar in the different T cell lines ($CR1^-CR2^-CR3^+CR4^-C5aR1^-C3aR^+CD46^+CD55^+CD59^+$). However, decreased signaling capacity of the TCR was related to increased C3aR and decreased CD46 and CD55 surface levels suggesting that TCR signaling may regulate these molecules basal levels on the surface. After TCR engagement, normal T cells selectively increased CD46, CD55 and CD59 levels (with CD69 as control), suggesting crosstalk between the TCR and all three Creg. $CD247^{+/-}$ T cells also induced CD69 expression, but, in contrast, did not induce any Creg, supporting TCR-dependence of expressed CReg induction. $CD247^{-/-}$ and $CD3D\Delta^{ex2/\Delta ex2}$ T cells were unable to induce CD69 or Creg. PMA+ ionomycin stimulation showed that normal, $CD247^{+/-}$ and $CD247^{-/-}$, but not $CD3D^{\Delta ex2/\Delta ex2}$, T cells were capable of TCR-independent CD69, CD55 and CD59 induction.

Therefore, our data provide evidence of specific TCR-to-CReg signaling which is dependent on TCR signaling capacity by genetic analyses, suggesting that T cells may receive CReg-dependent information of extracellular complement ligands during antigen engagement.

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The molecular basis for C1s inhibition by Lyme disease spirochetes

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Lyme disease is caused by an infection with tick-borne spirochetes of the Borreliella genus, including B. burgdorferi, B. garinii, and B. afzelii. Like all bloodborne pathogens, Lyme disease spirochetes must overcome complement-mediated attack. This is evidenced, in part, by the identification of at least a dozen different B. burgdorferi outer surface lipoproteins that directly interact with one or more complement components and interfere with their native activities. The complement evasion system of B. burgdorferi includes two distinct families of classical pathway-targeting protease inhibitors known as BBK32 and ElpQ/B. Previously we have shown that BBK32 is a highly specific inhibitor of C1r that relies on an active site targeting mechanism. In contrast, ElpQ and ElpB inhibit the classical pathway at the level of C1s using a novel exosite-targeting mechanism. Circular dichroism, AlphaFold2 modeling, and X-ray crystallography experiments revealed that the inhibitory C-terminal domain of ElpQ and ElpB exhibit helical bundle folds with a short C-terminal helical cap. To explore the molecular basis for C1s inhibition by ElpQ/B, we carried out a series of hydrogen-deuterium exchange mass spectrometry (HDX-MS) experiments. These experiments showed that the centrally positioned second alpha helix of ElpQ undergoes significant conformational change in the unbound state. Subsequent HDX-MS experiments involving ElpQ/C1s complexes identified putative C1s binding sites on ElpQ. To validate the HDX-MS findings, several site-directed ElpQ mutants were generated and evaluated for C1s binding using surface plasmon resonance, and for complement inhibition, using a classical pathway-specific ELISA assay. Ultimately, a single mutation was identified involving a residue within the dynamic second alpha helix of ElpQ which completely abrogated its C1s-binding and complement inhibition activities. Furthermore, analysis of the ElpQ binding site on full-length C1s was consistent with previous observations that the C4 exosite on C1s is bound by ElpQ, while the active site of C1s is not. These studies further our understanding of the molecular mechanisms associated with exosite-mediated C1s inhibition by B. burgdorferi ElpB and ElpQ and improve our knowledge of microbial complement evasion strategies.

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C1q-IgM interaction studies with recombinant globular C1q variants

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The classical complement activation pathway is initiated by the interaction of the globular head of C1q with the constant domain of an immunoglobulin/antigen complex, preferably IgM. To identify the amino acids and regions on the globular C1q that come into contact with IgM, we developed artificial globular C1q variants in single-chain format for recombinant expression in CHO cells and performed interaction and complement activation studies with different IgMs.

We designed three variants of the trimeric globular region of C1q, the globular heads of the A, B and C chains. The first globular construct (ACB) contains the three globular peptide chains consecutively connected with short linkers and expressed as a single-chain protein. The second comprises the first variant fused to human serum albumin domain 2 (ACB-AD2) via a glycine-serine linker. In addition, the third recombinant protein construct consists of two contiguous ACB domains connected via a flexible linker and a trimerization domain of T4 fibrin added at the C-terminus to mimic the hexameric form of native C1q ($(ACB)_2$) by trimerization of the paired ACB.

Stable recombinant cell lines of all three constructs were generated in CHO-K1 host cells and the proteins were purified using anti-FLAG affinity resin. Interaction studies with IgM and complement activation assays were performed in a 96-well plate format.

The competitive interaction assays showed that the recombinant constructs compete with the native C1q and bind to the IgM antibodies. Complement activation was also inhibited by the C1q variants. Interaction of the recombinant constructs with IgM will be verified by Bio-layer interferometry. The best performing C1q mimetic will be subjected to random mutagenesis and the best binders will be identified by yeast display with IgM panning to identify critical amino acid residues on the C1q globular head. This will provide valuable insight into the interaction between IgM and C1q.

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Complement regulator factor H is a cofactor for thrombin in both pro- and anticoagulant roles

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Background: Complement FH (FH) is a key regulator of complement activity whereas thrombin (FIIa) is central to hemostasis with both pro- and anticoagulant functions. Both have separately been shown to have auxiliary activities across the two systems. The purpose of this study was to determine the effect of FH on pro- and anti-coagulant functions and investigate the interaction between FH and thrombin.

Methods: Tail bleeding time and hemolysis were measured in FH-deficient mice (CFH-/-). Activated partial thromboplastin time (aPTT) was determined in FH-depleted human plasma. FH effect on fibrin clot generation was investigated in turbidity assays and on activated protein C (APC) generation. Binding affinity of thrombin with FH was determined using surface plasmon resonance (SPR).

Results: Tail bleeding time in CFH-/- mice was significantly prolonged compared to wild type mice. The aPTT in FH-depleted human plasma was elevated compared to normal plasma and restored by adding back FH to depleted plasma. Accordingly, FH enhanced thrombin-mediated fibrin clot generation by shortening lag time, increasing rate of clot formation and maximum turbidity, and affected clot structure. Despite this, FH also increased the rate of thrombin-mediated protein C (PC) activation, both in the presence and absence of soluble recombinant thrombomodulin (TM). Nanomolar affinity binding of FH with thrombin, but not prothrombin, was confirmed. Neither FH complement regulatory function nor complement haemolytic activity was altered by thrombin binding.

Conclusion: Complement FH binds thrombin with strong affinity and acts as a novel cofactor that enhances both pro- and anticoagulant actions of thrombin. These data highlight an important role for FH in hemostasis.

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Characterisation of CSMD1 – A complement regulator associated with schizophrenia risk

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Background: The CUB and sushi multiple domains 1 (CSMD1) gene encodes a complex transmembrane protein comprising 14 N-terminal C1r/s, uEGF, and BMP1 (CUB) domains interspersed with short consensus repeat (SCR) domains, followed by 15 tandem SCR modules, transmembrane domain, and short cytoplasmic tail. CSMD1 SCR17-21 and SCR23-26 are structurally comparable to many complement system regulators. CSMD1 SCR17-21 reportedly inhibits the classical pathway (CP) via Factor I co-factor activity and inhibits membrane attack complex (MAC) assembly through interaction with C7 and C8[1]. Genetic studies have robustly associated CSMD1 with schizophrenia risk[2]. Despite this, no further characterisation of CSMD1 has been performed, reliable antibodies for studying CSMD1 are scarce, and there is little literature exploring which cell types express CSMD1 in the brain.

Methods: Human CSMD1 SCR17-21 and SCR23-26 were expressed in Expi293F cells with IgG3-Fc tags to diminish interference with fragment function. Complement regulatory properties of both fragments were examined via various complement functional assays. Mice were immunised with CSMD1 SCR17-21, hybridoma

generated via fusion, and clones screened by indirect ELISA. Clones expressing CSMD1-specific monoclonal antibodies (mAbs) were expanded to monoclonality; mAbs were purified and validated in ELISA and western blot. CSMD1-expressing brain cell types were investigated via immunofluorescence.

Results: Both CSMD1 SCR17-21 and SCR23-26 inhibited complement activation in CP haemolytic assays but not in alternative pathway assays. Both fragments inhibited assembly of CP convertases but did not accelerate decay. Neither fragment inhibited MAC assembly in reactive lysis assays, even following pre-incubation with C7 and C8. CSMD1 mAbs were specific to SCR17-21 over SCR23-26 and recognised endogenous CSMD1 in western blots of human and mouse brain. CSMD1 mAbs immunostained astrocytes in human temporal cortex and whole mouse brain; immunocytochemistry with human induced pluripotent stem cell-derived astrocytes and cortical neurons also resulted in positive CSMD1 staining.

Conclusion: We demonstrate that CSMD1 possesses two complement regulatory domains which inhibit CP convertase assembly. We also present a panel of novel mouse mAbs that specifically target CSMD1 SCR17-21 and demonstrate novel evidence of astrocytic CSMD1 expression in human and mouse brain, a finding that may impact schizophrenia pathology.

Reference

1. PMID: 23964079.
2. PMID: 35396580.

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The functional consequence of two CFI ultra rare variants in complement-mediated diseases: Insights from *in vitro* splicing assay

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Background: The regulatory serine protease, complement Factor I (FI), plays a key role in controlling activity of the complement cascade by inactivating C4b and C3b. Low levels of plasma FI can result in increased baseline complement activity with a consequent decrease in plasma C3 thereby increasing risk for recurrent infections. FI haploinsufficiency also can be seen with complement-mediated renal diseases such as C3 glomerulopathy (C3G) and atypical hemolytic uremic syndrome (aHUS). It is therefore important to determine the functional impact, if any, of rare CFI variants identified in patients with complement-mediated diseases.

Methods: Using an in vitro minigene splicing assay, we identified the functional consequences of two CFI ultra-rare variants, c.1429G>C and c.1429+1G>C, located at the boundary of exon 11 and intron 11. These two variants were identified in five patients from our disease cohorts, one patient with recurrent infections (homozygous c.1429+1G>C), two patients with aHUS (heterozygous c.1429G>C), and two patients with C3G (heterozygous c.1429G>C and c.1429+1G>C, respectively). FI levels and other complement biomarkers were determined using Western blotting and a testing panel.

Results: The minigene assay showed a similar expression profile for both variants, resulting in four aberrant transcripts: two partial exon exclusions, c.1331_1429del, p.(Cys444-Asp477del) at 53% and c.1217_1429del,p.(Arg406-Asp477del) at 18%; an intron retention (p.Asp477GlyfsTer8) at 22%; and complete exon 11 skipping at 7%. Western blotting and ELISA confirmed that no aberrant transcript was expressed: the patient homozygous for c.1429 +1G>C lacked detectable FI protein and all heterozygous patients showed borderline low normal FI serum protein levels.

Conclusion: This study shows that CFI c.1429G>C, p.Asp477His (rs754972981), which is classified as a missense mutation, in fact has a substantial impact on pre-mRNA splicing. The c.1429G>C change leads to activation of cryptic splicing donor sites within exon 11 and results in haploinsufficiency, similar to the changes seen secondary to alteration of the neighboring highly conserved splicing donor site variant c.1429+1G>C. These findings highlight the importance of functional studies to understand the impact of rare genetic variants in CFI in patients with complement-mediated diseases. (Supported in part by National Institutes of Health R01 DK110023.).

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C5b-9 and Bb factor levels as potential novel biomarkers in crescentic IgA nephropathy

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Background: IgA nephropathy (IgAN) is the most common glomerulonephritis and the leading cause of end-stage kidney disease among them. Complement has a well-established role in the pathogenesis of IgAN through the activation of both lectin and alternative pathway, which converge at the generation of C5 convertase that initiates C5b-9 formation. C5b-9 pierces target cell membranes, causing cell activation and injury until cell lysis, with crescents formation in glomeruli. We hypothesized that the intensity of complement activation (CA) may influence the severity of IgAN.

Methods: We measured plasma C5b-9 and Bb factor, a marker of alternative pathway of CA in a cohort of patients referred for acute or chronic glomerulonephritis to the Service of Nephrology of the HVS (Hôpital du Valais).

Results: From this cohort, seventeen patients with primary and isolated IgAN on kidney biopsy have been identified since 2018. Plasma C5b-9 and Bb factor were compared in severe forms of IgAN associated with crescents to patients without crescents at the time of kidney biopsy, where forms of IgAN with crescents are associated with a severe prognosis. C5b9 and Bb levels were markedly higher in patients with crescents (514 + 236 vs 163 + 61 ng/ml and 1.7 + 0.6 vs 1.1 + 0.3 μg/ml, p of 0.002 and 0.02).

Conclusion: This pilot study supports our hypothesis of a stronger CA in severe forms of IgAN associated with crescents. Therefore, C5b-9 and Bb factor levels may represent easily accessible novel biomarkers of crescentic IgAN. This is of interest, especially in those cases in which the diagnostic kidney biopsy of crescentic IgAN is not immediately available. Moreover, the demonstration of

a strong correlation among CA and crescentic forms of IgAN can open the venue to complement blockade therapies, such as it is actually the case for C5a inhibitor in ANCA-vasculitis. However, these data are scanty and not prospectively collected. We need to confirm their robustness and reproducibility in a bigger sample of patients, possibly coming from different clinical contexts. We plan to conduct an observational cross-sectional, multicenter and international study investigating CA on patients with primary and isolated forms of IgAN in the future.

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Clinical and Histologic Correlations in C3 Glomerulopathy

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C3 Glomerulopathy (C3G) is characterized by complement dysregulation resulting in C3 deposition in glomeruli. More than 50% of patients progress to ESRF within 10 years. We reviewed the histologic characteristics of the baseline kidney biopsy in a cohort of patients with C3G to determine if any biopsy features correlate with the various clinical parameters.

Subjects were drawn from the University of Iowa's C3G Natural History Study. Criteria for entry: baseline native biopsy diagnosis of C3G with known serum C3 levels, UP/C, and a GFR >30. In addition, at 1 year, no history of anti-complement therapy. Other immune suppression and ACEI/ARB treatment was allowed for entry. Pearson correlation coefficients with two-tailed p values (95% confidence) were used to examine disease-related parameters. "Yes/No" parameters were coded 1/0 for correlation calculations.

Seventy-four subjects were identified. 5.6% of the cohort presented with moderate to severe crescent formation on baseline biopsy; interstitial inflammation was infrequent (2.7%). More severe glomerulosclerosis at onset was associated with a higher baseline C3 (R= 0.436, p= 4.49E-04) and stabilization or slight decrease of C3 over time (R= -0.325, p= 0.008). Lower baseline GFR was associated with more severe glomerulosclerosis (R= -0.647, p= 7.98E-10), interstitial fibrosis (R= -0.743, p= 1.22E-13), and tubular atrophy (R= -0.671, p= 1.52E-10). Endocapillary proliferation showed no significant correlation between changes in GFR (R= 0.113, p= 0.350) or UPC (R= -0.174, p= 0.160) with standard therapy. Immunosuppression had no significant association with changes in GFR (R= 0.106, p= 0.371) or UPC (R= -0.131, p= 0.282), although an association with C3 increase neared the threshold of 95% significance (R= 0.236, p= 0.055).

The lack of association with immunosuppression use and positive changes in clinical lab values support the notion of mixed success with these agents for use in C3G, and the need for more effective interventions. Future directions include the correlation of baseline biopsy criteria with complement biomarkers, as well as examining how pathological changes noted in subsequent biopsies are associated with patient outcomes.

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Using the C3 gain-of-function mouse model of aHUS as a complement therapeutic testbed to enable precision medicine for complement mediated atypical haemolytic uraemic syndrome

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Complement mediated atypical haemolytic uraemic syndrome (c-aHUS) arises from impaired regulation or gain-of-function changes in the alternative pathway (AP). Eculizumab is the gold standard therapy for c-aHUS but there is room for improvement in patient care. The unique mouse model of aHUS based on a single D1115N amino acid substitution in C3, ($C3^{N/N}$, PMID: 30714990) provides a testbed to evaluate targeted complement inhibition and we aimed to establish new effective therapies in c-aHUS.

Therapeutic blockade of the AP using anti-properdin monoclonal antibodies (mAb H4, 14E1), CR2-FH, homodimeric minimal-FH (HDM-FH - protein and gene therapy) or FH1-5, and terminal pathway (TP) blockade with an anti-C7 mAb or an oral C5aR antagonist (ACT-1014-6470) was carried out using previously published dosing strategies. A prophylactic (from weaning) or rescue strategy (after 2 days of haem at 25 ery/ul) was used as appropriate. Upon study completion, animals were culled, tissue collected, and analysed for histological evidence of renal disease, fibrin deposition and glomerular complement deposition. Plasma was analysed for renal impairment (BUN), haemolysis indices (Haemoglobin, reticulocyte and platelet count) and complement levels.

No $C3^{N/N}$ mice succumbed to aHUS during prophylactic use of the oral C5aR1 antagonist (up to 80 days) which mirrored genetic deletion of C5aR1 (as previously reported[1]). Similarly, anti-C7 mAb therapy rapidly reduced evidence of renal damage and no mice succumbed to disease. Anti-properdin mAb 14E1 (similar to genetic deletion of Cfp[2]) and HDM-FH (protein) were also highly effective in restoring renal function but CR2-FH (even when given daily) and FH1-5 largely failed to prevent acute TMA and renal damage in $C3^{N/N}$ mice. Therapeutic use of the oral C5aR antagonist and prophylactic gene therapy using HDM-FH is ongoing with no mice succumbing to TMA thus far.

Our data suggest that therapeutic targeting of either arm of the TP and disruption of the C5 convertase can significantly reduce renal damage and prevent c-aHUS (in the context of a C3 gain-of-function change) providing novel insight into disease mecha-

nisms as well as illuminating potentially new disease maintenance and treatment strategies.

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Inhibition of SARS-CoV-2 spike interaction with angiotensin converting enzyme-2 (ACE2) by specific antibodies is enhanced by complement, determined with a novel flow cytometry assay

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SARS-CoV-2, the causative agent of the Covid-19 pandemic, utilises its transmembrane Spike glycoprotein to adhere to host cells via binding to the angiotensin converting enzyme-2 (ACE2) receptor. Spike protein is composed of two subunits, S1 and S2. S1 contains the receptor binding domain (RBD) that binds ACE2 and S2 anchors Spike to facilitate cell membrane fusion. Engagement of ACE2 to the Spike protein activates downstream intracellular pathways that allows SARS-CoV-2 to fuse with the cell membrane and enter the cell. Anti-RBD antibodies block this interaction and neutralise the pathogen utilising Fc-mediated functions, such as complement deposition.

Numerous assays have been developed to investigate Spike-ACE2 binding and the efficacy of anti-spike antibodies blocking this interaction by direct competition. However, the blocking function of these antibodies through the recruitment of complement has yet to be investigated. We have developed an assay to evaluate the function of SAR-CoV-2 antibodies by quantifying the inhibition of Spike-ACE2 interaction due to the formation of antibody-complement immune complexes. This is a high-throughput bead-based flow cytometry assay that quantifies the ACE2 inhibitory units (AIU) of serum samples based on Spike-ACE2 binding in the presence of complement and variable levels of anti-spike antibodies. Addition of IgG- and IgM-depleted plasma as a complement source and recombinant ACE2 protein ensure reproducible results.

SARS-CoV-2 serum samples from non-infected, convalescent and vaccinated individuals were tested. In the absence of complement, quantifiable AIU values indicate ACE2-Spike blocking due to anti-RBD antibodies. Once complement is introduced, the AIU values increase, indicating that addition of complement potentiates the blocking of ACE2-Spike and may be crucial in the initial prevention of SARS-CoV-2 infection. Even if the mechanism of action is not known yet, these results suggest that complement is recruited over the RBD binding site or that antibodies potency is increased by the presence of complement. Although complement has been reported to potentiate pathogenesis of disease, this highlights that early recruitment may prevent SARS-CoV-2 infection.

Our ACE2 inhibition assay can be utilised to further profile the immune response to SARS-CoV-2, investigate vaccine efficacy and could be adapted to other pathogens of interest.

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Association of a new variant of complement regulator FHR2 with C3 glomerulopathy

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C3 glomerulopathy (C3G) is caused by a dysregulation of the complement system leading to C3b deposition and formation of glomerular deposits. Several C3G patients harbor mutations or copy number variations in the human Factor H (FH) and/or Factor H-Related (FHRs) genes. Therefore, FH and FHRs are emerging immune targets for inhibition of the complement cascade, as well as markers to monitor patients on complement regulatory drugs to test their efficiency. Here, we focused our study on FHR2, known to inhibit in vitro formation of the terminal complement complex. We identified new variants for the FHR2 gene in a cohort of C3G patients and performed detailed functional studies on the novel variant $FHR2_{Leu46}$, which has the Pro at position 46 replaced by Leu. Patients with $FHR2_{Leu46}$ variant presented increased FHR2 plasma levels, as compared to controls and displayed FHR2 deposits in glomeruli. We generated a recombinant $FHR2_{Leu46}$ mutant protein to gain insight into the effect of this novel FHR2 variant on complement regulation. As the amino acid exchange occurred in the first short consensus repeat (SCR1), we first tested if the Leu at position 46 altered FHR2 homodimerization and heterodimerization of FHR2 with FHR1 and FHR5. We observed that $FHR2_{Leu46}$ binds with significantly lower intensity to FHR2 and FHR1, but with higher intensity to FHR5. Furthermore, $FHR2_{Leu46}$ acquired the capacity to bind to cell surfaces by interacting with glycosaminoglycans heparin and malondialdehyde (MDA)-modified amino group (MAA) epitopes. $FHR2_{Leu46}$ also bound substantially more to necrotic cells as compared to wild-type FHR2 ($FHR2_{WT}$). In contrast, no difference was observed between $FHR2_{Leu46}$ and $FHR2_{WT}$ binding to C3 and C5. Taken together, the present study identified a novel $FHR2_{Leu46}$ variant in a C3G patient and suggests that the $FHR2_{Leu46}$ forms stable oligomers with FHR5 and enhances complement activation.

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Assessing Complement Factor B Function: A Comparison of Hemolytic and Immunofixation Electrophoresis-based Assays

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Background: Complement factor B (FB) is essential for the amplification of the alternative pathway (AP), which is critical for

host defense and elimination of damaged cells. However, overactivation of the AP due to FB variants can lead to complement-mediated diseases, such as atypical hemolytic uremic syndrome and C3 glomerulopathy. Various assays can be developed to assess the functional consequences of recombinant proteins carrying CFB variants, including the hemolytic-based assay and immunofixation electrophoresis (IFE).

Methods: This study compares the results obtained from the hemolytic-based assay with pre-decorated C3b on sheep erythrocytes and IFE using FB-depleted serum to determine their relative merits in FB functional assessment. The hemolytic-based assay measures the impact of factor B variants on the half-life of C3 convertase in the presence or absence of complement regulators, while IFE detects how factor B variants affect C3 convertase activity - the conversion of C3 to C3 activation products.

Results: We evaluated recombinant FB variants located in the Ba, vWFA, and serine protease domains. Our results indicate that IFE has higher sensitivity and specificity in detecting FB variant effects on the activation of C3 compared to the hemolytic assay. Specifically, IFE showed greater sensitivity in detecting variants located in all three FB domains, while the hemolytic assay was more sensitive to the variants located in the vWFA domain. Furthermore, the hemolytic assay allowed us to investigate interactions between FB variants and different regulators.

Conclusion: The choice of assay for FB functional assessment should be determined based on the specific research question and the variant location. This study provides valuable insights into the relative advantages and disadvantages of the hemolytic-based assay and IFE and can inform the selection of appropriate assays for FB functional assessment. (Supported in part by National Institutes of Health R01 DK110023.)

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Activation of complement pathways and formation of membrane attack complex contribute to the development of severe COVID-19

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Background: Extensive evidence indicates that activation of complement and formation of the membrane attach complex (MAC) during severe COVID-19 may participate in mediating endothelial cell (EC) damage, activating the coagulation pathway and platelets, and causing multiple organ damage. However, the causative roles of complement and the MAC in severe COVID-19 are largely unexplored.

Methods: To address this, we utilized C3 knockout mice (C3 KO) deficient in complement activation and MAC formation, mouse CD59 knockout (CD59 KO) mice deficient in regulating MAC formation (PMCID: PMC4280255), and used anti-C5 antibody (Eculizumab) to block the formation of C5a and MAC in mice. These mice were infected with mouse-adapted strain of SARS-CoV-2 (MA30), a strain that can infect C57BL/6 (B6) mice (PMCID: PMC9783543).

Results: The littermate black B6 wild type (WT) control mice infected with lethal doses of MA30 (1 X 10^5 and 5 X TCID50) developed severe COVID19 phenotypes, including progressive loss of body weight, loss of movement and shortness of the breath; lethality occurred with 6–7-days post infection and was associated with extensive lung edema and inflammation. The MA30-infected WT mice had significantly higher levels of systemic complement activity than non-infected WT mice, indicating increased complement activation in severe COVID-19. C3 KO mice infected with a lethal dose of MA30 (5 X 10^4 TCID50, a dose five times higher than minimal lethal dose) were protected against development of severe COVID19 as indicated by no progressive body weight loss and no lethality. The protection was associated with significantly reduced SARS-CoV2-mediated lung edema and inflammation at 6-days post-infection as compared with MA30-infected WT mice. No complement activity was detected in infected C3KO mice. Further, CD59 KO mice infected with sublethal dose of MA30 (1 X 10^4 TCID50) developed more progressive loss of body weight and shorter survival than MA30-infected WT mice. Pre-treatment with Eculizumab partially protected against severe COVID-19 phenotypes occurred in infected-CD59 KO mice.

Conclusion: Together, our results demonstrate activation of complement and formation of the MAC contribute to the development of severe COVID-19.

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Identification and characterization of a novel complement factor I-binding protein in Pasteurella multocida

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Pasteurella multocida is a Gram-negative microbe that causes serious infectious diseases in cattle and other ruminants. In North America alone, 75% of annual cattle death can be traced back to this bacterial infection, underlining the huge economic impact of this pathogen on the farming industry. Recurring outbreaks of *P. multocida* worldwide and the limitations of current vaccines have thus prompted us to search for a novel antigen that can elicit a cross-protective and broad-spectrum response to all circulating bacterial strains. Through an extensive bioinformatic analysis, we identified a surface lipoprotein, termed PmSLP, that is present in more than 95% of bovine *P. multocida* isolates (Hooda et al., 2017). The prevalence and conservation of PmSLP suggests that the protein is important for bacterial physiology and pathogenesis. We also showed that using PmSLP as a vaccine antigen could protect animals from invasive P. multocida infections (Islam et al., 2023).

To further understand the role of PmSLP in bacterial immune evasion, we used PmSLP as a bait and identified bovine complement factor I (FI) as the mammalian binding partner. We were able to isolate stable protein complexes through size exclusion chromatography and demonstrated high affinity binding between PmSLP and FI via kinetic studies. We also solved the structure of PmSLP using X-ray crystallography and identified several key resi-

dues required for binding function. Moreover, functional characterization of FI in complex with PmSLP showed that the enzyme is proteolytically active against both C3b and C4b without needing its native co-factors, factor H and C4BP, respectively. This result suggests that PmSLP serves as a promiscuous co-factor of bovine FI, allowing the bacteria to switch off all three branches of the complement cascade. Finally, we carried out gain-of-function experiments where PmSLP was expressed on the surface of a serum-sensitive strain of Escherichia coli. Interestingly, the presence of PmSLP alone on the cell surface was sufficient to promote E. coli resistance to complement-meditated killing.

Taken together, this study highlights a previously unknown mechanism used by *P. multocida* to evade the complement system.

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Bi-functional C5 antibody-fusion protein (LP-005) with potential best-in-class bioactivity for complement inhibition

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Background: Therapeutic antibodies targeting C5 proved to be a very effective approach for complement mediated disorders. However, due to the complexity of the complement system, including multiple pathways (CP, AP and LP) and potential cross-talk between different pathways, C5 antibody alone may not sufficiently block AP, LP, nor reduce the deposition of C3b on the cell surface produced by C3 activation.

The next-generation complement inhibitor emerges as C5 antibody fused with another functional part of complement inhibition activity, providing a potentially broader clinical indication (nephritis, neuromyelitis and etc) with better efficacy and controlled risk.

Methods: The sequence of Eculizumab, Ravulizumab, Crovalimab, Pozelimab, Narsoplimab, were obtained from INN. POT-4 (also called APL-1) was purchased from MedChemExpress.

LP-005 was generated from hybridoma, fused with sponsor's proprietary inhibitor (inCibitor^{TM}).

Ab-fH was generated by fusing fH 1-5 to the C terminal of anti-C5 antibody.

All antibodies were expressed by HEK293 cells.

For CP and AP inhibition assay, sheep or rabbit erythrocytes was used with 5-10% NHS (normal human serum);

For LP inhibition assay, WIESLAB® MBL pathway kit was used;

C3b deposition on rabbit erythrocytes was detected by flow cytometry, after AP activation;

Single i.v. dose of Surrogate antibody fusion protein (LP-005C) and Ab-fH fusion protein was administrated in Monkey PK/PD study, PK and PD (hemolysis) assay was conducted.

Results: In the present study, LP-005 shows the most potent inhibition in CP, AP and LP compared to Eculizumab, Ravulizumab, Crovalimab, Pozelimab and Narsoplimab (figure).

In addition, it also has the most efficient inhibition in C3b assay compared to Antibody-fH fusion and POT-4 (APL-1) (figure).

The monkey PK/PD study indicates that when fusing to the same C5 antibody, Longbio's unique inhibitor (inCibitorTM) shows a better PK/PD profile compared to the natural fH 1-5 fragment.

Conclusion: In summary, LP-005 is a novel bifunctional anti-C5 monoclonal antibody fusion protein, with highest bioactivity in CP, AP, LP and C3b deposition assays.

LP-005 is also engineered to change it surface charge (PI) and FcRn binding, which together has the potential to be a best-in-class drug candidate with improved pharmacokinetic properties and strongest in-vitro and in-vivo bioactivity.

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Complement activation during hypoxia-reoxygenation in human kidney epithelial cells

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Background and aims: Ischemia in combination with reperfusion leads to complement activation during kidney transplantation resulting in tissue damage. Prolonged ischemia leads to complement C3 production by proximal tubular epithelial cells and formation a membrane attack complex (C5b-9) The kidney has a capacity to protect itself from complement activation through cellular expression of complement regulatory proteins. In this study we investigated whether hypoxia and reoxygenation increases C3 and C5b-9 deposition and alters complement regulatory protein expression (CD46, CD59 and CD55) on tubular cells.

Methods: In vitro, HKC-8 renal proximal tubular epithelial cells were subjected to 24h of hypoxia (1% O2) and then reoxygenated for 4h (21% O2) in the presence of 40% normal human serum. qRT-PCR was used to estimate the level of VEGF-A (as a positive control), C3, CD46, CD59 and CD55 expression. Similar analysis was performed in untreated, normoxic cells. Immunofluorescence staining was used to determine C3 and C5b-9 deposition on the cell surface and intensity quantified by ImageJ. Experiments were repeated three times.

Results: qRT-PCR analysis of HKC-8 cells in hypoxia revealed significant increases in the expression of VEGF-A after 24h ($p=0.0113$). C3 mRNA expression following hypoxia ($p=0.42$) and reoxygenation ($p=0.24$) increased, but this was not significant. Increased expression of CD59 with the presence of serum was observed in epithelial cells ($p=0.026$). However, no significant change was seen in the expression of CD55 and CD46. Reoxygenation in human serum led to significantly greater deposition of C3 on hypoxic cells compared with normoxic HKC-8 cells ($p=0.0208$). Upon reperfusion with the presence of normal human serum HKC-8 cells showed significantly increased C5b-9 deposition ($p=0.0365$).

Conclusion: Our preliminary data suggests that hypoxia and reoxygenation significantly increases C3 and C5b-9 deposition on the proximal tubular cell surface. CD59, as one of the regulators of complement activation, is upregulated at the mRNA level after hypoxia.

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Complement genetics: implementation of a custom made panel for genetic testing of complement-associated kidney patients

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Background: Complement system is involved in the pathology of many diseases, including kidney disease. Evidence from genomics, animal models of kidney disease, and treatment with anti-complement drugs have confirmed a strong association of the complement cascade with kidney diseases such as immune complex-mediated glomerulonephritis, aHUS, C3G, ANCA vasculitis, renal ischemia-reperfusion injury, and rejection after kidney transplantation. Part of the factors influencing the occurrence and development of kidney diseases is also represented by genetic variants in complement system genes.

Methods: Our study group consists of 21 patients (11 women and 10 men) with various kidney diseases associated with the complement system. We have designed a custom AmpliSeq Custom DNA Panel (Illumina) comprising 396 amplicons covering 16 different genes (FH, CFHR1, CFHR2, CFHR3, CFHR4, CFHR5, CD46, FI, FB, C3, DGKE, THBD, PLG, VTN, MASP2, and CD36). Genomic DNA will be sequenced using the Illumina platform.

Results: Our goal is to identify and evaluate the genetic basis of the complement system in kidney disease patients. We will determine the diagnostic results of genetic analysis and the possible relationship between genotype and phenotype.

Conclusion: A major challenge is the functional evaluation of observed genetic variants. Nevertheless, genetic testing has its place in modern medicine because it is used for diagnosis, presymptomatic assessment of the probability of disease development, pharmacogenetic treatment planning, and allows better information in family planning.

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Treatment with warfarin but not Factor Xa inhibitors, is associated with dysregulated, noncanonical, complement activation

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Background: Warfarin treatment is associated with downregulation of C4b-binding protein (C4PB), the major soluble inhibitor of the classical and lectin pathways of complement, but the functional outcomes of warfarin and other anticoagulants on complement activation have not yet been fully addressed.

Objective and method: We performed a cross-over study on patients with venous thromboembolism, without antiphospholipid antibodies. Paired plasma samples from the same subjects were collected during and three weeks after cessation of treatment with either warfarin (n=22) or direct oral anticoagulants (DOACs) targeting FXa (n=33). Complement parameters were measured: C3a, sC5b-9, and C4BP (the total amount, the beta-chain containing fraction, and C4BP in complex with protein S).

In order to further dissect the mechanisms for complement activation we performed in vitro experiments where plasma samples from patients on warfarin and from healthy controls were incubated with tissue factor (TF), giving rise to proteolytically active FIXa and Fxa, both of which have been reported to cleave C3 and C5.

Results: Generation of C3a and sC5b-9 in combination with lowered levels of C4BP was found in patients on warfarin. Three weeks after cessation of treatment, there was a rebound effect leading to C4BP levels much over the reference range. C3a and sC5b-9 were still elevated, but did not correlate to the levels of C4BP. In contrast, C3a, sC5b-9 and C4BP were within the normal range without changes and remained stable in the DOAC group during and after treatment.

In vitro experiments showed that TF induced significant levels of C3a and sC5b-9 in plasma from patients on warfarin but not controls.

Conclusion: Significant activation of the complement system was seen in patients treated with warfarin, lingering for weeks after withdrawal, while no corresponding alterations were found in patients treated with FXa inhibitors. Results from in vitro experiments showed generation of C3a and sC5b-9 secondarily to coagulation activation by TF in plasma from warfarin treated patients. This observation suggests that warfarin treatment per se may induce dysregulated activation and/or lack of control so that coagulation proteases not only cleave their preferred physiological substrates but also complement components, thereby inducing non-canonical complement activation.

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Modeling complement activation on human glomerular microvascular endothelial cells

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Atypical hemolytic uremic syndrome (aHUS) is a rare kidney disease caused by dysregulation of the complement alternative pathway. The complement dysregulation specifically leads to damage to the glomerular endothelium. To further understand aHUS pathophysiology, we set up an ex vivo model for measuring complement deposition on human glomerular microvascular endothelial cells (GMVECs).

Endothelial cells were incubated with human test sera and stained with an anti-C5b-9 antibody to visualize and quantify complement depositions on the cells with immunofluorescence microscopy.

First, we showed that zymosan-activated sera resulted in increased endothelial C5b-9 depositions compared to normal human serum (NHS). This increase in C5b-9 deposition was comparable for conditionally immortalized (ci)GMVECs and primary GMVECs. The protocol with ciGMVECs was further validated and we additionally generated ciGMVECs from an aHUS patient. The increased C5b-9 deposition on control ciGMVECs by zymosan-activated serum could be dose-dependently inhibited by adding the C5 blocker eculizumab. The required eculizumab concentrations correlated with the target serum levels for aHUS. Next, sera from four aHUS patients were tested on control ciGMVECs. Sera from acute disease phases showed increased endothelial C5b-9 deposition levels compared to NHS. The remission samples of three patients showed normalized C5b-9 depositions, whether remission was reached with or without eculizumab in the blood. We also monitored the endothelial complement deposition of an aHUS patient with a hybrid complement factor H (CFH)/CFH-related 1 gene during follow-up. This patient had chronic kidney failure and an ongoing deterioration of kidney function despite absence of markers indicating an aHUS flare. Increased C5b-9 depositions on ciGMVECs were observed in all samples obtained throughout different diseases phases, except for the samples with eculizumab levels above the therapeutic target. We then tested the samples on the patient's own ciGMVECs. The C5b-9 deposition pattern was comparable and these aHUS patient ciGMVECs also responded similar to NHS as control ciGMVECs.

In conclusion, we demonstrate a robust and reliable model to adequately measure C5b-9-based complement deposition on human control and patient ciGMVECs. This model can be used to study the pathophysiological mechanisms of aHUS or other diseases associated with endothelial complement activation ex vivo.

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Citrullination of C1-inhibitor as a mechanism of impaired complement inhibition in rheumatoid arthritis

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Background: Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized by dysregulated complement activation, increased citrullination of proteins, and the production of autoantibodies against citrullinated proteins. Citrullination is induced by immune cell-derived peptidyl-arginine deiminases (PADs), which are overactivated in the inflamed synovium. The plasma-derived serpin C1-inhibitor (C1-INH) is the major inhibitor of both the complement pathway and the contact system by targeting C1r, C1s, mannose-associated serine proteases (MASP)-1 and -2, activated factor XII, and plasma kallikrein. In the current study, we investigated the effect of PAD2 and PAD4-induced citrullination on the ability of C1-INH to inhibit complement and contact system activation.

Methods: ELISA and Western Blot techniques utilizing a biotinylated phenylglyoxal probe were used to confirm the citrullination of C1-INH. To assess C1-INH-mediated inhibition of complement activation, a C1-esterase activity assay was conducted. Downstream inhibition of complement was studied by ELISA, using pooled normal human serum as a complement source, to measure C4b deposition on heat-aggregated IgGs. Inhibition of the contact system was investigated by chromogenic activity assays for factor XIIa, plasma kallikrein, and factor XIa. Additionally, autoantibody reactivity to native and citrullinated C1-INH was measured by ELISA in 101 RA patient samples.

Results: PAD2 and PAD4 efficiently citrullinated C1-INH. Citrullinated C1-INH could not bind the serine protease C1s and inhibit its activity. Citrullination of C1-INH abolished its ability to dissociate the C1-complex and hence inhibit complement activation. Subsequently, citrullinated C1-INH had a diminished capacity to inhibit C4b deposition via the classical and lectin pathways. Citrullination strongly reduced the inhibitory effect of C1-INH on the contact system components factor XIIa, plasma kallikrein, and factor XIa. In RA patient samples, autoantibody reactivity to PAD2- and PAD4-citrullinated C1-INH was detected. Significantly more

binding was identified in anti-citrullinated protein antibody (ACPA) positive than in ACPA-negative samples.

Conclusion: Citrullination of C1-INH by recombinant human PAD2 and PAD4 enzymes impaired its ability to inhibit both the complement and contact systems in vitro. Furthermore, citrullination seems to render C1-INH more immunogenic, and citrullinated C1-INH might be an additional target of the autoantibody response observed in RA.

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Complement-associated prothrombotic state is caused by MAC-induced lysis of cells

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Background: Complement-mediated cytolysis occurs in PNH eventually causing frequent thrombotic events which cannot be therapeutically addressed by common anticoagulants. The underlying mechanism for the induction of thrombosis remains mainly elusive. Since C5 blockade is protective, it appears obvious that terminal but not the proximal pathway plays a significant role. We hypothesized, that MAC-mediated lysis of cells drives the pathophysiology of complement-induced thrombosis.

Methods: Complement activation fragments were analyzed for their platelet-activating capability in whole blood, PRP and isolated systems by aggregometry, ROTEM and detection of cell activation markers on platelet surfaces by flow cytometry. In a hirudin-anticoagulated whole blood system, platelet activation was investigated by foreign cells (rRBC)-induced complement activation in absence and presence of proximal and terminal inhibitors. This was mimicked in a fully humanized model using the AB/0 blood group system for complement activation. Translationally, huRBC were infused into rats to induce intravascular hemolysis and effects on the hemostatic system were investigated.

Results: None of the anaphylatoxins or opsonins showed increased expression of cell activation markers on isolated platelets. Addition of anaphylatoxins into blood had no impact on ROTEM analysis but increased aggregometry potential which was abolished in PRP, indicating an indirect mechanism via blood leukocytes. Addition of rRBC in whole blood resulted in complement-mediated lysis and platelet activation which is reversed upon C3 and C5 blockade. Lysis of human AB RBCs in whole blood of blood group 0 was associated with platelet activation and could be reversed by C5 blockade. Addition of ADP receptor blockers disabled platelet activation although full complement activation took place, proving that complement-induced release of intracellular ADP is the main trigger for platelet activation. In an in vivo rat model of mismatch transfusion with human RBCs, intravascular hemolysis correlated with a decrease in platelet count and an increase of fibrin deposition in the lung, phenomenona that can be reduced by therapeutic modulation with OmCI or prior treatment with CVF.

Conclusions: By using in vitro and in vivo approaches, we demonstrate that prothrombotic platelet activation necessitate the release of danger signals, whereas individual complement activation fragments tested failed to induce prothrombotic platelet activation.

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Association between acute stage biomarker levels, severity and long-term outcome in COVID-19

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Background: COVID-19 disease course is unpredictable and patients show symptoms that vary widely, ranging from asymptomatic or mild symptoms to life-threatening conditions and death. A substantial portion of patients also suffer from long-term multi-organ dysfunction, known as Long-COVID. Clinically validated biomarkers for COVID-19 disease severity, long-term outcome, and Long COVID are lacking, thereby hampering accurate diagnosis and targeted therapies. Aim of this study was to identify biomarkers that show utility in the prognosis of COVID-19 including their potential for predicting disease severity, survival, long-term outcome and Long-COVID.

Methods: The biomarkers PTX3, C1q, C1-INH, C1s/C1-INH complex, FXII and sMR, all involved in the initial inflammatory response in COVID-19 but for which long-term effects are unknown, were selected. For all markers, EDTA-plasma concentrations were measured in 215 well-characterized COVID-19 patients and 47 controls using ELISA and investigated whether they are associated with disease severity, survival, immune recovery and Long COVID.

Results: Median plasma concentrations for PTX3, sMR, and C1s/C1-INH were significantly associated with disease severity ($p<0.0001$, $p<0.0001$ and $p=0.0004$, respectively) and survival ($p<0.0001$, $p<0.0001$ and $p=0.0259$, respectively). In the non-survival group, sMR levels remained elevated up to 90 days when compared to survivors (adjusted p-value=0.0152), whereas PTX3 levels normalized after 14 days. In addition, PTX3 and sMR were associated with long-term immune recovery ($p=0.0004$ and $p=0.0006$, respectively). AUC values indicated that sMR (AUC 0.8458; $p<0.0001$) and PTX3 (AUC 0.7796; $p<0.0001$) are good markers for predicting disease severity. No association between biomarker plasma levels and the development of Long COVID was observed.

Conclusions: These results confirm previously reported studies that PTX3 plasma levels are predictive for COVID-19 disease severity and survival. However, the strongest association between plasma levels and disease outcome in this cohort is observed for sMR. Soluble MR was not previously associated with COVID-19 disease severity and/or survival and may serve as a novel marker to predict disease course, also for a longer period of time after infection (>14 days). In that light, measuring sMR levels would be of added value for monitoring COVID-19 disease course next to PTX3.

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Decreased Expression of CR1 on Peripheral White Blood Cells During Acute Dengue Infection

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Background: Dengue virus (DENV) infection can lead to severe symptoms, and the involvement of the complement system in dengue pathogenesis has been suggested. Complement receptor 1 (CR1) is involved in immune complex clearance and regulation of complement activation. Altered expression of CR1 during dengue infection may contribute to complement-mediated immunopathogenesis.

Methods: Blood samples were collected from 12-16 confirmed DENV-positive patients during acute and convalescent phases of infection, as well as from 6 healthy controls. CR1 expression levels on granulocytes, monocytes, and B cells were analyzed by flow cytometry.

Results: CR1 expression on granulocytes, monocytes, and B cells was significantly decreased during the acute phase of dengue infection, compared to the convalescent phase and healthy controls.

Conclusion: The down-regulation of CR1 expression on phagocytes during acute dengue infection may impair complement regulation and immune complex clearance, leading to severe disease and associated pathogenesis.

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The complement C1s cleaves HMGB1, generating a potent anti-inflammatory product

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Background: A crosstalk between HMGB1 and the complement system has been recently evidenced [1,2]. The HMGB1 protein was discovered as a nuclear DNA-binding protein. However, it can be released in the extracellular medium by dying and immune cells, where the immune system detects it as an alarmin. The two DNA-binding domains of HMGB1, the A- and B-box, differently modulate the immune response. While the full-length alarmin and its B-box exert pro-inflammatory effects through TLR4 signaling, the isolated A-box triggers antagonist effects. Here, the focus is on HMGB1 as a non-canonical target of the complement C1s protease, and how this could modulate inflammatory responses.

Methods: HMGB1 digestion by the C1s protease was characterized by SDS-PAGE and Western Blot analyses combined with mass spectrometry studies. The influence of the molecular environment of both proteins in this enzymatic reaction was also assessed. The three N-terminal cleavage fragments were produced and purified to test their impact on the secretion of pro-inflammatory cytokines. IL-6 and TFNα concentrations were quantified upon stimulation of RAW264.7 cells with LPS in complex with HMGB1 or its fragments.

Results: HMGB1 is cleaved at three positions by the C1s protease. The shorter N-terminal fragment, F3, includes a large part of the A-box. When complexed with LPS, HMGB1 shows pro-inflammatory effects by enhancing pro-inflammatory cytokines secretion. The same effect is observed with the A-box. In contrast, F3 inhibits the LPS-induced secretion of these pro-inflammatory cytokines. The presence or absence of a disulfide bridge in HMGB1, and the accessibility of C1s (free or within the complement C1 complex) modulate the amount of F3 released by C1s cleavage and, thus, indirectly impact the inflammatory response.

Conclusion: HMGB1 facilitates TLR4 dimerization through its B-box. According to our results, we hypothesize that F3 acts as a competitive inhibitor, preventing HMGB1/TLR4 binding. Hence, it would inhibit the receptor dimerization and its downstream signaling leading to the secretion of pro-inflammatory cytokines.

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Targeted complement inhibition using engineered bispecific antibodies that bind local antigens and endogenous complement regulators as a novel therapeutic approach

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Background: Complement activation provides protection against infection but also contributes to tissue damage in many diseases. Clinically used complement inhibiting drugs inhibit complement systemically, which is associated with adverse effects, including the increased risk for infections. To avoid this risk, we developed a novel class of bispecific antibodies (bsAbs) to target endogenous complement regulators towards defined (auto)antigens, in order to locally inhibit complement activation at the site of action.

Methods: Controlled Fab-arm exchange was used to generate sets of bsAbs of which one Fab-arm binds to a site-specific/pathology associated target and the other Fab-arm binds to either complement regulator factor H (FH) or C4b-binding protein (C4BP). The bsAbs were engineered in such a way that their Fc-domain did not allow additional complement activation or triggering of Fc-Receptors. These engineered bsAbs and their controls were tested in functional complement assays to determine their capacity to inhibit complement activation and target cell lysis.

Results: Compared to the parental antibodies only the bsAbs were able to bind both the model-antigen and human complement regulator FH or C4BP. Mass spectrometry confirmed that all bsAbs were formed correctly and had the desired bi-specificity.

In functional tests we observed that the bsAbs indeed inhibited complement activation and lysis as compared to their control bsAbs. In plate-bound assays the bsAbs were able to localize the inhibitors to the target and strongly inhibit complement activation initiated by either the classical, lectin, or alternative pathway. We established that the concentrations of endogenous FH and C4BP in serum were sufficiently high to mediate local inhibition. Next the bsAbs were tested for their capacity to inhibit complement activation on liposomes, erythrocytes and on primary human cells. The bsAbs also protected liposomes from classical pathway-mediated lysis as well as erythrocytes in classical and alternative pathway assays. Finally, the bsAbs protected human leukocytes from complement-mediated lysis.

Conclusion: Local complement inhibition by bsAbs binding to specific (auto)antigens as well as human endogenous FH or C4BP provides a completely novel therapeutic approach for locally targeted treatment of complement-mediated diseases.

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LPS O1-antigen-mediated complement-resistance of Klebsiella pneumoniae clinical isolates

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Introduction: The Gram-negative bacterium Klebsiella pneumoniae is an opportunistic pathogen associated with diseases like pneumonia and sepsis. It has previously been reported that Klebsiella can prevent detection and killing by the human complement system through the expression of capsule and LPS O-antigen modifications. The aim of this study is to understand the influence of LPS O1-antigen on bacterial survival in serum.

Methods: Several clinical Klebsiella pneumoniae strains expressing different O-antigens were collected. Complement-mediated killing was assessed using a membrane impermeable DNA-dye that becomes fluorescent upon damage of the bacterial inner membrane. Analysis of complement activation was performed using either fluorescence-labelled complement components or specific antibodies using flow cytometry.

Results: Serum-susceptibility was assessed by colony forming units (CFU) and membrane damage upon serum exposure. Strains expressing LPS O1-antigen were more serum resistant than strains expressing LPS O2-antigen. LPS O1-strains deficient for capsule (ΔwbaP) showed no decrease in survival after exposure to serum. In contrast, deletion of the whole O-antigen (ΔwbbO) or O-antigen cap (ΔwbbYZ) from an O1 strain increased serum-susceptibility. All the different knockout strains activated complement at C3b level. Deposition of the terminal complement proteins C5b-9 (membrane attack complex; MAC) was highest in O-antigen deficient strains, decreased in O1-antigen cap knockouts, and was lowest in presence of the full O1-antigen. Our data show that LPS O1-antigen expression renders Klebsiella pneumoniae resistant to killing via the complement system by interfering with deposition of C5b-9 complex.

Conclusion: We show that Klebsiella presenting LPS O1-antigen are more serum-resistant than Klebsiella presenting LPS O2-antigen. To achieve serum resistance, fully expressed O1-antigen is required, as O-antigen cap mutants are serum-sensitive. The O1-antigen then prevents killing via complement by preventing effective deposition of the membrane attack complex. The exact mechanisms by which the O1-antigen prevents effective MAC formation remains to be elucidated. Understanding bacterial evasion of complement is essential to explore novel antimicrobial strategies.

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CD59 isoforms IRIS-1 and IRIS-2 are expressed in human and mouse brains, required for neurotransmitter release, and have a decreased expression in Alzheimer[StQuote]s disease patient[StQuote]s brains

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Background: We previously described that non-GPI anchored CD59 splice isoforms IRIS-1 and IRIS-2 (Isoforms Rescuing Insulin Secretion 1 and 2) are required for insulin secretion from pancreatic β-cells and that the expression of both IRIS-1 and 2 is significantly reduced in islets isolated from human type 2 diabetic (T2D) patients, as compared to healthy controls. While assessing the expression of IRIS-1 and IRIS-2 in various human tissues we found high RNA expression levels of these isoforms in the human brain, however, their protein expression or role in the brain was unknown.

Methods: Using confocal microscopy, noradrenaline ELISA, proximity ligation assay (PLA), and expression studies on primary human and mouse brain sections, and neuronal cell lines we assessed the localisation and function of IRIS-1 and IRIS-2.

Results: Both IRIS-1 and IRIS-2 exist at mRNA and protein levels in human and mouse brains, where they localize in astrocytes and neurons, but not in microglia. IRIS-1 and IRIS-2 are found within the cytosol of the neuroblastoma cell line (SH-SY5Y), and their expression significantly increases in neuroblastoma cells differentiated into mature neurons with retinoic acid. Knockdown of IRIS-1 and IRIS-2 in SH-SY5Y cells greatly reduces the SNARE complex formation (needed for synaptic vesicle exocytosis). As a result, cells with IRIS-1/2 knockdown display significantly reduced secretion of noradrenaline. We have also shown that neuronal IRIS-1 and IRIS-2 expression is reduced in patients with Alzheimer's disease (AD) and that phosphorylated Tau expression (a key pathological feature of AD) is much higher in SH-SY5Y cells with IRIS-1/2 knockdown.

Conclusions: Our data prove the existence of non-GPI anchored CD59 splice isoforms IRIS-1 and IRIS-2 in human and mouse brains and indicate that these isoforms are required for synaptic vesicle exocytosis. Additionally, the knockdown of IRIS-1/2 in SH-SY5Y cells results in increased activity of cyclin-dependent kinase 5 (CDK5) which causes Tau protein hyperphosphorylation and the accumulation of phosphorylated Tau in these cells, accelerating AD pathology.

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Role of Dengue NS1 and its Antibodies in Complement Activation in Hepatocytes

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Background: Dengue virus (DENV) non-structural protein 1 (NS1) is a glycosylated protein involved in viral replication, immune evasion, and viral pathogenesis. Hepatic dysfunction is common in severely ill patients, but the underlying causes are not fully understood. We hypothesized that NS1 attaches to the surface of uninfected hepatocytes, and its recognition by specific antibodies leads to complement activation and liver damage in dengue patients.

Methods: Immortalized hepatocyte-like cells were used to test 16 clones of chimeric monoclonal antibodies to NS1. Confocal microscopy was used to determine NS1 binding to the cell surface and the efficiency of complement activation leading to the deposition of C3d and C5b-9 on the uninfected cells.

Results: The clone 2C5 of chimeric monoclonal antibodies, recognizing the wing of NS1, showed the highest efficiency in recognizing NS1 attachment to the cell surface, resulting in complement activation and deposition of C3d and C5b-9. Some clones of chimeric monoclonal antibodies that recognized the β-ladder of NS1 showed higher NS1 intensity but lower efficiency in complement activation, leading to the deposition of C3d but not C5b-9.

Conclusion: NS1 attachment to uninfected cells exposed different epitopes for recognition by antibodies, leading to complement activation. Knowledge of NS1 epitopes and the specific antibodies responsible for complement activation may lead to a better understanding of the NS1 specific regions that contribute to the protection and pathogenesis of dengue.

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C3d-Directed Factor H Delivers Potent and Durable Local Complement Inhibition, Disease-Modifying Efficacy, and Decreased Renal-Derived C5b-9 Without Inhibiting Systemic Complement

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Background: ADX-097 is an antibody fusion protein that targets the alternative pathway (AP) complement negative regulator factor H to diseased tissue. ADX-097 was designed to bind tissue-fixed C3d-containing C3 activation fragments and inhibit complement in diseased tissue while minimizing systemic blockade.

Methods: Having demonstrated C3 activation and C3d deposition in human renal and skin disease biopsies, we generated and characterized ADX-097, a humanized anti-C3d monoclonal antibody linked to five N-terminal repeats of the AP inhibitor factor H (fH_{1-5}). We further evaluated circulating and tissue PK/PD of ADX-097 in mouse and non-human primate (NHP) models of tissue complement activation. Finally, we tested ADX-097 PK/PD and efficacy in the rat Passive Heymann Nephritis (PHN) model of membranous nephropathy.

Results: We characterized in vitro binding of ADX-097 to C3d, demonstrating that at high C3d densities, use of a bivalent anti-C3d antibody yields binding affinities that are ~1000x greater than those of comparable monovalent binders, suggesting a substantial avidity-based targeting advantage. Consistent with this highly efficient targeting, in rodent and NHP models of local complement activation, ADX-097 dosed subcutaneously (SC) as low as 1 mg/kg distributes to C3d+ tissue and inhibits complement activation. In factor H-/- mice, a single 1 mg/kg SC dose achieves >75% glomerular complement C3 inhibition for at least 7 days while avoiding systemic complement blockade. In a rat model, 1 mg/kg dosed SC or IV inhibited glomerular complement activation and significantly reduced urine protein-creatinine ratios without inhibition of systemic complement, and with an efficacy that was equivalent to full systemic complement blockade using daily injections of cobra venom factor. We also show that urinary C5b-9 (uC5b-9) correlates strongly with decreased glomerular C3 activation and is independent of systemic blockade or proteinuria-associated renal leakage, suggesting the utility of uC5b-9 as a biomarker for local complement regulation in the kidney.

Conclusions: These data demonstrate the therapeutic potential of ADX-097 and show that C3d-mediated tissue targeting of fH_{1-5} in preclinical models results in potent, durable, and efficacious local AP complement blockade without systemic complement inhibition, and that this is an effective strategy for re-regulating tissue complement in disease.

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Targeting terminal pathway in brain reduces complement activation and synapse loss in a mouse model of dementia

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Background:
Neuroinflammation is a critical component of Alzheimer's Disease (AD). Dysregulation of complement leads to excessive inflammation, direct damage to self-cells and propagation of injury. This is likely of particular relevance in the brain where inflammation is poorly tolerated and brain cells are vulnerable to direct damage by complement. The membrane attack complex (MAC) is a highly pro-inflammatory product of complement activation, killing cells by lysis and/or causing sublytic damage, including initiating NLRP3 inflammasome activation, and provoking other damaging responses leading to death of vulnerable brain cells.

Methods: The role of MAC in AD was investigated in MAC-deficient animals and by using a newly developed anti-C7 monoclonal antibody (mAb) that efficiently inhibits formation of the MAC in vitro and in vivo. Impact of C7 deficiency on brain complement dysregulation, synapse loss, amyloid load and cognitive decline was examined by comparing APP^{NL-G-F} mice back-crossed to C7 deficiency (APP^{NL-G-F}xC7) with unmodified APP^{NL-G-F} mice. To assess the effect of therapeutic C7 blockade, unmodified APPNL-G-F mice were treated systemically (for four weeks) with anti-C7 mAb or control IgG and the same set of parameters of complement dysregulation, pathology and cognition measured.

Results: C7 deficiency in App^{NL-G-F} mice reduced levels of complement activation markers, reduced amyloid load and increased synapse density with a commensurate improvement in cognitive test performance. Systemic treatment of App^{NL-G-F} mice with a blocking anti-C7 mAb caused reduced brain levels of complement activation markers, reduced amyloid load and increased peri-plaque synapse density and cognitive performance when compared to controls.

Conclusions: We demonstrate that complement dysregulation occurs in brain in the App^{NL-G-F} mouse model of AD. C7 deficiency reduced brain complement dysregulation, reduced synapse loss and improved other pathological parameters and cognitive function. Systemic anti-C7 therapy reduced complement dysregulation and protected from synapse loss in the model. Current efforts to modify the anti-C7 mAb for brain delivery will enhance efficacy in the model. The findings highlight the potential for complement inhibition at the level of MAC as a therapy in AD.

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Elucidating the role of anaphylatoxins and their receptors in osteoarthritis regarding cartilage calcification

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Cartilage trauma leads to complement activation and subsequent generation of anaphylatoxins C3a and C5a by cleavage of C3 and C5. Previous studies indicated that anaphylatoxins and the corresponding receptors C3aR and C5aR1 are associated with the calcification of blood vessels and involved in osteogenic differentiation (OD). Therefore, we investigated the involvement of anaphylatoxins in cartilage calcification.

Human cartilage from donors undergoing knee replacement (n=55) was either used for histology (IHC or Alizarin Red staining), qPCR or isolation of articular chondrocytes (hAC). Anaphylatoxin generation was assessed by ELISA after incubation of C3 or C5 for 4h w/ or w/o hAC in presence or absence of cartilage homogenizate (HG) or conditioned media of impacted or unimpacted cartilage. For OD, hAC were cultured for 21 days in differentiation media in presence or absence of C3a, C5a, SB290157 (C3aR inhibitor) and/or PMX53 (C5aR1 inhibitor). Calcification was evaluated by Alizarin Red staining. The expression of C3aR and C5aR was analyzed by flow cytometry and IHC. Statistical analysis: Student́s t-test and one-way ANOVA.

We found that hAC can cleave C3 ($P<0.0001$) and C5 ($P=0.0009$) which was further enhanced by HG (C3a: 3.7-fold, $P<0.0001$; C5a: +20%, $P=0.054$). Additionally, C3a ELISA revealed that cartilage-conditioned media contained 4-fold higher C3a concentrations (median: 19.81ng/ml) relative to the previous experiments. Furthermore, 1000 ng/mL C3a (+12%, $P=0.048$) and 50ng/mL C5a (+14%, $p=0.027$) increased calcium deposition during OD, which could be reduced by simultaneous inhibition of C3aR (-7.6%, $p=0.059$) and C5aR1 (-28.6%, $p=0.001$). We also observed that gene expression of anaphylatoxin receptors was upregulated by 4-fold (C3aR: $P=0.043$; C5aR1: $P=0.044$) in highly degenerated (OARSI$\geq$3) human cartilage compared to macroscopically intact (OARSI$\leq$1) tissue. IHC staining confirmed this on protein level (C3aR: +73%, $P=0.0006$; C5aR1: +31%, $P=0.0004$). In addition, expression of C3aR and C5aR1 on hAC was significantly associated with both calcium deposition during in vitro OD and calcification of native cartilage tissue.

Our results indicate that both, amplified C3a and C5a generation in a posttraumatic environment (mimicked by HG) and increased C3aR and C5aR1 expression might contribute to cartilage calcification and thus cartilage degeneration and subsequent OA progression.

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Lack of MASP-1 results in delayed onset of albuminuria and prolonged survival in lupus-prone MRL/lpr mice

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Background: Mannose-binding lectin-associated serine protease-1 (MASP-1) and MASP-3, transcribed form the common Masp1 gene, play critical roles in the lectin and alternative complement pathway activation, respectively. We previously reported that Masp1-knockout (deficient for both MASP-1 and MASP-3) lupus-prone MRL/lpr mice showed significantly reduced glomerulonephritis and albuminuria compared to their wild-type littermates. The data suggested that MASP-1 and/or MASP-3 play an important role in the development of lupus-like glomerulonephritis in these mice via activation of the lectin and/or alternative pathways. Here, we aimed to clarify the role of MASP-1 in the development of renal disease in lupus by analyzing disease expression in MRL/lpr mice monospecifically deficient for MASP-1.

Methods: MASP-1-deficient mice were generated by the CRISPR/Cas9-mediated genome editing in the C57BL/6 background and then backcrossed to the MRL/lpr background for eight generations. Sera and urine were collected biweekly from groups of MASP-1-deficient and wild-type MRL/lpr mice starting at 12 weeks of age. Serum C3, anti-dsDNA IgG antibody and urinary albumin excretion levels were measured by ELISA. Circulating immune-complex levels were measured by ELISA utilizing anti-C3 and anti-mouse IgG antibodies. Mice were sacrificed at 24 weeks of age and kidneys were collected for histopathological analysis.

Results:

MASP-1-deficient MRL/lpr mice showed no significant differences in serum C3, anti-dsDNA IgG antibody and circulating immune-complex levels compared to their wild-type littermates. Wild-type MRL/lpr mice developed albuminuria as early as 12 weeks of age, whereas MASP-1-deficient MRL/lpr mice developed albuminuria around 16 weeks of age. Wild-type MRL/lpr mice started to die as early as 16 weeks of age, whereas no MASP-1-deficient MRL/lpr mice died until 20 weeks of age ($p = 0.019$ at 19 weeks of age). However, histopathological analysis of the kidneys collected at 24 weeks of age showed no significant differences in C3 deposition levels and in the development of lupus-like glomerulonephritis including proliferative, necrotic and crescentic changes between the two groups.

Conclusion: Our results suggest that MASP-1 accelerates the development of albuminuria and mortality in MRL/lpr mice, most likely via activation of the lectin pathway.

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Ex situ porcine liver machine perfusion activates the complement system and increases cytokines independent of pre-induced liver injury

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Background and goal of study: Ischemia-reperfusion injury (IRI) is a key challenge in liver transplantation leading to short- and long-term failure of the transplanted liver. Machine perfusion (MP) has proven to limit the metabolic consequences of IRI and has the potential to rescue discarded livers. However, the effect of MP on the innate inflammatory response is unknown. We aimed to investigate complement activation and downstream effects that may be targeted to reduce IRI and inflammation during MP.

Method: Porcine livers (n=24) were exposed to either biliary injury (n=8), global liver injury (n=8), or no liver injury (n=8). Ex situ liver MP was performed with 1h hypothermic, 1h rewarming, and 4h normothermic perfusion. Belzer preservation solution was used during the hypothermic phase. Thereafter, heparinized leukocyte- and platelet-depleted homologous blood was used as perfusate. Perfusate and tissue samples were collected at set time points and analysed for terminal complement complex (TCC) and cytokines (TNF, IL6, IL-1β, IL8 and IL10) using ELISA and Multiplex.

Results: During normothermic MP, TCC increased significantly from start (median 11 interquartile range [5.14-17]) to end MP (40 [15-60], $p<0.0001$). There was no statistical difference between the biliary injury, the global injury, and the no liver injury groups ($p>0.5$). During normothermic MP cytokines increased significantly in plasma (TNF, IL 6, IL10), in liver tissue (TNF, IL-1β, IL6, IL8) and bile tissue (TNF, IL-1β, IL6, IL8), all $p<0.0001$ Friedmann test/Mann Whitney test.

Conclusion: Complement and downstream cytokines are strongly and persistently activated during normothermic MP independently of pre-induced liver injury. Cytokines increase significantly despite a leukocyte-depleted perfusate and are thus probably due to liver-derived cytokine production. Future studies should evaluate the source of cytokine production and if complement inhibition can suppress cytokine production during liver MP. Inhibition of complement activation might be a therapeutic option during MP.

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Design and in vitro Evaluation of Collectin-11 Antagonists for the Treatment of Ischemia-Reperfusion Injuries

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Background: Ischemia-reperfusion injury (IRI) is a complex pathophysiological process that is induced by temporarily restricted blood flow and can lead to the activation of immune responses, tissue injury and reduced function or even rejection of the graft. Recent studies revealed a key role for collectin-11 (CL-11), a circulating PAMP/DAMP receptor associated with the complement system's lectin pathway (LP), in the pathology of renal IRI [1]. CL-11 is a C-type lectin, which recognizes aberrant l-fucoseresidues on endothelial cells upon hypoxic stress or hypothermia [1], induces complement activation, and contributes to acute tissue injury and fibrosis. While competitive antagonism of CL-11 with soluble l-fucose was able to prevent IRI damage in mice[2], monosaccharides are not well suited for future therapeutic purposes. In this study, we established a design-make-test platform to enable the development of glycomimetic CL-11 antagonists.

Methods: The carbohydrate recognition domain (CRD) of CL-11 was recombinantly produced in E. coli and its functional activity was validated by testing the binding to carbohydrate ligands. Glycomimetic lead compounds were rationally designed in silico based on structural analyses. Polymer-based competitive binding assays and nanoDSF analysis were employed to assess the binding and antagonistic activity of natural and synthetic CL-11 ligands.

Results: Upon optimization of the CL-11 construct (neck region) and expression conditions, recombinant CL-11-CRD could be produced with yields and purity suitable for screening efforts and showed the expected selectivity for mannose and fucose. Our assay platform revealed that several of the newly designed CL-11antagonists showed improved binding affinity and inhibitory efficacy when compared to l-fucose or mannobiose.

Conclusion: Owing to their high polarity and low monovalent affinity, the development of carbohydrate-based therapeutics is challenging and relies on finely tuned design-make-test cycles. The platform established in this study enabled us to conduct initial structure-activity relationship experiments for a series of α-D-mannosides and derivatives thereof that show promising activity profiles for further development.

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Investigation of complement-related integrin receptors using a screening platform and newly developed tool compounds

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Background: Complement receptors CR3 and CR4 are members of the β2-integrin family. They are involved in complement-dependent phagocytosis and leukocyte adhesion, migration, and activation, thereby playing important roles in immune surveillance and inflammation. The direct involvement of β2-integrin receptors in autoimmune, inflammatory, and age-related diseases render them interesting drug targets. However, they often act as both immune activators and suppressors in a complex interplay that is not well understood. In addition, CR3 has been shown to interact with a vast number of ligands [1], with unknown biological implications. To address this unmet need, we are developing a platform of recombinant αI domains of β2-integrins and assays to test their interactions with endogenous and synthetic ligands. In this study, we employed this platform to assess newly developed tool compounds that may help to disentangle the promiscuous ligand binding profile of CR3 and its implication in pathologies.

Methods: We expressed recombinant ligand-binding domains (αI) of all β2-integrin members in E.coli. These αI-domains were used to investigate the interaction of C3-derived opsonins with members of the β2-integrin family in direct binding (SPR) and bead-based adhesion assays. Using phage display screening, we have identified tool compounds that bind to CR3 and are able to compete with the interaction of endogenous ligands.

Results: The αI-domains of all four β2-integrins could be uniformly expressed in E. coli, in both wildtype and high-affinity conformation. Recombinant CR3 αMI and CR4 αXI showed distinct binding profiles for C3-derived opsonins that were dependent on activation states and cofactors. Phage display screening against CR3 αMI identified cyclic peptides as CR3 ligands, which showed direct binding to the αMI domain, partially competed with opsonin recognition, and exerted modulating activities in functional adhesion assays.

Conclusion: The platform established here is suitable to characterize the interaction profile of β2-integrins with their ligands and develop tool compounds able to distinctly modulate integrin-ligand interactions. The availability of integrin/ligand-specific modulators will facilitate the elucidation of the β2-integrin receptor's intricate functional interplay in health and disease and may enable the development of therapeutic integrin modulators that block specific -ligand interactions while maintaining immune defense functions.

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Investigating the Role of Complement in Cognitive Decline after Repetitive Mild Closed Head Brain Injury

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Background: Repetitive mild closed head injury (rmCHI) results in the development of cognitive deficits and may lead to neurodegenerative disease later in life. The underlying neuroimmune mechanisms linking rmCHI to cognitive decline are not well understood, and the role of the complement system in this context is unexplored.

Methods: We developed a mouse model of rmCHI and examined pathophysiological and cognitive outcomes in injured vs. non-injured animals in the context of complement inhibition. Flow cytometry was used to characterize local and peripheral immune cell recruitment after injury. Immunofluorescence microscopy was used to analyze complement involvement, as well as to characterize microglial morphology and activation status. A multi-omics approach including proteomics and RNAseq coupled with CyTOF was employed to further dissect the neuroimmune response and the role of complement in brain pathology and animal behaviour after rmCHI.

Results: Of various rmCHI paradigms investigated, a 12 hit model was found to be optimum in terms of measurable outcomes. Following 12 hit rmCHI over 24 days, animals exhibited worsened spatial learning and memory retention. Flow cytometry revealed increased infiltration of various innate and adaptive immune cells. Immunofluorescence staining showed elevated complement deposition in the hippocampus of injured animals compared to non-injured. Microglial ramification was reduced after 12-hits, which was also associated with decreased microglial cell volume. In a therapeutic approach, we treated animals with an injury site-targeted complement inhibitor, CR2Crry, which inhibits all activation pathways of complement at C3 cleavage. CR2Crry treatment of injured animals improved cognitive outcomes, which was associated with changes in the abundance of distinct microglial subpopulations as revealed by mass cytometry. RNAseq and proteomic analysis revealed major changes in neurodegenerative associated pathways after rmCHI, with many of these alterations moderated by complement inhibition.

Conclusion: We developed and characterized a closed head repetitive injury model and demonstrated a role for complement in cognitive decline and the upregulation of multiple neurodegenerative markers/pathways post rmCHI. Targeting the complement system as a therapeutic approach in repetitive brain injuries requires further investigation.

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Proteolytic activity of secreted proteases from pathogenic leptospires and effects on phagocytosis by murine macrophages

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Leptospirosis is a zoonosis caused by spirochete bacteria that belong to the genus Leptospira. This disease represents a serious public health problem, especially in developing countries with tropical and subtropical temperatures. Pathogenic leptospires escape from the Complement System, a property that permits them to survive in vitro when in contact with normal human serum (NHS). In a previous study carried out by our group, it was observed that culture supernatants from different pathogenic species of leptospires (SPL) contain proteases that cleave many Complement proteins, including the central molecule C3 and its fragments C3b and iC3b. Our hypothesis is that these proteases, could decrease the phagocytic clearance of leptospires. Using flow cytometry, we observed decreased amounts of CR3 and CR4 in murine peritoneal macrophages treated with SPL for 24 h. By confocal microscopy, we observed reduction in TLR2, CD11b and CD206 levels when these cells were treated with SPL and recombinant thermolysin for 24 h. Furthermore, opsonins such as C3b/iC3b deposited on the surface of pathogenic leptospires were observed to be completely degraded in the presence of SPL or recombinant thermolysin. Finally, we decided to investigate the phagocytosis of pathogenic leptospires by macrophages in the presence of these proteases. We observed an increase of phagocytosis of leptospires opsonized with normal mouse serum even when macrophages were treated with the proteases. However, when opsonized bacteria were also incubated with SPL, recombinant thermolysin and recombinant leptolysin., there was a decline in leptospires phagocytosis. This suggests that the proteolytic activity can affect phagocytosis by peritoneal macrophages mainly through the degradation of opsonins deposited in the membrane of leptospires. These observations lead us to suggest that proteases secreted by pathogenic leptospires could degrade opsonins present in normal serum or deposited in the bacterial membrane as well as cleave or inhibit macrophage surface molecules. Therefore, these proteases could interfere with the recognition and internalization by murine macrophages, favoring the spread of leptospires in the host.

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Distinctive dosage requirements between C3 and Factor D (FD) in the activation of the alternative complement pathway

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Background: Alternative pathway (AP) contributes substantially to complement activation through its powerful amplification loop. Factor D (FD) is the smallest in size and lowest in concentration among AP components. We and others have established that a relatively small amount of FD is required to activate the AP. The role of FD function in combination with other AP components is further investigated in this study.

Methods: Crry/FD double knockout (DKO) and Crry/FD/C3 triple knockout (TKO) mice were used to study AP function. Serum or purified FD was transferred into DKO or TKO and Western blots were employed to quantitate C3 consumption. A rabbit erythrocyte hemolysis assay was utilized to monitor AP functional activity.

Results: Using hemolysis assay, 180 ng/ml of purified FD (approximately 1/100 of normal) rescues the AP defect in FD KO mice while 360 μg/ml of C3 (about 1/3 of normal) is required to rescue the AP in C3 KO mice. C3 consumption occurs in the Crry KO mouse due to a lack of AP control but Crry/FD DKO mice have a normal level of C3. Injection of purified FD into the Crry/FD DKO mouse leads to a rapid decrease (within 15 minutes) of C3 in blood. Transferring 200 μl of WT serum into the Crry/FD/C3 TKO mouse results in the virtual disappearance of blood C3, being consumed in an environment lacking the membrane regulator Crry. Infusion of FB KO serum [without initial C3 (H2O) Bb convertase] versus WT serum into Crry/FD/C3 TKO leads to equivalent C3 consumption. The defect in hemolysis of Crry/FD/C3 TKO serum was not rescued by adding a higher concentration of FD or C3. A low dose of FD at 100 ng/ml and doses of C3 from 40 to 360 μg/ml were added to the serum of Crry/FD/C3 TKO mice. C3 at 360 μg/ml was again required to achieve about 90% of WT hemolysis.

Conclusion: Our results demonstrate that a low amount of FD but C3 at more physiological levels are required to activate the AP. These experimental data have implications for treating diseases with anti-FD therapeutics.

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Complement System and leptospirosis: understanding the role of C3 during the renal fibrosis caused during chronic infection by pathogenic leptospires in murine experimental model

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Leptospirosis is a neglected zoonosis affecting approximately 1 million people each year worldwide and causing near 5% deaths.

Most patients are asymptomatic or have only mild clinical manifestations. However, some patients develop severe chronic and end-stage kidney disease responsible, which may be fatal. We are investigating the role of C3 in the chronic leptospirosis by using C57BL/6 wild-type (WT) and C57BL/6 C3-knockout (C3KO) male mice infected with 10^8 L. interrogans serovar Copenhageni strain Fiocruz L1-130 (LIC) or PBS (negative control) after 15, 30, 60, 90 and 180 d.p.i.. We observed the following results: i) all infected mice survived. However, more leptospires were observed in the kidney of LIC-C3KO mice up to 90 d.p.i., when compared to LIC-WT mice, by immunohistochemistry analysis; ii) LIC-infected mice lost more body weight during the first 5 days of infection when compared to PBS, independent of the presence of C3; iii) no significant differences in the spleen/total body weight ratio were observed between LIC-WT and LIC-C3KO after 15 d.p.i. or 30 d.p.i.; iv) LIC-C3KO mice presented higher scores of histopathological alterations in the liver and kidney, when compared to LIC-WT mice at 30 d.p.i up to 180 d.p.i.; v)) higher percentage of fibrosis was observed in the kidney from LIC-C3KO mice when compared to LIC-WT at 30 d.p.i., after Red Sirius staining, which reveals the presence of collagen I and III in the tissue. In conclusion, C3 did not affect the survival of LIC-infected C57Black/6 mice but it was important for the in vivo killing of leptospires. Leptospira remains for longer time in the liver and kidney of LIC-C3KO mice when compared do WT in the same conditions. In addition, the lack of this protein may trigger renal fibrosis in the infected mice at 30 d.p.i. suggesting that the presence of local C3 may control tissue repair after damage by pathogens.

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Complement protein autoantibodies and increased Factor H related protein four levels may contribute to pathogenesis in IgA nephropathy

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IgA Nephropathy (IgAN) is the most common form of primary glomerulonephritis world-wide. Patients develop chronic kidney disease and up to 30% advance to end stage renal failure (ESRF). IgAN pathophysiology remains poorly understood. However, the lectin and alternative pathways of the complement system have been implicated. Indeed, altered Factor H (FH) and FH related protein 1 (FHR1) levels have been shown to associate with disease severity. Given the immune predisposition in IgAN, we decided to assay for the presence of autoantibodies to complement proteins (FH, FB, etc) and for altered expression levels of FHR proteins.

EDTA plasma samples were obtained from healthy blood donors (~200) via the Newcastle blood donor service and NRCTC. Over 500 biopsy-proven IgAN patient plasma samples were obtained from the Glomerulonephritis DNA Bank (UKGDB). Patients were classified as either non-progressors (with stable serum creatinine levels) or progressors (where serum creatinine doubled or patients reached ESRF) within 10 years of diagnosis. The standardised anti-FH autoantibody ELISA protocol was modified to test for anti-complement protein autoantibodies, and in house assays developed to measure FH and FHRs.

Approximately, 5% of IgAN samples were positive for anti-FH autoantibodies (> 100RU) and 1% had detectable Factor B autoantibodies but no significant anti-C3b reactivity was detected in the IgAN samples. Strikingly, mean FHR4 serum concentration was 2.3-fold greater in a cross section of the IgAN population (2.205±1.393μg/mL) compared to controls (1.248±0.639μg/mL), $p<0.0001$. Interestingly, samples from patients deemed to be progressors, had significantly higher levels of FHR4, compared to non-progressors ($p=0.0009$). As well as this, FH/FHR4 molar ratios were significantly decreased compared to healthy samples (progressors ($p=0.0076$) and non-progressors ($p=0.0089$)). FHR4 levels did not correlate with age and gender.

Our research into the reasons why the alternative pathway may be dysregulated in IgAN has identified the presence of anti-complement protein autoantibodies (although generally at a relatively low titre) and a suggestion of skews in the FH/FHR4 ratio. Strikingly, FHR4 levels were significantly increased in progressors compared to non-progressors, suggesting FHR4 may provide some utility as a potential biomarker when determining patient risk status, although expansion and replication of this work is needed.

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The mannose-binding lectin (MBL) in MIS-C: relationship between MBL genotype, levels, and functional activity against SARS-CoV-2

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Multisystem inflammatory syndrome in children (MIS-C) is a rare complication of SARS-CoV-2 infection with clinical features common to Kawasaki disease and overactivation of the immune system. We performed whole-exome sequencing from a patient who developed MIS-C with fatal consequences, and we extended this analysis to the family. We identified the p.Arg52Cys polymorphism in heterozygosis in the MBL2 gene (encoding the mannose-binding lectin, MBL), and a frameshift variant in FCN3 (encoding ficolin-3), suggesting a role of the lectin pathway of complement in MIS-C pathogenesis. The p.Arg52Cys variant (known as "D", while the wild-type allele is referred as "A"), has been associated with lower levels of plasma MBL levels and with loss of function. We recently reported that MBL binds to the spike protein of SARS-CoV-2 and has antiviral activity. Upon interaction with spike, MBL was found to activate the complement pathway. However, comprehensive data examining the interaction between structural MBL variants and SARS-CoV-2 infection are lacking. In this study

we investigated the binding of recombinant MBL carrying the "D" variant to SARS-CoV-2 spike protein. Compared to the wild-type protein, Arg52Cys substitution impaired MBL oligomerization, but it did not affect its interaction with SARS-CoV-2. Then, we examined the interaction of native MBL with SARS-CoV-2 using biological samples obtained from the relatives of the patient affected by MIS-C. We quantified functional MBL levels using a mannan-binding assay, and we measured the spike-dependent activation of the complement pathway using a C4-deposition assay. Serum-derived MBL A/D did not bind to spike, and it did not activate the complement, compared to the MBL A/A from the father. Surprisingly, MBL A/A from the brother did not recognize SARS-CoV-2 spike protein. Thus, genetic variations in MBL2 locus, yet to be identified, affect the antiviral activity of MBL and may contribute to hyperinflammation in MIS-C.

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Differential complement iC3b levels in baseline plasma of individuals at clinical high risk for psychosis

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Background: Early identification and treatment of individuals at clinical high-risk (CHR) for psychosis is critical but there are currently no objective biomarkers for the diagnosis of those at risk who go on to develop psychosis. Proteomic investigations have identified altered blood protein levels in the immune complement and coagulation pathways in people with CHR who subsequently develop psychosis. This study quantified complement activation fragments in CHR plasma with the aim to identify pathway-specific pathophysiological mechanisms, complement activation fragments correlating with clinical symptoms and distinguishing those CHR individuals who transition to psychosis.

Methods: This study included n=230 participants from the NEURAPRO study of which n=26 had a known outcome of psychosis and n=204 CHR who did not transition to psychosis after 6 months. Plasma concentrations of complement activation fragments C4a, C4d, C3a, iC3b, Bb, C5a, and sC5b-9 (TCC) were quantified at baseline using standardised commercial ELISA kits (Quidel). Outliers removed >+3SD. The main outcome of interest was transition to psychosis (Mann Whitney u test) and secondary outcome measures included the Scale for the Assessment of negative Symptoms (SANS), and Social and Occupational Functioning Assessment Scale (SOFAS).

Results: Plasma levels of iC3b were significantly lower in those CHR individuals who transitioned (T) to psychosis at 6-months follow up (T:19.70±22.06 vs NT:23.56±21.21 mg/ml, p=0.0313). Plasma iC3b levels are significantly different in high versus low negative symptoms (SANS) subgroups (18.48±15.88 vs 28.24±24.33 μg/ml, p<0.0001). Baseline SOFAS correlated significantly with iC3b (r=0.217, p=0.001), C3a (r=0.143, p=-0.032), C4d (r=0.153, p=0.022), and SANS with iC3b (r=-0.185, p=0.006) and sC5b-9/TCC (r=-0.226, p=0.001).

Conclusions: Complement activation and regulation can be monitored by measuring pathway-specific complement activation and cleavage fragments. Cleavage of C3b into iC3b leads to down-regulation of complement amplification in plasma and on cell surfaces, where it can also act as an opsonin promoting phagocytosis. This study found an interaction between complement cleavage fragment iC3b with symptom severity and future transitioning to psychosis in clinical-high risk individuals. Further investigations are needed to determine the feasibility of complement activation fragments as biomarkers and to investigate underlying pathophysiological mechanisms to facilitate targeted therapeutic development.

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Complement receptor 1 long homologous repeats (A, B, and C) as a novel tool to study immune-adherence functionality of human erythrocytes

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Complement receptor 1 (CR1) is an integral membrane protein that consists of 30 extracellular complement control proteins (CCPs) comprising four (A, B, C, and D) long homologous repeats (LHRs). CR1 harbours not only the binding sites for C3b, iC3b and C4b, but also the domains that are responsible for both, the decay accelerating activity (DAA) and cofactor activity (CA). The DAA (Site 1) of CR1 is harboured in CCP(1-3) of LHR-A, while the cofactor activity (Site 2) maps to CCP(8-10) of LHR-B and CCP(15-17) of LHR-C. CR1 is implicated in the phenomenon of immune adherence in which materials tagged with complement opsonins adhere to the erythroid CR1 molecules and thus get transported throughout the body while bound to erythrocytes.

This study set out to express and functionally characterise recombinantly produced CR1-ABC as a tool to study mechansistic requirements of the immunoadherence phenomenon. CR1-ABC was expressed in the heterologous host Pichia pastoris and purifed to homogeniety using previosuly established methodology. 69 mg of a highly pure protein was obtained. The regulatory activity of this protein was assessed through a standard alternative pathway mediated haemolysis assay of rabbit erythrocytes (rRBCs). To test the applicability of soluble CR1-ABC protein as a tool to disrupt the binding of C3b-coated cells to CR1 on human erythrocytes (hRBCs), which mimicks the basic concept of immunadherence, a competition assay was performed. C3b opsonized rRBCs were stained with a flourecenct dye and mixed in different ratios (1:1, 1:10, and 10:1) with hRBCs labelled with a flourecent antibody. The flourescence intensity was measured by using flow cytometry.

Highest mutual adherence of the C3b-opsonised rRBC to hRBCs was observed when the cells were mixed in 1:1 ratio. The addition of solube CR1-ABC markedly reduced the mutual adherence of the C3b-opsonised rRBC to hRBCs proving that soluble CR1-ABC can compete with erythroid CR1 on hRBCs for binding to C3b-opsonised rRBCs.

In conclusion, CR1-ABC could be efficently produced as recombinant protein using the heterologous expression host Pichia pastoris. Addition of CR1-ABC as a soluble reagent is able to disrupt the immuneadherence-like interaction of C3b-coated rRRBs cells with hRBCs.

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Atypical haemolytic uraemic syndrome in the era of complement inhibiting therapy: the UK national cohort experience

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Background: In complement mediated atypical haemolytic uraemic syndrome (aHUS) dysregulated complement activation occurs on endothelial cell surfaces and results in thrombotic microangiopathy. Historically management comprised predominantly supportive care with or without plasma exchange, and outcomes were poor. Trials of the terminal complement inhibitor eculizumab published in 2013 suggested efficacy and revolutionized management. Given the high cost of the drug a national specialized service was commissioned by NHS England. We report the real life experience of treating individuals with suspected aHUS with eculizumab in a national cohort and compare outcomes with a control cohort.

Methods: 1956 individuals have been referred with suspected aHUS. Of these, 243 were treated with eculizumab. The control cohort comprised 279 individuals referred with suspected aHUS prior to the availability of eculizumab in whom a pathogenic complement gene mutation or autoantibody was identified. Clinical data were collected in collaboration with local clinicians and from RaDaR. Outcomes were compared using Kaplan-Meier analysis (IBM Statistical Package for Social Sciences (SPSS)). Multivariate analysis used the logistic regression model (R).

Results: The 5-year cumulative estimate (Kaplan-Meier) of end stage kidney disease (ESKD) free survival was 39.5% in the control cohort and 85.5% in the eculizumab treated cohort subgroup with a mutation or FHAA; HR 4.95 (95% CI 2.75-8.90), p=0.000, number needed to treat 2.17 (NNT) (95% CI 1.81-2.73). The magnitude of the improvement with eculizumab depends upon mutation type or autoantibody. The relapse rate upon eculizumab withdrawal was 1 per 9.5 person years for those with a pathogenic mutation. No relapses were recorded in 67.3 person years off eculizumab in those with no rare genetic variant.

Conclusion: Eculizumab costs ~£328000 per person (adult) per year and increases the risk of meningococcal sepsis. It was introduced into clinical practice on the basis of small single-arm clinical trials. In this genotype matched study of aHUS we show ESKD-free survival is significantly improved with eculizumab, and we believe that the magnitude of the improvement in outcomes justifies the high cost and potential complications of terminal complement blockade. We also demonstrate that eculizumab withdrawal in aHUS is safe and relapse is predicted by genotype.

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Characterising complement in amyloid-beta plaques

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Background: Complement is a major component of the innate immune response as it orchestrates immunological and inflammatory processes. Measurements of complement in plasma are highly predictive of Alzheimer's disease (AD) progression and variants in complement genes have been associated with an increased risk of AD in genome-wide association studies. Some animal models also support a role for complement in AD pathogenesis. Amyloid-β (Aβ) plaques are a pathological hallmark of AD in the grey matter with several plaque subtypes being described. Complement activation and plaque formation are mutually promoting mechanisms; aggregated Aβ binds to C1q; further enhancing Aβ aggregation and fibril formation. Previous studies have shown complement bound to Aβ plaques, however, the timing of when complement binds to these plaques and whether different complement components bind different plaque subtypes remains unknown.

Methods: Using immunohistochemistry, we characterised complement expression and activation on Aβ plaques (6E10) and peri-plaque cells (within 50 μm of the plaque) from post-mortem tissue of five human Braak stage VI AD patients. The tissue was digitally scanned so that a similar area of the tissue was analysed, enabling us to identify plaques from different complement components of the classical, alternative, and terminal pathways.

Results: Expression of the complement activation and recognition fragments C1q, C3b/iC3b, and C9neo as well as the alternative pathway regulator, factor H, were present on Aβ plaques throughout the cortical grey matter. Complement-positive microglia/astrocytes were also found closely apposed to Aβ plaques. C1q was present on Aβ plaques or peri-plaque cells in 92.1% of Aβ plaques analysed. The proportion of C3b/iC3b-positive Aβ plaques and peri-plaque cells was 88.8%. The proportion of Aβ plaques and peri-plaque cells immunostained for the membrane attack complex (C9neo) was 33.8% and was found on diffuse, dense-core, neuritic, and burnt-out plaques. Factor H was immunopositive on 38.3% of Aβ plaques and the peri-plaque cells analysed.

Conclusion: Expression of complement activation, recognition fragments, and regulators of the classical, alternative, and terminal pathway are present on Aβ plaques and peri-plaque cells and indicates that the complement cascade is activated at all stages of Aβ plaque evolution.

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Differential complement activation in the blood of individuals at clinical high risk for psychosis compared with healthy controls

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Background: Early identification and treatment of individuals at clinical high-risk (CHR) for psychosis is critical but there are currently no objective biomarkers for the diagnosis of those CHR who go on to develop psychosis. This study quantified complement activation fragments in CHR and control plasma with the aim to identify pathway-specific pathophysiological mechanisms, complement activation fragments correlating with clinical symptoms and distinguishing those CHR individuals who transition to psychosis compared to non-converters and healthy controls (HC).

Methods: This study included n=153 participants from the multi-site North American Prodrome Longitudinal Study (NAPLS2) study at baseline of which n=45 had a known outcome of psychosis and n=108 CHR who did not transition to psychosis after 24-months, compared to n=80 healthy controls (HC). Plasma concentrations of complement activation fragments C4a, C4d, C3a, iC3b, Bb, C5a, and sC5b-9 (TCC) were quantified using standardised commercial ELISA kits (Quidel). Outliers removed >+3SD. The main outcome of interest was comparison of HC with CHR and with those CHR who had a known outcome of psychosis (Mann Whitney u test). Secondary outcome measures included the Scale of Prodromal Symptoms (SOPS) (Pearson's correlation).

Results: Baseline plasma levels of complement iC3b (HC 52.25±27.25 μg/ml, CHR 23.12±21.29μg/ml, p<0.0001), C3a (HC 289.1±207.7 ng/ml, CHR 109.4±80.04 ng/ml, p<0.0001), C4d (HC 6.301±3.μg/ml, CHR 5.331±2.306 μg/ml, p=0.0356) and Bb (HC 1.094±0.2758 μg/ml, CHR 1.225±0.2965 μg/ml, p=<0.0001) were significantly different in CHR versus HC. No significant difference was found when comparing CHR non-converters with those who had a known outcome of psychosis at follow up. Further, complement Bb (r=0.206, p=0.011, baseline) and C4d (r=-0.238, p=0.02, 18 months) correlated significantly with SOPS and trends were observed for iC3b and C3a.

Conclusions: Complement activation and regulation are monitored by measuring pathway-specific complement activation fragments. We found evidence of differential complement activation in CHR compared with controls, possibly reflecting prior in vivo activation. We further observed complement activation correlation with prodromal symptoms. These findings identify complement activation fragments as markers for early identification of CHR and support further investigations into complement-targeting therapeutic development.

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Cell-based assay for the measurement of complement convertase activity

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Introduction: The C3 to C3b transition is a nodal point in the complement cascade. This process is amplified by dedicated enzymatic complexes called convertases. Importantly, most of the known complement inhibitors act at the level of convertases, preventing them from excessive activity with potentially detrimental consequences. Dysregulation of complement convertases takes place by various mechanisms including loss-of-function mutations in inhibitors, variations in complement inhibitor genes' copy number, gain-of-function mutations in convertase components that render them insensitive to inhibitors and spontaneous decay, and the presence of convertase-stabilizing autoantibodies. Very often, a single mutation in complement genes has limited penetration, and the occurrence of disease onset depends on combinations of several aspects like risk polymorphisms and humoral factors. Therefore, functional assays of patients' sera aid the elucidation of complement system abnormalities and support doctors' decisions on the targeted therapy. Herein, we propose a cell-based assay for the assessment of convertase activity. The overall idea is that one could use it as a single screening test to reveal whether the patient shows functional impairment of either alternative pathway (AP) or classical/lectin pathway (CP/LP) convertases.

Methods: Complement-dependent cytotoxicity in human CD20 + Ramos cell line sensitized by anti-CD20 antibodies was previously shown to depend on either CP or AP that supports an amplification loop (Felberg et al. Cancer Immunol Immunother. 2019). We used this model to set up a convertase assay, which was performed with a combination of C5 blockers: eculizumab and crovalimab.

Results: We examined the convertase activity curve upon the addition of wild-type factor B and C2 proteins, as well as upon the addition of acknowledged gain-of-function variants on both proteins: K323E and D279G for factor B, and R249C and S250C for C2, respectively. The results confirmed that our system can detect the presence of these pathogenic variants that dominantly affect the function of convertases.

Conclusions: We showed a proof of concept that our cell-based convertase assay detects impairment of both CP/LP and AP convertases. Importantly, it is operable in the physiological milieu of whole serum and offers a physiological surface of human cells equipped with typical complement inhibitors.

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Autoantibodies targeting HMGB1 and the main C1s generated HMGB1 fragment in systemic lupus erythematosus: Prevalence and characterization

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Background: Systemic lupus erythematosus (SLE) is a chronic autoimmune disease, characterized by the presence of circulating autoantibodies directed against self-antigens. Default in dying cells clearance is suggested to be involved in SLE pathogenesis. Recent studies showed involvement of HMGB1, a nuclear protein which can act as an alarmin when released into the extracellular environment and anti-HMGB1 autoantibodies (Abs) among SLE patients. On the other hand, the C1s complement protease, the catalytic arm of the complement system, is able to cleave HMGB1. According to these data, we aimed to assess anti-HMGB1 Abs relevance in SLE patients and to evaluate Abs specificities against the main HMGB1 fragment produced by C1s cleavage.

Methods: Anti-HMGB1 Abs were measured in 146 SLE patients' sera by ELISA. 66/146 patients had quiescent disease. Of 80 patients with active disease, 40 had nephritic exacerbation and 40 had rheumatoid exacerbation. Antibody specificity was also investigated using different fragments of HMGB1 (the main C1s cleavage AB fragment and its two separate DNA binding fragments A and B boxes).

Results: Of the 146 SLE patients, 41% (59/146) presented anti-HMGB1 Abs, the majority of them with active disease (39/80). Interestingly, active disease was significantly associated with the presence of anti-C1s Abs (p=0.03). Titers of anti-HMGB1 Abs were significantly higher in rheumatoid exacerbation than in quiescent patients (p=0.007); no statistical difference was observed between nephritic exacerbation and quiescent patients (p=0.53). Moreover, anti-HMGB1 Abs positively correlated with anti-dsDNA Abs (r=0.2; p=0.019) but not with SLEDAI (r=0.17; p=0.05). Regarding antibodies specificities, all positive for anti-HMGB1 Abs targeted HMGB1 AB Boxes, A Box and B Box fragments. Interestingly, 24% negative for anti-HMGB1 Abs presented Abs against these fragments, this could suggest that HMGB1 cleavage by C1s reveals epitopes for patients' Abs binding.

Conclusion: Anti-HMGB1 Abs might be interesting new biomarkers for diagnosis and follow-up as they were found more frequently in active than in quiescent SLE patients. Moreover, these Abs could impact the HMGB1 inflammatory functions and this part needs to be investigated, as well as the interconnection with C1s.

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The temporal role of complement in severe trauma and hemorrhagic shock

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Background: Polytrauma as a life threatening multiple injury leads to rapid activation of the cross-talking coagulation- and complement systems. However, excessive complement activation can contribute to systemic inflammation and organ dysfunction. In the case of sepsis, blockade of central complement components and TLR molecules appeared to be beneficial as we also documented recently in a pig model with 72 hrs observation using C5 and CD14 inhibition. Therefore, we hypothesized that absence of the central complement component C3 and the TLR 4-coreceptor CD14 will improve cellular and organ function post trauma in genetically modified mice.

Methods: Polytrauma plus hemorrhagic shock (HS) was modelled in C57bl/6 mice by blunt traumatic brain injury, thoracic trauma, femur fracture and soft tissue injury. After the trauma impact, animals were hemodynamically monitored in our mouse intensive-care unit. Sham procedures and polytrauma/HS was applied in genetic absence or presence of C3, or CD14 or both, C3/CD14 (n=8/group). As read-outs, we investigated the hemodynamic and inflammatory response aas well as multi-organ function 4 h after injury. Statistical analyses were performed by ANOVA with post hoc testing.

Results: The hemodynamic changes after the polytrauma/HS did not significantly alter between the C3/CD14 sufficient and deficient mice. Systemic inflammation assessed by IL-6 plasma concentrations, multi-organ performance measured by the established biomarkers CC16, IFABP, GFABP, NGAL, among others, and clinical outcome (reflected by mortality rate) did not significantly alter between the C3 deficient, CD14 deficient and C3/CD14 double knock out mice in the early observation period up to 4 h post polytrauma/HS. Of note, the polytrauma-induced increase in the endothelial damage marker syndecan-1 was significantly reduced in C3-/- mice.

Conclusion: Whereas long-term effects after severe tissue trauma seem to benefit from blockade of complement (C5) and TLRs (CD14) , our current data in mice suggest, that very early after polytrauma/HS neither complement nor TLR inhibition alter the inflammatory and organ response, except for the endothelial damage. These differential-temporal findings indicate that the therapeutic effect of this inhibition is not observed early, but might have a major impact at a later stage.

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A Role for P-selectin and complement in the pathological sequelae of Germinal Matrix Hemorrhage

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Background: Germinal Matrix Hemorrhage (GMH) is a devastating disease of pre-term infancy that commonly results in post-hemorrhagic hydrocephalus, periventricular leukomalacia, and subsequent neurocognitive deficits. We demonstrate vascular expression of P-selectin after GMH and investigate a strategy to specifically target complement inhibition to sites of P-selectin expression to mitigate the pathological sequelae of GMH.

Methods: Two fusion proteins were prepared consisting of different anti-P-selectin single chain antibodies (scFv's) linked to the complement inhibitor Crry. One scFv targeting vehicle (2.12scFv) blocked the binding of P-selectin to its PSGL-1 ligand expressed on leukocytes, whereas the other (2.3scFv) bound P-selectin without blocking ligand binding. Post-natal mice on day 4 were subjected to collagenase induced-intraventricular hemorrhage and treated with 2.3Psel-Crry, 2.12Psel-Crry, or vehicle.

Results: Compared to vehicle treatment, 2.3Psel-Crry treatment after induction of GMH resulted in reduced lesion size and mortality, reduced hydrocephalus development, and improved neurological deficit measurements in adolescence. In contrast, 2.12Psel-Crry treatment resulted in worse outcomes compared to vehicle. Improved outcomes with 2.3Psel-Crry were accompanied by decreased P-selectin expression, and decreased complement activation and microgliosis. Microglia from 2.3Psel-Crry treated mice displayed a ramified morphology, similar to naïve mice, whereas microglia in vehicle treated animals displayed a more ameboid morphology that is associated with a more activated status. Consistent with these morphological characteristics, there was increased microglial internalization of complement deposits in vehicle compared to 2.3Psel-Crry treated animals, reminiscent of aberrant C3-dependent microglial phagocytosis that occurs in types of adult brain injury (although pruning of C3-opsonized synapses by microglia is also a feature of normal development). Also, following systemic injection, 2.3Psel-Crry specifically targeted to the post-GMH brain. Likely accounting for the unexpected finding that 2.12Psel-Crry worsens outcome following GMH, we found that this construct interfered with coagulation, and specifically with heterotypic platelet-leukocyte aggregation, which express P-selectin and PSGL-1, respectively.

Conclusion: GMH induces expression of P-selectin, the targeting of which with a complement inhibitor protects against pathogenic sequelae of GMH. A dual functioning construct with both anti-coagulative and complement blocking activity worsens outcomes in this hemorrhagic model, but has potential for treatment of conditions that incorporate pathological thrombotic events, such as ischemic stroke.

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Elevated expression of complement factor I in lung cancer cells associates with shorter survival – Potentially via non-canonical mechanism

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Background: Normal cells are equipped with numerous membrane-bound complement inhibitors that protect them from misguided complement attack. However, soluble complement inhibitors are rarely produced in locations other than the liver. Previously, we reported the expression of complement factor I (FI) in non-small cell lung cancer (NSCLC) cell lines.

Methods: FI expression in cancer biopsies from lung adenocarcinoma and squamous cell carcinoma patients was assessed by immunohistochemical staining and associated with clinicopathological characteristics and clinical outcomes. To approach the question of whether the expression of FI by tumor cells was aimed to protect tumor cells from host innate immunity, the deposition of C4d - the end degradation product of FI-supported inactivation of active complement component C4b was analyzed in the same tissue. To elucidate the role of FI in lung cancer cell physiology, three human non-small lung cancer cell lines naturally expressing FI were engineered with CRISPR/Cas9 technology and the transcriptome of FI-deficient and FI-sufficient clones was compared in each cell line. These cells were also compared in in vitro colony-formation assay.

Results: FI immunohistochemical staining intensity did not correlate with age, smoking status, tumor size, differentiation grade, stage, T cell infiltrates or PD-L1 expression, but was associated with progression-free survival (PFS) and disease-specific survival (DSS). Multivariate Cox analysis of high vs. low expression of FI revealed HR 0.55, 95% CI 0.33-0.95, p=0.031 for PFS and HR 0.35, 95% CI 0.15-0.78, p=0.011 for DSS. Importantly, only negligible C4d staining was found in cancer tissue. RNA sequencing in FI knockout and wild-type NSCLC cells revealed differentially expressed genes of potential importance for intracellular signaling pathways controlling proliferation, apoptosis, and responsiveness to growth factors. In vitro colony-formation assays showed that FI-deficient cells formed smaller foci than FI-sufficient NSCLC cells, but their size increased when purified FI protein was added to the medium.

Conclusions: We postulate that a non-canonical activity of FI influences cellular physiology and contributes to the poor prognosis of lung cancer patients.

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Complement Factor I protein concentration in human plasma and ocular tissue samples stratified by AMD genotypes and phenotypes

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Increased susceptibility to age-related macular degeneration (AMD) associates with single nucleotide polymorphisms (SNPs), or combinations of SNPs (haplotypes), in genes that play a fundamental role in alternative pathway (AP) complement regulation. AMD genome wide association studies (GWAS) have identified SNPs within or near CFH, CFHR3/1, CFI, C3, C9 and C2/CFB genes that influence risk for – or protection against – AMD. Similarly, rare and highly penetrant mutations have been identified in numerous complement genes, further underscoring dysfunctional complement regulation as an instigator of disease. Two key proteins, complement factor I (FI) and complement factor H (FH), are vital negative regulators of AP amplification in fluid and on cellular and extracellular surfaces.

We developed an ELISA to quantitatively measure and compare relative FI protein concentration in human donor retina, retinal pigment epithelium (RPE), Bruch's membrane (BM) and BM-choroid tissues. To identify associations between common AMD-related SNPs and FI protein concentration, we compare plasma, vitreous humor, retina and RPE-choroid tissue samples stratified on CFH-CFHR5 (Chr1) diplotypes and common CFI quantitative trait loci (QTL) SNPs.

FI protein concentration is higher in RPE and BM-choroid than in BM and retina tissues. FI protein levels are not significantly altered in plasma or RPE-choroid tissue when stratified by homozygous Chr1 diplotypes with and without AMD. An increase in FI protein is detected in retina derived from Chr1 risk donors as compared to non-risk donors and a modest decrease in FI protein is detected in vitreous humor from AMD donors as compared to non-AMD donors. Finally, the CFI QTLs rs10033900 and rs13117504 do not significantly impact FI protein concentration in plasma or ocular tissue samples.

The absence of significant variation in FI protein levels in plasma and ocular tissue samples suggests FI is not dramatically altered in subjects with common CFI SNPs, in contrast to published rare CFI mutations (PMID 25788521). It is conceivable that subtle reduction of FI protein, in specific ocular compartments (e.g. sub-RPE space), may result in suboptimal cofactor functions during age-related processes. The association between FI protein concentration and predilection to AMD susceptibility have important implications for therapeutic FI augmentation strategies.

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Complement drives chronic inflammation and progressive hydrocephalus in murine neonatal germinal matrix hemorrhage

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Background: Germinal matrix hemorrhage (GMH) is a pathology that occurs in infancy, with often devastating long-term consequences. Post-hemorrhagic hydrocephalus (PHH) can develop acutely, while periventricular leukomalacia (PVL) is a chronic sequala of GMH. There are no pharmacological therapies to treat PHH and PVL. We used the complement inhibitor CR2-Crry in a therapeutic paradigm to investigate the role of complement in acute and chronic outcomes after murine neonatal GMH induced at post-natal day 4 (PND4).

Methods: GMH was induced by intraventricular injection of collagenase at PND4. Animal groups were: Wild-type Naïve (no GMH), Vehicle (GMH with intraperitoneal PBS treatment), and CR2-Crry (GMH with intraperitoneal CR2-Crry treatment). Study endpoints were survival, and PND7 and PND45 for histopathological and immune analysis.

Results: Red blood cell (RBC) infiltration and lysis is a feature of GMH, and the cytolytic complement membrane attack complex (MAC) colocalized with RBC's acutely after GMH in vehicle, but not CR2-Crry treated animals. MAC deposition on RBC's was associated with heme oxygenase-1 expression and heme and iron deposition, which was reduced acutely with CR2-Crry treatment, and which resolved in all animals by PND90. Complement inhibition also reduced hydrocephalus rate and improved survival. Following GMH, there were structural alterations in specific brain regions linked to motor and cognitive functions, and these changes were ameliorated by CR2-Crry treatment, as measured at various time points through PND90 post-GMH. Astrocytosis occurred in both treatment groups acutely after GMH, but was significantly reduced in CR2-Crry treated animals by PND90. Furthermore, at PND90, myelin basic protein and the lysosomal marker LAMP-1 colocalized, indicating chronic ongoing phagocytosis of white matter, which was reduced by CR2-Crry treatment.

Conclusion: The data indicate a role for the MAC acutely in post-GMH PHH via RBC lysis, and that acute MAC-mediated iron-related toxicity and inflammation exacerbates chronic effects seen after GMH.

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Finding optimal targets for complement-based cancer therapy

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Despite the substantial preclinical evidence for the role of complement in cancer, complement-based cancer therapies have not yet advanced to the clinic, likely, because of the failure to identify the optimal targets within the complement system and patients that will benefit from complement-based interventions. To address these needs, we identified the subsets of renal cell carcinoma (RCC) that are associated with upregulation of complement genes and can be potentially regulated by complement-mediated mechanisms. These mechanisms include complement anaphylatoxins-mediated inhibition of antitumor immunity and acceleration of angiogenesis in mouse model of cancer. The upregulation of complement genes in patients was associated with T cell exhaustion and myeloid cells' transcriptomic signatures linked to immune suppression. The complement-dependent subsets of RCC responded poorly to immune checkpoint inhibitors (ICI) and antiangiogenics. Abundance of plasma complement proteins was associated with the response to ICI, pointing to plasma complement as a potential predictive biomarker. In low grade RCC tumors, complement protein expression and deposition were limited to stroma and infiltrating cells, consistent with complement functions in the regulation of infiltrating immune cells. However, tumor cells in aggressive high grade RCC expressed large amounts of C3. In addition, we found expression of complement proteins and evidence of complement activation in several cellular organelles of tumor cells from common human cancers. Downregulation of these genes led to reduced tumor cell proliferation and downregulation of the mammalian target of rapamycin complex 1 (mTORC1) downstream signaling, consistent with the role of intracellular complement activation in T cells. Interestingly, tumors with hyperactivation of mTORC1 expressed high levels of complement proteins, suggesting the signaling regulatory loop involving complement and mTORC1. In conclusion, complement in human malignancies appears to regulate both host components of the tumor microenvironment that contribute to immunosuppression and tumor cell signaling essential for tumor cell growth. Thus, cancer patients are likely to benefit from targeting extracellular complement to improve antitumor immunity and complosome to stop tumor cell proliferation.

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Complement propagates visual system pathology following traumatic brain injury

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Introduction: There is a clinical need for a better understanding of the mechanisms driving chronic dysfunction after traumatic brain injury (TBI), as well as therapeutic approaches to alleviate them. These chronic disabilities include motor, cognitive and visual dysfunction. The development and worsening of visual deficits following TBI occurs over time, suggesting that ongoing neuroinflammatory changes rather than the initial injury contribute to their development. We investigated whether complement activation within the visual system after TBI contributes to ongoing visual decline, and whether treatment with an injury site-targeted complement inhibitor (CR2-Crry) can prevent visual system decline.

Methods: Mice were subjected to open-skull controlled cortical impact and treated with 16 mg/kg CR2-Crry or saline (vehicle) intravenously 1 hour after injury. Changes in visual acuity were assessed using the optokinetic response and visual cliff. C3 deposition, microgliosis and synaptic changes in the visual system were assessed by immunohistochemistry. Motor and cognitive dysfunction after TBI was confirmed, and was improved in CR2-Crry treated animals.

Results: C3 was deposited on retinogeniculate synapses in both the ipsilateral and contralateral dorsal lateral geniculate nuclei (dLGN) within 3 days after TBI, and was reduced in CR2-Crry treated animals. Microglia exhibited quantifiable morphological changes in both the ipsilateral and contralateral dLGN, with a more amoeboid phenotype after TBI in vehicle vs. CR2-Crry treated animals. Microglia in vehicle treated animals also had a greater internalized VGlut2+ synaptic volume after TBI compared to CR2-Crry treated animals, reminiscent of aberrant phagocytosis seen in perilesional areas of the brain after TBI and stroke. There were no changes in the thickness of the inner retina, retinal nerve fiber layer or retinal ganglion layer. These pathologies were accompanied by reduced visual acuity in both the ipsilateral and contralateral eyes at 10 and 35 days after TBI; vision was absent in the majority of contralateral eyes in both groups of animals, but complement inhibition preserved visual acuity in the ipsilateral eye.

Conclusion: These data suggest that complement plays a role in the development of visual deficits after TBI, and that inhibition of complement acutely after TBI has therapeutic potential for ameliorating visual and other deficits.

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Low Gene Copy Numbers (GCN) of complement C4 and C4A deficiency are highly significant genetic risk factors for idiopathic inflammatory myopathies and its major subgroups

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Idiopathic inflammatory myopathies (IIM) are a group of autoimmune diseases with chronic muscle weakness and fatigue. It is characterized by inflammation, infiltrations of leukocytes into muscles and/or the skin, deposition of complement membrane attack complexes leading to vasculopathy and necrosis of skin or muscle fibers. The etiology for IIM is largely unknown. Through collaborations with the Myositis Genetics Consortium and the UK Myositis Network, we studied complement C4 diversities in 1,650 Caucasian patients with IIM from the Great Britain, Sweden, Czech Republic, Belgium, and the US, plus 3,526 matched healthy controls. Plasma protein levels for C4 and C3, HLA-DRB1 allelic polymorphisms, IIM disease subgroups, and the presence of myositis-related autoantibodies were analyzed with reference to GCN variations of total C4 (C4T), C4A, C4B, long genes (C4L) and short genes (C4S), which were determined and verified by TaqMan-based quantitative real-time PCR with five independent amplicons. Complement protein concentrations were determined by single radial immunodiffusion. Low GCNs of C4T (C4T=2 or 3) and C4A deficiency (C4A=0 or 1) were present in close to half of IIM patients. Complement deficiencies were strongly correlated with increased risk of IIM with odds ratios (OR), and 95% confidence-intervals equal to 2.58 (2.28-2.91), $p=5.0x10^{-53}$ for C4T; and 2.82 (2.48-3.21), $p=7.0x10^{-57}$ for C4A deficiency. Similar findings were observed in all four major subgroups of IIM (JDM, DM, PM and IBM). Among patients with IBM and C4A deficiency, 98.2% have HLA-DR3 with an OR of 11.02 (1.44-84.4), p=0.0012. Intra-group analyses of IIM patients for C4 protein levels and IIM-related autoantibodies revealed that those with anti-Jo-1 or with anti-PM/Scl antibodies had significantly lower C4 plasma concentrations than those without these autoantibodies (anti-Jo +/-: 283.7±89.0 mg/L vs 324.7±408.9 mg/L, $p=9.6x10^{-5}$; anti-PM/Scl +/-: 268.4±72.2 mg/L vs 321.5±108.1 mg/L, p=0.0005). In conclusion, low gene copy numbers of total C4, C4L and C4A deficiency are strong risk factors of IIM and its four subgroups. IBM patients had the lowest mean copy number of C4 genes. Patients with IBM and C4A deficiency almost uniformly had HLA-DRB1*03:01, with high effect size (reference: Ann Rheum Dis 2023; 82:235-245).

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Dual inhibition of the complement system and toll-like receptors prevents systemic and local kidney inflammation in mice experiencing brain death

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Background: Brain death (BD) induces a potentially harmful systemic inflammation, which may reduce organ quality for transplantation. The complement system (CS) and Toll-like receptors (TLRs) are key for the innate immune system both for recognition and response. The cluster of differentiation 14 (CD14) is a co-receptor for several TLRs, necessary for TLR signaling. We hypoth-

esized that dual inhibition of CS and TLRs by complement protein 5 (C5) and CD14 inhibition will prevent innate immune-mediated inflammation during BD.

Methods: BD was induced with a fluid-filled intracranial balloon in wild-type C57/BL6 mice. Prior to BD, mice were left untreated (n=8), treated with a C5 inhibitor (n=7), a CD14 inhibitor (n=7), or both inhibitors (n=7). Sham mice did not experience BD and were left untreated (n=8). Blood and kidneys were collected three hours after BD. Inflammatory plasma cytokines were analyzed using a 23-plex immunoassay, kidney mRNA expression by qPCR.

Results: In plasma, BD significantly induced expression of interleukin-6 (IL-6), human IL-8 homolog, IL-12, monocyte chemoattractant protein (MCP-1), macrophage inflammatory protein MIP-1α, and MIP-1β compared to sham (all $p<0.01$). In kidneys, BD significantly induced IL-6, IL-8, TNF, MCP-1, P-Selectin, and VCAM-1 (all $p<0.01$). C5 and CD14 single inhibition significantly reduced BD-induced activation of all markers in plasma (all $p<0.01$) and in kidneys ($p<0.01$, except C5 inhibition for P-Selectin $p=0.06$). Dual inhibition of C5 and CD14 further reduced all plasma cytokines to levels comparable with sham animals (all $p>0.05$). In kidneys, double inhibition was comparable to single inhibition.

Conclusion: The innate immune system is crucial for inducing inflammatory reactions during BD. Inhibition of both the CS and TLRs is necessary to efficiently prevent BD-induced systemic inflammation and to reduce local kidney inflammation. CS and TLR inhibitors are clinically available and clinical studies should be performed on deceased BD donors to enhance donor organ quality.

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Multiple pregnancy can induce preeclampsia-like changes in complement factor H-point mutation mice

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Preeclampsia is a severe placenta-related pregnancy disorder, its pathogenesis has still not been fully elucidated. Studies suggested a link between complement activation and preeclampsia. In view of that normal pregnancy can induce appropriate activation of the complement system, we assumed that patients with preeclampsia may have increasing genetic susceptibility to overactivation of complement system, and under the burden of pregnancy, overactivation of the complement system occurs, in turn leading to the occurrence of preeclampsia. In order to confirm our hypothesis, we mated the female and male mice with heterozygous mutations in complement factor H through five times and assessed clinicopathological indicators of preeclampsia. Along with complement activation, multiple pregnant heterozygous complement H mutation mice recapitulated the key features of human preeclampsia: hypertension, proteinuria, elevated blood urea nitrogen, lactic dehydrogenase, reduced placental weight, restricted fetal growth, typical histology change in placenta and kidney of preeclampsia, placental imbalance of angiogenesis and renal endothelial cell injury. This study provided direct evidence that pregnant status can induce overactivation of complement system in mice with mutation in complement regulator gene, which could lead to the development of preeclampsia. This model provided a new option for the investigation of the pathogenesis of preeclampsia.

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Complement system activation is mediated by COVID-19 severity, inducing endothelial cell injury and cell permeability

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Background: Coronavirus Disease 2019 (COVID-19) is a respiratory disease that has been classified as mild, moderate, severe, and critical.

The complement system (CS) is a key component of the innate immune response. The activation of the CS involves a series of enzymatic transformations, where C3b acts as a mediator, and the final step is the formation of a membrane attack complex (C5b-9) on the plasma membrane (PM) of cells.

There is evidence linking CS activation and endothelial injury to organ damage and complications that increase the risk of mortality in COVID-19.

We hypothesized that endothelial cell injury resulting from complement activation contributes to COVID-19-associated vascular and organ injury thus, influencing the overall patient outcome.

Methods: Clinical information and sera from SARS-CoV-2 positive patients with mild and severe COVID-19 were obtained from the Canadian COVID-19 Prospective Cohort Study (CANCOV).

Complement activation was induced using an established protocol. Briefly, primary human umbilical vein endothelial cells (HUVECs) were treated with adenosine diphosphate, followed by treatment with serum. We quantified the deposition of C3b and C5b-9 on the PM of HUVECs via immunofluorescence (IF).

Changes in cell permeability were measured using a transwell model. Briefly, a monolayer of HUVECs seeded on the top part of the insert was treated with patient sera, and a fluorescent tracer was placed on top of the monolayer, and then followed to the bottom chamber.

Results: We were able to detect complement activation/deposition when exposing the cells to COVID-19 sera. As for C3b deposition, cells exposed to patient sera showed no difference compared to those treated with sera from healthy donors. However, we observed increased C5b-9 deposition on the PM of cells treated with patient sera compared to those treated with sera from healthy donors, with further increased deposition when cells are treated with deceased patient sera.

We observed a trend of increased fluorescence on the transwell bottom chamber when cells were treated with deceased patient sera compared to healthy donors.

Conclusion: Complement is activated in COVID-19. Deposition of complement activation products reflects disease severity. Complement activation also leads to a clinically relevant feature by increasing cell permeability.

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Mesangial cells derived complement factor H inhibits complement activation and inflammation in IgA Nephropathy

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Background: IgA nephropathy (IgAN) is the commonest primary glomerular nephritis in the world, with glomerular IgA and C3 deposition, inducing mesangial cell proliferation and inflammatory injury. Complement factor H (CFH), a key regulator of alternative pathways, was reported as an important factor for the development and progression of IgAN, mainly focused on the complement inhibition function of circulating factor H. Recent studies reported non-canonical function of CFH in both retinal pigment epithelium and kidney endothelial cells[1,2]. Here, we investigate the potential role of mesangial cells derived CFH in IgAN.

Methods: CFH expression at mRNA and protein levels were firstly detected in primary human glomerular mesangial cells (pHGMC) treated with or without IgA1-containing immune complexes derived from IgAN patients (IgAN-IgA1-IC). Then, inflammatory factors secretion, cell proliferation and complement activation were evaluated under modified expression of CFH in pHGMC, including siRNA targeting CFH and overexpression of CFH.

Results: Expression of CFH at mRNA and protein levels were observed in pHGMC, and IgAN-IgA1-IC decreased CFH expression. In vitro, we found C3c and C5b-9 deposition and increased IL-6 secretion in pHGMC after IgAN-IgA1-IC treatment. Moreover, we found that inhibited production of CFH using siRNA in pHGMC led to increased secretion of IL-6 ($P<0.001$), deposition of C3c ($P<0.001$) and C5b-9 ($P<0.001$), and decreased cell proliferation ($P=0.001$), while overexpression of CFH in pHGMC decreased IL-6 secretion, C3c and C5b-9 deposition, and promoted cell proliferation.

Conclusion: Glomerular mesangial cells derived CFH contributes to renal complement activation, as well as mesangial cell proliferation and inflammation in IgA nephropathy.

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External quality assurance program for diagnostic complement laboratories: Evaluation of the past six year[StQuote]s results

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The external quality assurance (EQA) program was first organized in 2010 by a group of researchers working in diagnostic complement laboratories. Starting in 2016, INSTAND eV, a German, non-profit interdisciplinary scientific medical society dedicated to provide expert EQA programs for medical laboratories started organizing the EQAs for complement diagnostic laboratories together with the same group of experienced scientists and doctors who also work as EQA experts. The aim of the current work is to provide descriptive analysis of the past six years' complement EQA results and evaluate time-line changes in proficiency testing.

Methods: Each year, in March and October, coded samples (normal, pathological) were sent to the participating diagnostic laboratories, where complement parameters were evaluated exactly as in daily routine samples. Since no reference method/target values exist for these parameters, and participants used different units for measurement, the reported results were compared to the stable mean (Algorithm A) of the participants using the same method/measurement units. A reported result was qualified as "passed", if it fell into the 30-50% range around the mean (depending on the given parameter).

Results: While the number of participating laboratories increased in the past years (from around 120 to 370), the number of complement laboratories providing multiple determinations remained rather unchanged (around 30 worldwide). C3, C4, C1-inhibitor antigen and activity determinations provided the best proficiency results, with >90% passing quotas in the past years, independently of the applied method. Determination of the functional activity of the three pathways was good in general, but results showed large variance, especially with the pathological samples. Complement factor C1q and regulators FH and FI are determined only by a few laboratories, with variable outcomes (in general in the 85-90% pass range). Activation products sC5b-9 and Bb were determined in 20 and 10 laboratories, respectively, with typical passing quotas in the 70-90% range, without a clear tendency over the past years.

With these accumulated data from the past six years it is now possible to assess sample-, method-, and evaluation related aspects to further improve proficiency testing and protocolize diagnostic complement determinations.

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Complement C1q and von Willebrand Factor interaction in atherosclerosis of human carotid artery

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Background: Atherosclerosis is an inflammatory disease of the vessel wall characterized by the deposition of cholesterol crystals (CC) within atherosclerotic plaques. CC can trigger complement activation and hemostasis with growing evidence on the cross-talk between both systems, including the interaction between complement C1q and von Willebrand factor (vWF). The interaction of C1q and vWF also occurs on CC surfaces in vitro, forming CC-C1q-vWF complexes, which modulate the downstream anti-inflammatory effects on human macrophages. The aim of this study was to explore whether C1q-vWF complexes also occur in human atherosclerosis ex vivo.

Methods: We used immunofluorescence and proximity ligation assay (PLA, Duolink®) to examine the presence, localization and co-localization of C1q and vWF in frozen sections of human carotid arteries with atherosclerosis (autopsy material, diseased DA, n=7) or without atherosclerotic changes (autopsy material, healthy HA, n=7) as well as material from thrombendarteriectomy (TE, n=7).

Results: Overall, we observed stronger signals for C1q and vWF with significant higher levels in healthy tissue compared to diseased material ($p<0.001$ for C1q and vWF, respectively) and a greater co-localization in the PLA in healthy samples than in diseased samples (HA vs DA $p<0.001$, HA vs TE $p<0.001$). However, signal intensities for C1q and vWF correlated stronger in atherosclerotic vessels (R=0.63 versus R=0.38). In healthy arteries, co-localization was predominantly localized in the endothelium and subendothelial space. In diseased samples, signals were highest in locations encompassing atheroma and foam cells. While there was overall reduced signal in areas with CC, staining was spotty and there was evidence for co-localization on individual CC.

Conclusion: In this study, we demonstrate the presence of C1q-vWF in human carotid arteries ex vivo. Although this interaction can also be demonstrated in areas with CC, it is most abundant in healthy endothelial and subendothelial space. Based on our previous in vitro finding of at least partial anti-inflammatory effects of CC-C1q-vWF complexes on human macrophages, it is feasible that C1q-vWF interaction conveys a protective effect which is lost during plaque progression.

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CA19-9 versus an inflammatory profile in blood samples from pancreatic cancer patients

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Background: Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal disease as it is usually diagnosed at a non-curative advanced stage where the cancer has already metastasized. The carbohydrate antigen 19-9 (CA19-9) is the standard diagnostic marker for pancreatic cancer. Elevated serum levels are associated with tumour growth and bad prognosis, but may also result from other disorders. Therefore, the prognostic value of CA19-9 alone is limited. Persistent inflammation is a hallmark of cancer. Inflammatory mediators may both promote and decline tumour growth, by differentially orchestrating innate and adaptive immunity, both locally and systemically. Here, we examined the putative correlation between CA19-9 and inflammatory mediators, including complement, in blood samples from PDAC patients.

Methods: Nine PDAC patients and nine healthy age-matched controls were included in the study. Blood samples. from patients were collected at the time of diagnosis, i.e. before cancer treatment. Healthy controls were anonymized blood donors at Nordland Hospital blood bank. EDTA-plasma, serum and total blood RNA were stored at -80°C until analyses. Serum CA19-9 was measured by radioimmunoassay, plasma cytokine levels by Cytokine 27-plex immunoassay, and complement components by ELISA. RT-qPCR was performed with Taqman chemistry. Statistical analyses were performed using GraphPad Prism and SPSS. This study is limited by a small sample group, and is based exclusively on blood samples.

Results: Eight patients had elevated CA19-9 serum levels (>37U/mL; >5000U/mL for n=4) and metastases to liver, lung or intestine. In blood samples from all PDAC patients compared to healthy controls, plasma levels were significantly elevated for IL-6, IL-8, MIP-1α and G-CSF, and significantly reduced for IL-1RA. mRNA expression was significantly upregulated for IL-6, as well as for MMP9; and reduced for C1qBP albeit in high CA19-9 patients (n=4), only. The results for plasma levels of complement components, e.g., TCC, C1q and CFB, are pending.

Conclusion: Here, we established a blood profile of various inflammation markers in samples from PDAC patients compared to healthy controls. In line with the immune hallmarks of cancer and the differential anti- or pro-tumour functions of the tested inflammatory mediators, we expect both positive, negative and non-linear correlation with CA19-9 serum levels.

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Escherichia coli-induced platelet aggregation in human whole blood is partly dependent on complement C3b

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Background and aim: Platelets are important players in the immune system by interacting with leukocytes and connecting the cascade systems including complement. For sepsis treatment, confound knowledge about the mechanism of platelet activation is essential for the development of supportive adjunctive therapies. Earlier studies have shown that complement C3a increased the ADP-induced platelet aggregation. We aimed to study whether C3a plays a similar role in bacteria-induced platelet aggregation, and, therefore, examined the interactions between platelets and complement during Escherichia coli (E. coli)-induced platelet aggregation.

Methods: Platelet aggregation in whole blood was studied by Multiplate® (Roche). Using an in-house program, we studied platelet aggregation in presence of different inhibitors, i.e. compstatin (C3-inhibitor), anti-C3a antibody, C3a receptor (C3aR) antagonist, anti-C3b antibody, tirofiban (glycoprotein (GP) IIb/IIIa-inhibitor), eculizumab (anti-C5), and anti-CD14 antibody. Data are given in mean value of area under the curve (AUC). Flow cytometry was used to measure the activation of GPIIb/IIIa with a FITC labelled anti-PAC-1 antibody.

Results: E. coli increased the platelet aggregation significantly from 2344 AUC to 17744 AUC. C3 inhibitor compstatin significantly reduced the E. coli-induced platelet aggregation to 9947 AUC. Inhibition of C3a or C3aR did not reduce the E. coli-induced platelet aggregation, and addition of C3a had also no effect. However, a blocking anti-C3b antibody reduced the aggregation to the same level as compstatin. Compstatin also significantly reduced the E. coli-induced activation of GPIIb/IIIa on platelets by 42%. In another set of experiments, GPIIb/IIIa inhibitor tirofiban non-significantly reduced the E. coli-induced platelet aggregation from 9905 AUC to 5931 AUC, while compstatin significantly reduced it to 3801 AUC. Importantly, the combination of tirofiban and compstatin completely abolished the E. coli-induced platelet aggregation (1471 AUC). Inhibition of C5 and CD14 had no effect.

Conclusion: Complement C3b is important in the E. coli-induced platelet aggregation. Platelet GPIIb/IIIa is also involved in this mechanism.

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Antibodies targeting human complement receptor C3aR

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The complement C3a receptor (C3aR) is the receptor ligand for the complement component C3a. C3aR is a seven-transmembrane domain receptor that belongs to the G-protein coupled receptor family. C3aR is broadly expressed in various organs and tissues, including but not limited to leukocytes, kidney and lung cells, and cells of the central nervous system and cardiovascular system. C3aR expression levels vary depending on the specific tissue, cell type, and the physiological or pathological conditions.

C3a/C3aR is a key player in the pathogenesis of various diseases, which makes it a relevant therapeutic target. Antibodies are attractive therapeutic agents, given their high target specificity, relatively long half-life, and minimal toxicity. However, antibodies with a therapeutic potential against human C3aR are limited.

In this study, we aimed to generate monoclonal antibodies for therapeutic targeting of human C3aR. Mice were immunised with peptides mimicking different parts of the extracellular loops of C3aR, followed by the establishment of hybridoma cell lines. We utilized a recombinant C3aR cell based reported system to assess C3aR recognition and validated obtained results via a human whole blood model to select candidate antibodies. We have selected a panel of antibodies against human C3aR that will be further characterized and evaluated for C3aR inhibition, as well as compared to state-of-the-art C3aR inhibitors.

In conclusion, it is possible to obtain antibodies against C3aR suitable for functional characterisation using a peptide immunization strategy.

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A novel selective leukocyte depletion human whole blood model reveals the specific roles of monocytes and granulocytes in the cytokine response to *Escherichia coli*

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Background: The lepirudin-based human whole blood model is a well-established holistic ex vivo system to characterize inflammatory responses. However, the contribution role of individual cell

populations to cytokine release in whole blood has not been investigated. Thus, we modified the model by selectively removing cellular subpopulations to elucidate the contribution of individual leukocyte populations to the inflammatory response. Methods: Lepirudin-anticoagulated whole blood was depleted from monocytes or granulocytes using StraightFrom® Whole Blood Microbeads. Reconstituted blood was incubated with Escherichia coli (108/mL) for 2 hours at 370C. Flow cytometry was performed to detect CD11b, CD62P and CD63. Complement- (C3bc, sC5b-9), leukocyte- (CD11b) and platelet activation (CXCL-4, CXCL-7) were measured by ELISA. Cytokines were quantified by multiplex assay. Results: A significant ($p < 0.05$) and specific depletion of the monocyte (mean 86%; CI: 71–92) and granulocyte (mean 97%; CI: 96–98) population was obtained. Background activation induced by the depletion protocol was negligible for complement (C3bc, sC5b-9), leukocytes (CD11b) and platelets (CXCL-7). Upon Escherichia coli incubation, release of 10 of the 24 studied cytokines were solely dependent on monocytes (IL-1b, IL-2, IL-4, IL-5, IL-17A, IFN-γ, G-CSF, GM-CSF, MIP-1a, and FGF-basic), whereas eight were dependent on both monocytes and granulocytes (IL-1ra, IL-6, IL-8, IL-9, IL-10, MIP-1b, TNF, and eotaxin). Six cytokines were not monocyte- or granulocyte-dependent, of which PDGF and RANTES were mainly platelet-dependent. Conclusion: We document an effective model for selective depletion of individual leukocyte populations from whole blood, without causing background activation, allowing in-depth cellular characterization. The results confirmed the major role of monocytes in cytokine release, and expand on current knowledge regarding the role of granulocytes.

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Characterization of the interaction of mannose-binding lectin with variant SARS-CoV-2 spike proteins

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The classical and lectin pathways of the complement system are activated by pattern recognition molecules (PRMs) that recognize conserved molecular patterns on pathogens and induce a cascade of proteolytic reactions that recruit immune cells. One such PRM, mannose-binding lectin (MBL), has been found to recognize and neutralize SARS-CoV-2, the virus responsible for the COVID-19 pandemic, in an antibody-independent manner. We aimed to elucidate the impact of Wild-type compared with variants of concern (Delta and Omicron BA.1 and BA.2) on recognition by MBL and to investigate potential binding sites for MBL on the SARS-CoV-2 spike protein. To find the critical glycan positions recognized by MBL, we performed site-directed mutagenesis of 12 experimentally confirmed N-glycan positions within the spike protein. We produced recombinant proteins harboring individual or combined mutations. ELISA, WB, nano differential scanning fluorimetry and an ACE-2 binding assay confirmed the identity and integrity of the mutated spikes. To study the interactions, recombinant MBL and MBL in plasma were used. Moreover, we assessed the complement activation capabilities of MBL bound to spike. MBL appears to associate with spike proteins from different mutations present in variants of concern unimpeded and with no significant variation. MBL bound to the full-length spike and to the N-terminal domain, but not to the RBD. Mutation of single glycan sites was not sufficient to abolish MBL binding. However, we identified N717 and N801 as critical for the structural integrity of the spike protein. MBL bound to spike activated the complement assessed as deposition of C4, C3 and the terminal complement complex. In conclusion, MBL activates complement on spike. The recognition of SARS-CoV-2 spike is conserved against the Delta and Omicron BA.1 and BA.2 variants of concern and does not seem to rely on the recognition of a single glycan residue. The broad glycan recognition and the importance of glycans in spike integrity make it unlikely that future SARS-CoV-2 variants will substantially escape MBL binding.

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Autoantibodies against complement C1q induced by Epstein Barr Virus antigen exacerbate renal disease in lupus-prone mice

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Background: Systemic lupus erythematosus (SLE) is a complex autoimmune disorder characterized by the production of autoantibodies to self-antigens, including complement C1q. Anti-C1q autoantibodies (anti-C1q) have been associated with lupus nephritis, and recent studies identified a major linear epitope located on the C1q A-chain (called 'A08'), which is cross-reactive with several antigenic structures of different pathogens, including Epstein-Barr virus (EBV). The aim of this study was to investigate whether EBV-antigen induced anti-C1q can exacerbate renal disease in mice having a mild autoimmune phenotype.

Methods: To investigate the induction of a specific autoimmune response to an EBV-derived peptide, MerTK deficient mice (having a defect in the clearance of apoptotic cells) were subcutaneously immunized at the tail base with an EBNA-1 of EBV derived peptide emulsified in Complete Freund's Adjuvant (CFA) and compared to immunization with CFA only. Serum samples were collected and evaluated for the presence of anti-EBNA-1 peptide, anti-A08 and anti-C1q by ELISA. Additionally, assessment of anti-dsDNA, kidney function, histological analyses of kidney sections and elution of anti-C1q from the renal cortex were performed.

Results: Immunization of MerTK deficient mice with the EBV-derived peptide resulted in the induction of anti-A08 (about 2/3 of immunized mice) and anti-C1q (about 1/3 of mice) respectively. No anti-C1q were observed in the controls. Anti-C1q/anti-A08 double positive mice showed altered kidney function and increased glomerular IgG, C4 and C3 deposition when compared to anti-A08 negative mice or anti-A08 positive mice without anti-C1q. Anti-C1q recognizing mouse as well as human C1q could be eluted from kidneys of affected mice but not from controls. No accompanying increase in anti-dsDNA was observed after the immunization process.

Conclusion: Our findings demonstrate that an EBV-derived antigen can induce anti-C1q in the context of a defect in the clearance of apoptotic cells. Induced anti-C1q were found to be strongly associated with altered kidney function and increased glomerular immune deposits supporting a pathogenic role of anti-C1q in vivo.

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A targeted binding and activation of native C3 without proteolytic cleavage induced by contact with biosurfaces

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Background: According to the state-of-art, the alternative pathway (AP) is initiated by spontaneous proteolysis of C3 to C3b in the fluid phase. Continuous formation of C3(H2O) has been suggested to be the nidus of this reaction. However, we have previously shown that fluid phase C3(H2O) is a poor initiator of the AP compared to the in vivo continuously formed C3b [1]. As an alternative AP activation pathway, we have in this study investigated the ability of native C3 to specifically bind and form an AP convertase on various biosurfaces.

Methods: Adsorbed native C3, C3b and preformed C3(H2O), e.g., methylamine treated C3 (C3(met)) was investigated on various biosurfaces, and the conformation and convertase activity of the adsorbed C3 was evaluated using methods such as ELISA, flow cytometry, and quartz crystal microbalance with dissipation monitoring (QCM-D).

Results: Purified C3, C3b and C3(H2O), all bound to activated platelets, but it turned out that native C3 bound more efficiently to platelets compared to C3b and C3(H2O). Similarly, the generation of C3a in the presence of factor B, factor D and properdin was most efficient with platelet-bound native C3, compared to C3b and C3(H2O). Native C3 also bound to apoptotic cells. Competitive binding of C3 and Annexin V indicated that they compete for the same binding site, i.e., phosphatidyl serine, which is exposed on the cells during apoptosis. As assessed by QCM-D, C3 bound to all types of biomaterial surfaces tested, likely without covalent binding via the thioester. However, C3 only formed C3 convertases on surfaces with certain properties, e.g., rigid polymer surfaces, hydrophobic polystyrene surfaces, and negatively charged liposomes containing cholesterol. The convertase formation was correlated to an preceding conformational change of C3 to C3(H2O) on the specific surfaces and required properdin for the activation complex assembly.

Conclusion: These results propose an alternative targeted activation mechanism of C3 that leads to opsonization of altered self and foreign material surfaces, and formation of initial C3 convertases.

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FH desialylation as an acquired cause of Complement dysregulation in atypical Haemolytic Uraemic Syndrome

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Background: Mutations in the complement regulator Factor H (FH), or autoantibodies against its C-terminal region, predispose to Atypical Haemolytic Uraemic Syndrome (aHUS), a rare disease where microvascular endothelial cells are damaged by autologous complement. We previously reported a transient desialylation of FH carbohydrates in several aHUS patients with Streptococcus pneumoniae infections, which led to a reduction in its capacity to prevent sheep erythrocyte lysis in vitro (Gómez-Delgado et al., Front Immunol. 2021; 12:641656). Now, we have compared the capacity of FH and desialylated FH (dFH) to regulate complement activation on human endothelial cells.

Methods: FH was desialylated in vitro with C. perfringens neuraminidase. A human serum partially depleted of FH was used to opsonize human microvascular endothelial cells (HMEC-1) and sheep erythrocytes with C3b. The proteolysis of C3b-coated microtitre plates by FI at different incubation times was analysed by Western blotting, using in-house antibodies recognizing C3b fragments.

Results: Immunofluorescence experiments revealed decreased binding of dFH to HMEC-1 cells, as well as a decreased capacity to prevent Complement activation and C3b deposition on the endothelial surface. However, when C3b had been previously deposited on the endothelial cells, dFH displayed increased binding than FH. A similar finding was observed by flow cytometry when using sheep erythrocytes opsonized with C3b, as competition experiments suggested a higher affinity of dFH for the C3b-opsonized erythrocytes. To reconcile these apparently contradictory results, we analysed the FI-cofactor activity of FH and dFH on the proteolysis of C3b coated on microtitre plates. Western blotting analysis of C3b fragments showed no differences between FH and dFH at short incubation times (2-15 minutes), but after 30 minutes of incubation, dFH showed less cofactor activity than FH. Experiments to compare the cofactor activity of FH and dFH on C3b-opsonized HMEC-1 cells are ongoing.

Conclusion: We have observed that loss of sialic acids in FH reduces its ability to prevent complement activation on cell surfaces, which could be caused by a reduced capacity to allow the proteolytic inactivation of C3b by FI. Our results support that FH desialylation by pneumococcal neuraminidases also contributes to the pathogenic mechanism of SP-HUS.

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C1q dependent synaptic pruning in sepsis-associated encephalopathy (SAE)

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Sepsis-associated encephalopathy (SAE) is a severe and frequent complication of sepsis characterized by delirium and long-term cognitive dysfunction. We studied transcriptional and translational changes in the complement system in sepsis using hippocampal tissue from human autopsy samples and from the murine peritoneal contamination and infection (PCI) sepsis model. Human feces were injected into the peritoneal cavity, inducing peritonitis and sepsis.

The analysis in the CA1 region of human hippocampal tissue showed enhanced soma size of iba1-positive cells as well as an increase of the lysosomal and activation marker CD68 indicating activation of microglia. On day 3 (d3) and day 10 (d10) following PCI in mice, we performed RNA sequencing of hippocampal tissue. GO-term (biological processes) and KEGG pathway analysis of upregulated genes revealed categories including 'synapse pruning', 'phagosome', and 'complement and coagulation' in both time points. Among the top 40 upregulated genes 3 days after sepsis induction, C1qa, C1qb, and C1qc were prominent that together encode for complement factor C1q.

Immunohistochemical stainings in the post-mortem hippocampus of sepsis patients showed increased C1q-positive cells. In parallel, we performed immunofluorescence co-staining of Iba1, CD68, Homer1, and C1q on d3 and d10 after PCI in mice using high-resolution 3D airyscan imaging to evaluate PCI-induced synaptic pathology. We observed C1q labeled Homer1 synapses were co-localizing with the lysosomal marker CD68 in iba1-positive microglia in the CA1 region.

We depleted microglia by continuous CSF1-R inhibitor PLX5622 oral treatment at d3 after PCI during sepsis recovery in mice. Neurocognitive outcome was measured with the aid of the novel object recognition test where PLX5622 treated mice showed reduced learning impairment. Additionally, we found an increase in synapses and less C1q tagging of synapses in PCI-treated animals after microglia depletion. In vivo, experiments using the intra-hippocampal injection of a specific C1q blocking antibody with a silenced Fc part showed a reduction of engulfed Homer1 spots indicating that synapse engulfment by microglia in after PCI is C1q-dependent.

In conclusion, microglia mediate neuronal injury through C1q-dependent synaptic pruning after PCI. Thus, targeting the complement system might be a promising treatment strategy for preventing long-term neurocognitive deficits in SAE.

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A transcriptome array-based approach to link SGLT-2 and tubulointerstitial synthesis of complement C5 in IgA nephropathy

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Background: Immunoglobulin A (IgA) nephropathy was initially described more than 50 years ago and is considered as the most common primary glomerulopathy in many countries. Recently, the efficacy of sodium-glucose cotransporter-2 inhibition (SGLT-2i) to reduce progressive kidney disease in IgA nephropathy has been reported. Because complement is increasingly recognized in the pathogenesis of IgA nephropathy, SGLT-2i may also exert nephroprotection by affecting complement system activation and signaling in IgA nephropathy to achieve such beneficial effects. We pursued a transcriptome array-based approach to link intrarenal SGLT-2 and the synthesis of distinct complement components in IgA nephropathy.

Methods: Publicly available datasets for SLC5A2 (encoding SGLT-2) and complement system components were extracted specifically from microdissected tubulointerstitial (n=25) and glomerular compartments.

Results: First, we compared tubulointerstitial and glomerular log2 SLC5A2 mRNA expression levels and confirmed a predominant synthesis within the tubulointerstitial compartment. While complement components C2, C3, C5, C5AR2 and CFB were predominantly expressed in the tubulointerstitial compartment, complement components C3AR1, C5AR1, CFD, CFH, CFP, and CR1 were predominantly expressed in the glomerular compartment in IgA nephropathy. Finally, we analyzed the association between tubulointerstitial SLC5A2 mRNA expression levels and various complement components and receptors. Interestingly, the only significant finding was a positive association between SLC5A2 and the tubulointerstitial synthesis of the complement component C5 (p=0.0006).

Discussion: Because new therapeutics targeting the complement system are emerging in IgA nephropathy, we here provide the first evidence to link SGLT-2 and intrarenal synthesis of complement C5.

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Antibodies against Streptococcus pneumoniae pneumolysin correlate with modified HDL levels and complement activation markers in peripheral arterial disease patients

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Streptococcus pneumoniae is a bacterial pathogen that causes pneumonia and systemic infections such as sepsis. Pneumolysin (PLY) is a cholesterol dependent pore-forming cytolysin that is an important virulence factor for S. pneumoniae. Earlier we found that PLY changes the structure and function of HDL leading to reduction in atheroprotective properties of HDL. Antibodies against PLY can be detected from healthy individuals and patients with pneumonia. Occasionally pneumonia may cause acute complications in atherosclerosis patients. Therefore, we next investigated the role of anti-PLY antibodies in peripheral arterial disease (PAD) patients in in vitro functional assays and in ex vivo atherosclerotic plaques. We found a positive correlation between plasma anti-PLY IgG and malondialdehyde (MDA) modified HDL levels. Interestingly, isolated IgG from PAD patients with high anti-PLY levels significantly inhibited PLY-induced hemolysis. Plasma levels of complement activation markers correlated positively with anti-PLY and negatively with MDA-HDL levels. Atherosclerotic sections revealed complement activation and inhibition in different locations of femoral artery plaques. These results indicate that anti-PLY IgG might have both protective and inflammatory roles in atherosclerotic processes and therefore, detailed mechanistic function of this antibody needs to be further explored.

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Intrarenal synthesis of complement C3 localized to distinct vascular compartments in ANCA-associated renal vasculitis

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Background: Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a small vessel vasculitis affecting multiple organ systems, including the kidney. The activation of the complement system contributes essentially to its pathogenesis by autoantibody-antigen recognition directed against host cells in ANCA-associated renal vasculitis. We herein provide evidence for intrarenal synthesis of complement C3 localized to distinct vascular compartments in ANCA-associated renal vasculitis that associated with distinct inflammatory signaling pathways.

Methods: A total number of 43 kidney biopsies with ANCA-associated renal vasculitis were retrospectively included and evaluated for presence/absence of C3 deposits localized to distinct vascular compartments in association with clinicopathological biopsy findings. In addition, intrarenal C3 mRNA expression levels specifically from microdissected tubulointerstitial and glomerular compartments were extracted from transcriptome datasets.

Results: C3 deposits were present in the glomerular tuft, interlobular arteries, peritubular capillaries, and venules in ANCA-associated renal vasculitis. Most C3 deposits are localized to the glomerular tuft overlapping with peritubular capillaries. The presence of C3 deposits in the glomerular tuft correlated with impaired kidney function and overall short-term survival. Intrarenal complement C3 deposits were not associated with consumption of respective serum levels, supporting the concept of intrarenal C3 synthesis. Finally, intrarenal synthesis of complement C3 was linked to distinct inflammatory signaling pathways in the kidney that is especially relevant in ANCA-associated renal vasculitis.

Conclusion: Considering recent advances in AAV therapy with the emergence of new therapeutics that inhibit complement activation, we here provide novel insights into intrarenal complement synthesis and associated inflammatory signaling pathways in ANCA-associated renal vasculitis.

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SARS-CoV-2 nucleocapsid protein is not responsible for the activation of complement lectin pathway

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The nucleocapsid (N) protein of SARS-CoV-2 is a very strong immunogen and it is present in high concentration in the blood during viral infection. Its role is to protect the viral RNA in the virion through strong nucleic acid-protein interaction. Previously it was proven that SARS-CoV-2 activates the complement system, however the details of the mechanism are yet to be revealed. Controversial data were published about the participation of lectin pathway in the initiation of complement response. We hypothesised that the activation might be due to the presence of nucleic acids inherently bound to N protein. Since the binding of nucleic acids is a major intrinsic feature of N protein, we had to evolve a purification method to produced recombinant, nucleic acid-free N protein. The complement activation in human sera triggered by N protein was followed by ELISA deposition assays. We observed strong C3b deposition on N protein regardless of nucleic acid content. However, C4b deposition due to lectin pathway activation was detected only if the immobilized N protein carried bound nucleic acids. In the literature we have found data suggesting that lectin pathway activation could be explained by the direct interaction between N protein and mannose-binding associated serine protease-2 (MASP-2) enzyme, which is involved in the initiation step of lectin cascade. To verify this claim we performed in vitro binding assays using biolayer interferometry (BLI). Our experiments could not support the direct interaction between MASP-2 and N protein neither in the presence nor in the absence of nucleic acids. Furthermore, the potentiation effect of N protein on the enzymatic activity of MASP-2 could not be detected although it was previously suggested by others. Hence, we have

concluded that N protein activates the complement cascade mainly through the alternative pathway, while the contributions of lectin and classical pathways are only minor if any.

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Association between loss of immune checkpoint programmed cell death protein 1 and local synthesis of complement factor B in active ANCA-associated renal vasculitis

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Background: Immune checkpoint inhibitors (ICIs) have made an important contribution on the survival of patients with certain cancers. ICIs interrupt co-inhibitory signaling pathways mediated by programmed cell death protein 1 (PD-1), programmed cell death protein-ligand 1 (PD-L1), and cytotoxic T lymphocyte-associated antigen (CTLA-4) that result in the elimination of cancer cells by stimulating the immune system. However, immune-related adverse events have also been described and attributed to an enhanced immune system activation. Recent observations have suggested dysregulation of immune checkpoints in active antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV).

Methods: We here analyzed intrarenal PD-1 and PD-L1 by immunostaining in a total number of 15 kidney biopsies with ANCA-associated renal vasculitis in correlation with glomerular and tubulointerstitial lesions. For independent validation, publicly available datasets were analyzed for PD-1 expression (encoded by PDCD1).

Results: We here observed a predominant tubulointerstitial expression of PD-1 that is decreased in ANCA-associated renal vasculitis. Moreover, loss of tubulointerstitial PD-1 correlated with active ANCA-associated renal vasculitis. Consistent to the observed association with active glomerular and tubulointerstitial lesions, we identified that interstitial PD-1 correlated with tubular and/or glomerular PD-L1 positivity. Finally, PD-1 was associated with decreased local synthesis of complement factor B. Interestingly, we did not observe a correlation between PD-1 and complement C5 or its C5a receptor.

Conclusion: Combined with our observations, this may implicate a link between impaired PD-1/PD-L1 signaling, complement factor B, and active ANCA-associated renal vasculitis. These findings could be of relevance because experimental data have already been described that PD-1 agonism can be used therapeutically to attenuate autoimmunity in multiple disease models. Furthermore, targeted therapy against complement C5/C5a receptor and factor B are both available and currently evolving in the treatment of AAV. Therefore, this pilot study expands our current knowledge and describes a potential interplay between immune checkpoints and the alternative complement pathway in active ANCA-associated renal vasculitis.

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Complement system remains unaltered in an acute schizophrenia

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Background: Immunological alterations have been observed in schizophrenic patients. It is hypothesized that systemic inflammation is involved in the development of schizophrenia due to the possible deleterious effects on the central nervous system and brain homeostasis [1]. Therefore, the aim of our study was to screen the systemic complement system proteins and function of the classical pathway in a well-characterized first-episode (FEP) drug naïve and relapsed schizophrenia (sz) patients who had not been treated for at least six weeks prior admission. Samples were collected from patients presenting acute psychosis (T0) and after treatment period of 6 weeks (T6).

Methods: Serum C4, plasma C5a and sTCC were assessed with an ELISA method. Functional analysis was conducted with sheep red blood cells (shRBCs) tagged with rabbit anti-sheep antibodies for the classical pathway activation. In a follow-up experiment, C4d deposition of the surface of shRBCs was quantified by flow cytometry measurement.

Results: The static complement levels remained unaltered between the groups (FEP, sz, and controls). Furthermore, a subgroup analysis with patients presenting high neutrophil count and release of neurofilament light chain (NfL) did not reveal any differences between the complement levels. The classical pathway activity was stable between the groups, and the C4d deposition was not altered.

Conclusions: The involvement of the complement system in schizophrenia has been hypothesized over the last decades, but the overall conclusion has remained weak due to high variability between the study results [2]. Therefore, our aim was to explore the role of complement from an extensive patient cohort which was compared to pairwise-collected, sex-, and age-matched healthy controls. Static levels, function, and deposition of C4d remained unaltered between the groups stating that the complement system was not consumed nor inhibited during an acute psychosis.

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Assessing function and inhibition of a synthetically assembled C3 convertase using a novel functional assay

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Independent on the initial trigger for complement activation, the Alternative Pathway (AP) amplification loop accounts for the majority of terminal complement activity. The positive AP regulator, Properdin (a.k.a. Factor P, FP), can significantly contribute to prevention or promotion of disease pathology. Complement dysregulation is being linked to an increasing number of diseases, emphasising the potential for complement modulating therapeutics to ensure patient homeostasis; thus, functional determination of complement pathways, complexes, and regulators is increasingly interesting.

Utilising a novel assay for functional determination of Properdin (Functional Factor P assay, Svar Life Science, Malmö, Sweden), the function of properdin was assessed in healthy controls. Moreover, through slight modification to assay protocol, the assay was used to study inhibition of the C3 convertase, C3bBb(P), through addition of a selection of complement inhibitors.

The assays dynamic range allowed for assessment of both increased and decreased Properdin function, compared to a standardised FP calibrator (positive control, human serum derived). There was a significant variation of properdin function within the healthy control group (n=120), ranging 89,6 – 176,6 % relative activity in samples normalised against properdin concentration.

Utilising a modified protocol, in which the standardised FP calibrator was used as the source of Properdin, inhibition of C3bBb activity was assessed. The inhibitors were efficient in decreasing the apparent convertase activity through different modes of action, thus quenching assay signal.

Beyond the assays ability to determine the integrity of, and variation in, Properdin function in serum samples, the assay could be modified to study inhibition of C3bBb(P), through inhibition of assembly or activation, or through dissociation of the synthetically assembled C3 convertase, formed in the assay's activation step. Imbalances caused by dysregulation of Properdin, or opposing regulators, may have severe consequences for patient health, emphasising the need for functional complement assays. The Wieslab Functional Complement ELISAs are an example of such assays, enabling functional study of all three complement pathways; the presented findings suggest that the described Functional Factor P assay is a versatile tool, extending our ability to specifically study function and inhibition of the AP amplification loop and its critical components.

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Visualizing complement activation and regulation in the tumor microenvironment

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The role of the complement system in the tumor microenvironment (TME) is ambiguous. Complement activation could result in antitumor responses through complement-dependent cytotoxicity of tumor-targeting antibodies. However, complement may also promote tumor development by supporting chronic inflammation and hampering antitumor T cell responses. These opposing effects of complement in the TME seem to be context and cancer type dependent. To better understand these opposing effects it is important to determine local complement activation and regulation. We aim to visualize local sites of complement activation and regulation in the TME by distinguishing the presence of C3 activation fragment C3b from C3b regulation fragments iC3b and C3dg.

In order to distinguish the different C3 fragments, highly-characterized anti-C3 antibodies (Abs) with known specificity for C3b, iC3b and C3dg are needed. To this end we developed a novel anti-C3 Ab screening tool using C3b, iC3b and C3dg of which the thioester was linked to biotin, allowing physiological presentation of each purified fragment. To do so, C3 was activated into C3b through cleavage by trypsin in the presence of biotin. The produced biotinylated C3b was further cleaved into iC3b and/or C3dg and purified. Subsequently, each C3 fragment was captured via their biotinylated thioester on streptavidin coated surfaces to screen anti-C3 Abs for their selectivity and affinity via ELISA and SPR.

This set-up enabled us to identify specific Abs against different C3 fragments. These Abs are used to analyze patients' tissues for complement activation and regulation. Combined with co-staining for the presence of immune cells, other complement proteins and local conditions of the TME (e.g. hypoxia markers and vascularization), this will shed light on the role of the complement system in the TME and tumor progression.

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Inhibition of terminal pathway activation by Diclofenac

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Background: Diclofenac is a widely used non-steroidal drug which has anti-inflammatory, antipyretic and analgesic properties in the treatment of a variety of clinical conditions. However, the effects of diclofenac go beyond inhibiting cyclooxygenases, such as blocking acid-sensing ion channels and having antimicrobial properties. Some former studies also raised the assumption of pos-

sible effects of diclofenac in a yeast-induced edema putatively mediated through complement inhibition.

Methods: The inhibitory activity of diclofenac was evaluated in complement haemolysis assays addressing both classical (2% serum) and alternative pathways (20% serum). In addition, surface deposition of the central components opsonins C4b and C3b was assessed after classical pathway activation on sheep erythrocytes using flow cytometry. In parallel, anaphylatoxin concentrations detected by Western blot and quantified by ELISA. Interaction of diclofenac with complement components was investigated by native PAGE and surface plasmon resonance. Additionally, the effect of diclofenac was tested in fluid-phase-based cleavage assays on C5 activation mediated by extrinsic proteases.

Results: In the range of 1 mM to 250 μM, diclofenac revealed a clear concentration-dependent inhibition of classical pathway-mediated haemolysis. By detecting opsonins on cell surfaces, we ruled out inhibitory effects at the proximal complement pathway which was confirmed by unaltered C3a levels. However, C5 activation seemed to be completely abolished since no C5a generation took place. To elucidate the underlying mechanism, we performed SPR-based binding studies and uncovered binding of diclofenac to C3b, which eventually decreased C5 binding to C3b. However, in direct cleavage assays, diclofenac also reduced cleavage of C5 by plasmin in complete absence of C3b.

Conclusions: In high concentrations, diclofenac is able to inhibit complement at the level of terminal pathway activation. However, the exact mechanism remains elusive and requires further investigation.

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Activation of the complement system early on during brain death management

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Introduction: Brain death (BD) results in an inflammatory environment, including activation of the complement system. Currently, the clinical impact of prolonged duration of BD on the donor organ is still unknown. Here, we investigated how different BD durations impact complement activation levels both systemically and locally within renal tissue.

Methods: The QUOD biobank was used to obtain EDTA-plasma samples and kidney biopsies from BD donors. Samples were routinely taken at several time points during BD management and grouped based on short (≤14h), average (15-22h) or long (≥23h) BD duration. Furthermore, groups were divided based on the presence of delayed graft function (DGF) (6 groups, n=20 per group for plasma, n=10 per group for biopsies).

Results: ELISAs were used for specific quantification of C4d, C3c and C5b-9 in plasma samples. All three complement activation factors showed high levels at the start of donor management (DB2). C4d levels decreased over time, and were significantly lower in samples taken just before organ retrieval (DB4) compared with DB2 (median 5365 ng/mL vs. 2664 ng/mL, p=<0.001). C3c and C5b-9 also showed a similar trend towards lower complement activation levels at the end of donor management (DB3) and at DB4 compared with DB2. Preliminary analysis between different BD duration groups did not show a difference in C4d, C3c and C5b-9 levels. C4d, C3c and C5b-9 levels, at any point during BD management, did not appear to influence the development of DGF. Biopsies (collected at the time of organ retrieval) have been stained for C4d, C3d and C5b-9 and showed clear signs of local complement activation at different compartments, including glomerular, tubular and peritubular regions. The quantification of different staining patterns is currently under investigation.

Conclusion: In conclusion, the complement system is activated in BD donors already early on during the management period, and is decreasing over time. Prolonged duration of BD does not appear to be associated with increased levels of C4d, C3c and C5b-9. Future studies on administering complement inhibitors to BD donors could benefit from starting therapy early on during management period.

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From parasite to therapeutic: Expression, functional characterization, and therapeutic potential of the leech-derived complement inhibitor gigastasin

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The complement system serves as a first-line defense mechanism against pathogens, but inappropriate complement activation also contributes to various clinical conditions such as ischemia-reperfusion injury (IRI), autoimmune-hemolytic anemia (AIHA) and other autoimmune reactions. Therapeutic complement inhibition has therefore emerged as a promising clinical approach and parasitic host defense inhibitors may serve as templates for novel therapeutics as they often block early-response pathways. Gigastasin, a serine protease inhibitor from the giant Amazon leech, was shown to impair both the classical (CP) and lectin (LP) pathway of complement activation. The complexity of the disulfide-rich protein has so far limited the recombinant production of gigastasin in yields suitable for preclinical development. Here we describe the expression of gigastasin in a prokaryotic system and present studies that show the inhibitor's potential for future therapeutic applications.

Gigastasin (6xHis-TEV-gigastasin) was expressed in E. coli under optimized conditions and purified using affinity chromatography. The protein was characterized concerning identity and purity (SDS-PAGE,WB, MS) and thermal stability (nanoDSF). To further enhance purity and remove endotoxins, the protein was processed with reversed-phase HPLC. Endotoxin was measured under GMP conditions. The 6xHis-Tag was cleaved via TEV to receive tag-free gigastasin. Target binding was tested using SPR and the activity of the protein was assessed by monitoring direct C1s inhibition and impairment of complement activation in human, monkey, and mouse serum using CP and LP ELISAs.

We successfully expressed recombinant gigastasin in E. coli at high yield and purity. The protein bound to C1s, but not proC1s, with nanomolar affinity, indicating selectivity towards the active

enzyme. Gigastasin displayed strong activity in substrate cleavage and human complement activation assays, and the observed activity in mouse and monkey serum indicates translational potential. Recombinant gigastasin showed a profound thermal stability that enabled reversed-phase HPLC purification, lyophilization and storage while maintaining full activity. Importantly, this step also drastically reduced endotoxin to levels suitable for clinical applications.

Our studies establish gigastasin as promising preclinical candidate for the development of therapeutics to treat complement-mediated diseases such as IRI during transplantation or stroke, and as molecular template for derivatives with enhanced affinity and selectivity profiles.

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Structure-guided design of derivatives of the complement inhibitor compstatin with improved species specificity profiles

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The compstatin family of peptide-based complement C3 inhibitors, discovered in 1996 at the University of Pennsylvania, has been continuously optimized and found broad applications in biomedical research and as complement therapeutic. Pegcetacoplan, a PEGylated compstatin derivative, has meanwhile been approved by the FDA for PNH and GA (Empaveli/Syfovre, Apellis) and next-generation analogs with enhanced PK/PD properties are in clinical development (AMY-101, Amyndas). While facilitating clinical development, compstatin's narrow species specificity for human/primate C3 prevents its evaluation in many preclinical disease models restricting translational studies. We therefore aim to identify and develop compstatin derivatives with complement-inhibiting activity in rodent models, and to gain more insight into their pharmacokinetic properties.

By taking advantage of recent structural insight from the clinical candidate compstatin Cp40 and combining it with experimental and homology models of mouse/rat C3b, we describe molecular determinants of compstatin's species specificity and use in silico methods to predict derivatives with activity for rodent C3b. These rational design efforts are supplemented by directed evolution approaches based on phage display library screening against C3b from different species. Promising candidates are produced using solid-phase peptide synthesis and tested for binding affinity for mouse/rat C3b (using SPR and BLI) and complement-inhibitory activity (e.g., using ELISA).

Our structural analysis revealed that the narrow species specificity of compstatin is determined by a reduced number of drug-target contacts in the binding pocket rather than by steric hindrance. Key interactions between compstatin and human C3b were shown to be absent in the other species, and efficacy optimization achieved in Cp40 even accentuates the specificity profile. By selectively substituting amino acids in sequence of the first-generation compstatin analog Cp01 guided by our structural models, and performing phage display screening, we are currently selecting lead peptides that show binding to rodent C3b. To account for missing drug-target contacts, we explore extended binding sites and chemical modifications to regain affinity. In addition to providing novel C3 inhibitors for translational studies, the in-depth structure-activity relationship analysis increases our understanding of specificity, selectivity, and activity determinants of the compstatin family that expedite the next generation of clinical C3 inhibitors.

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Functional characterization of two novel Complement Factor B variants in patients with primary Immune-Complex-mediated MPGN

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Primary Immune-complex-mediated MPGN (IC-MPGN) is a rare chronic nephropathy characterized by glomerular IgG and C3 deposits. Dysregulation of the complement alternative pathway (AP) plays a role in IC-MPGN; among the genetic factors, variants in complement gene CFB, encoding a component of the AP C3 convertase (C3bBb), have been reported. We characterized two FB novel variants, identified in two unrelated IC-MPGN patients: the G161R heterozygous variant in a patient with low C3 levels and high sC5b-9 levels; the homozygous R679W variant in a patient with low C3 levels that normalized at remission.

Recombinant (r)FB were expressed in HEK293. C3 convertase, C3bBb(Mg2+), and C3 proconvertase, C3bB(Mn2+), were formed incubating C3b-coated wells with rFB ± FD, then detached and quantified through the B(C3bB) or Bb(C3bBb) bands on WB, respectively. C3bBb formation was also evaluated by measuring the Ba in the supernatant. Spontaneous or FH-mediated decay of C3bBb was monitored by further incubation with buffer or FH. In fluid phase experiments, rFB, C3 and FD were mixed and, formation and activity of the C3bBb was evaluated by Ba and C3a ELISA or by WB for the occurrence of C3b a'-chain band, respectively.

Secretion of 161R mutant was comparable to wt. Solid phase assays revealed that variant 161R, localized in the Ba domain, formed more C3bB (250% vs wt) but much less C3bBb (20% vs wt), and decay of mut C3bBb was 2-fold greater than wt C3bBb. Conversely, in the fluid phase experiments, C3bBb formation and activity was greatly increased with FB 161R as compared to FB wt (200% increase vs wt C3bBb). These results are consistent with finding that the patient had strong fluid phase AP activation. The second variant R679W, was secreted at reduced levels (20% of FB

wt) but induced an enhanced formation of C3bBb on wells (200% vs wt C3bBb).

In summary, G161R and R679W are gain of-function variants which however impact differently on AP. The 161R variant causes chronic activation of AP in fluid phase, while the 679W variant appears to impact mainly in solid phase, an effect likely tempered by its impaired secretion.

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CD59, C9-lock and horse C9 all inhibit MAC assembly through similar mechanisms

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Background: Five plasma proteins, C5b, C6, C7, C8 and C9, assemble in a stepwise manner to form the membrane attack complex (MAC) which inserts into target cell membranes to cause lysis. C5b captures C6 and C7 while attached to the convertase, the C5b-7 complex is released and binds adjacent membranes. C8 and multiple copies of C9 are sequentially recruited, adopting an extended conformation that traverse membrane to form the lytic pore. CD59 binds nascent C5b-8, permitting the first C9 to bind but preventing its unfolding, further C9 recruitment and polymerisation into the lytic pore. A disulphide-locked C9 ('C9-lock'; $C9_{F262C/V405C}$) lacked haemolytic activity while horse C9 showed restricted haemolysis in standard assays.

Methods: Here we compare mechanisms of MAC inhibition by soluble CD59 (sCD59), C9-lock and horse C9 (hoC9). C9lock and sCD59 were generated recombinantly, hoC9 was affinity purified from plasma. Binding and haemolytic assays were used to identify and compare the modes of action of MAC inhibition by sCD59, C9lock and hoC9.

Results: sCD59, C9lock and hoC9 all inhibited haemolysis in classical and alternative pathways. To test mechanism, C5b-8 sites were pre-formed on guinea pig erythrocytes (gpE) using purified MAC components, incubated with sCD59, C9-lock or hoC9 and haemolysis developed by adding an excess of C9. All three inhibitors bound C5b-8 and blocked lysis. Preincubation of each inhibitor with C9 prior to addition to C5b-8-gpE prevented MAC formation, demonstrating competition for C5b-8 between C9 and each inhibitor. To test species cross-reactivity, rat or mouse C5b-8-coated gpE were incubated with the inhibitors prior to addition of human C9 (lytic on rodent C5b-8); preincubation with either sCD59, hoC9 or C9-lock blocked lysis in this heterologous system.

Conclusion: The data demonstrate that sCD59, C9-lock and hoC9 all stably bind human or rodent C5b-8 sites to inhibit C9 recruitment and subsequent lysis. While sCD59 prevents C9 unfolding by steric hindrance, we suggest that hoC9, like C9-lock, fails to unfold and hence blocks subsequent C9 binding to the complex. These findings provide simple tools for analysis of MAC assembly and mechanisms of MAC inhibition.

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VIS954 is a Potent Anti-C5aR1 Antibody for the Treatment of ANCA-Associated Vasculitis

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The binding of anaphylatoxin C5a to its cognate receptor C5aR1 is one of the terminal events in the complement pathway that can lead to the activation and transmigration of neutrophils and the release of inflammatory mediators. The C5a-C5aR1 axis, because of its pervasive role in immune regulation, is implicated in numerous diseases, including the autoinflammatory condition anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitis (AAV). Complete ablation or small molecule-based pharmacologic inhibition of C5aR1 has been shown to limit AAV progression, validating C5aR1 as a therapeutic target, but a potent and specific inhibitor of C5aR1 with appreciable half-life has yet to be developed for AAV. Visterra has therefore created VIS954, a human monoclonal antibody and orthosteric antagonist of human C5aR1. Through in vitro assessments using primary human neutrophils, VIS954 potently and specifically inhibits C5a-C5aR1 signaling and subsequent neutrophil activation. The in vivo potency of VIS954 against C5a-mediated neutrophil activation was demonstrated in pharmacodynamic assessments using transgenic human C5aR1 knock-in mice. In a murine model of AAV, VIS954 abrogated pathological and histological hallmarks of disease. Moreover, VIS954 reduced C5aR1-mediated neutrophil activation in ex vivo human AAV patient samples. These data, coupled with the favorable pharmacokinetic profile of VIS954, support the pursuit of VIS954 for C5aR1-based inflammatory diseases like AAV.

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Ficolin-2 high consumption is a hallmark of ischemic stroke with an etiology of large-artery atherosclerosis

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Background: Ischemic stroke represents 87% of all strokes being a major cause of death and permanent disability worldwide. Prevention is still the primary approach to reduce stroke socio-economic burden. Atherosclerosis characterized by fatty deposits (plaques) in the inner walls of major arteries is the main risk factor for

ischemic stroke. We reported that elevated circulating levels of ficolin-2 are associated with vulnerable atherosclerotic plaques, those that upon their rupture increase stroke risk. We here aimed at describing ficolin-2 kinetics after an acute ischemic stroke, with the primary endpoint of identifying etiology-linked changes, namely atherosclerotic vs. others.

Methods: 300 patients (68.6y ± 16) were enrolled at Lyon Stroke Center for an acute ischemic stroke due to large vessel occlusion treated with mechanical thrombectomy. Blood was withdrawn at H0: admission, H6: hour 6, H24: hour 24, H48: hour 48, M3: month 3. Ficolin-2 circulating levels were measured by ELISA.

Results: Considering the whole cohort, circulating ficolin-2 levels were 5620 ± 2006 of ng/mL ± SD at H0 and decreased at H6 (4821 ± 1936, p<0.001 vs. H0), H24 (4928 ± 1811, p<0.001) and H48 (5182 ± 1851, p<0.05). Data indicate an early consumption of ficolin-2 after acute ischemic stroke, due to target binding and lectin pathway activation. A large-artery atherosclerosis (LAA) etiology regarded 40 patients (13%), showing lower circulating ficolin-2 at H6 (3810 ± 1775) compared to other causes of stroke (4971 ± 1917). The area under curve (AUC) reflected different ficolin-2 levels over time, namely AUC of 1.077e+007 ± 2997205 in stroke patients with LAA vs. 1.195e+007 ± 2914745 in those with other etiology (p=0.02, Unpaired t-test). Contingency analysis by Fisher's exact test showed that lower ficolin-2 levels at H0 and H6 were associated with LAA. Ficolin-2 levels were seemingly independently associated with LAA since other risk factors including ASPECTS score, obesity, dyslipidemia, coronopathy smoke, diabetes and hypertension were not.

Conclusion: Our data indicate increased ficolin-2 usage in patients having a symptomatic atherosclerotic plaque, in line with the association of ficolin-2 circulating levels with vulnerable plaques. As such ficolin-2 circulating levels could predict the risk of stroke in atherosclerotic patients standing as a new biomarker with future diagnostic value.

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Single cell sorting and cloning of human anti-C1q autoantibodies reveals specificity for solid-phase C1q and capacity to engage Fc receptors

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Anti-C1q autoantibodies are present in several autoimmune diseases and are known to associate with nephritis in systemic lupus erythematosus (SLE). However, these antibodies have also been detected in up to 10% of healthy individuals. Importantly, analysis of sera suggests that the anti-C1q autoantibodies in both healthy subjects and SLE patients are selective for ligand-bound, solid-phase, C1q, and do not bind to fluid-phase C1q. This is underscored by the observation that in patients with anti-C1q, C1q levels tend to be in the normal range and the autoantibodies are thus not depleting.

To study the nature of human C1q autoantibodies at the molecular level, we isolated C1q-reactive B cells and cloned 9 monoclonal antibodies (mAb) from 4 individual healthy donors. The mAb were produced recombinantly and characterized in biochemical and cellular assays.

The isolated clones were of IgG isotype, contained a highly mutated variable domain and showed high affinity to the Collagen-Like Region (CLR) of C1q. Binding of anti-C1q mAb was observed only to solid-phase C1q that was bound on a range of natural ligands such as IgG, IgM, CRP, antibody-opsonized cells and necrotic cells. The binding to solid-phase C1q was not inhibited by fluid-phase C1q. Electron microscopy confirmed that multiple anti-C1q mAb can bind to a single solid-phase C1q molecule. In competition experiments, we observed 2 separate groups of human anti-C1q mAb indicating that at least 2 epitopes are targeted. Both epitopes are also targeted by anti-C1q from SLE sera, implying that the cloned anti-C1q mAb are representative for anti-C1q autoantibodies found in SLE. The presence of anti-C1q on C1q-containing immune complexes did not enhance complement activation. However, the presence of anti-C1q on C1q-opsonized immune complexes greatly enhanced triggering of cellular Fc receptors, a process that is normally impaired when C1q is bound to the immune complex. Indeed we observed increased phagocytosis of C1q-opsonized beads and bacteria after addition of anti-C1q mAb.

Thus, anti-C1q autoantibodies are specific for solid-phase C1q, do not enhance complement activation, but importantly enhance Fc-receptor triggering which may contribute to the immunopathology of autoimmune diseases.

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Role of nutraceuticals in ameliorating inflammation in Alzheimer's disease

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Introduction: Alzheimer's disease (AD) affects almost 50 million people worldwide. There are currently few effective therapies. In this context the anti-inflammatory activity of plant derived molecules offers potentially promising, cheap alternatives which may be able to slow disease progress if used in a prophylactic manner. The aims of the work presented were to test the ability of the plant derived polyphenolic compounds; Green Tea Catechin (GTC) and Hydroxytyrosol (HT) to counteract inflammation relevant to AD using in vitro and in vivo models.

Method: In vitro, an inflammatory activator, amyloid peptide (Aβ1-42), was applied to BV2 microglial cells together with GTC and or HT. Cell function was assessed measuring, apoptosis, cell viability, ROS production, protein expression and gene expression.

In vivo, high fat fed the APPKI -AD mouse were used to test whether HT and GTC alone or in combination could suppress inflammation and slow the onset of AD. Parameters measured included: lipid profiles, cytokine expression by ELISA and qPCR, immunostaining of inflammatory markers related to AD .

Statistical analysis: Statistical analyses were carried out using either independent sample T-tests or One-way ANOVA as appropriate to the specific dataset. Averaged data represents a minimum of three individual experiments.

Results: (1) In vitro, using BV2 cells, we show that GTC and HT, either singly or in combination, were able to reduce the reactive oxygen species and nitric oxide production caused by amyloid peptide (Aβ1-42). By flow cytometry and qPCR, they also downregu-

lated the expression of microglial markers CD45, CD11b and IBA-1. (2) In vivo, using the APPKI -AD mouse model we show that both HT and GTC alone or in combination were able to suppress inflammation caused by high fat diet, suppressing cytokine expression and various other markers of inflammation.

Conclusion: The data presented in this poster provides evidence that HT and GTC are able to reduce the microglial activation induced by amyloid peptide (Aβ1-42) and reduce the pro-inflammatory effect of high fat diet in the APPKI -AD mouse. We may thus conclude that both polyphenols might serve as a potential therapeutic agents for treating neurodegenerative diseases which have strong neuroinflammatory components.

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IQGAP1 deficiency promotes complement activation on podocytes: new insights into the pathogenesis of kidney complement-mediated disease

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Background: C3 glomerulopathy (C3G) is a rare kidney disorder associated with complement alternative pathway (AP) dysregulation and C3 deposition within the glomerulus.

Proteinuria and nephrotic syndrome are present at onset in 90% and 30% of C3G patients, respectively. A morphological hallmark of nephrotic syndrome is effacement of foot processes in podocytes, highly specialized cells that play a main role in glomerular permeability.

Through exome sequencing, we identified in a proband from a trio with C3G and nephrotic syndrome a variant in a new candidate gene, IQGAP1, encoding for the IQ Motif Containing GTPase Activating Protein 1 (IQGAP1). IQGAP1 is a scaffold protein largely expressed in the podocytes, where it interacts with proteins of the slit-diaphragm complex and cytoskeleton, regulating podocytes morphology, motility and permeability. The novel de novo heterozygous 1-base pair deletion leads to a frameshift with the introduction of a premature stop codon (p.L159Rfs*4).

This preliminary finding led us to investigate the link between IQGAP1 alterations and complement activation in podocytes.

Methods: Conditionally immortalized human podocytes were transfected with null or IQGAP1 small interfering RNA (siRNA). IQGAP1 mRNA expression levels were evaluated by real-time PCR, and IQGAP1 and cytoskeleton proteins localization by immunofluorescence. C3 deposition was evaluated, using confocal microscopy, by incubating transfected podocytes with 10% normal human serum (NHS).

Results: Transfection of podocytes with IQGAP1 siRNA, which recapitulates the effect of the frameshift identified in C3G patient, induced a decrease of IQGAP1 mRNA expression by 80% and caused a reorganization of podocyte cytoskeleton proteins, such as integrin-linked kinase (ILK) and podocin, and a cortical accumulation of F-actin. Incubation of siRNA transfected podocytes with NHS induced a three-fold increase in C3 deposition as compared to podocytes transfected with null siRNA (n=5; IQGAP siRNA: 1259 pixels^2; null siRNA:377 pixels^2; p=0.01). C3 deposits were abrogated by sCR1.

Conclusions: Altogether, these findings support the hypothesis that intrinsic defects altering the podocyte structure and/or function may predispose to a secondary complement attack on the glomerular cells, contributing to C3G onset. Further studies are needed to validate this hypothesis and to shed light on the molecular players involved in C3G.

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The complement system is dysregulated in long covid patients

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Introduction: Long COVID (LC) is characterised by a heterogeneous set of unresolved symptoms lasting many months after the initial acute phase of SARS-CoV-2 infection. Complement dysregulation is an important contributor to inflammation and tissue damage in COVID-19, driven largely by the alternative pathway. The impacts of complement in LC have yet to be explored. Herein, we sought to investigate whether dysregulation of complement is observed in LC, to identify which parts of the complement cascade are dysregulated and explore utility of complement biomarkers for diagnosis and monitoring.

Methods: We undertook a comprehensive study of complement in LC, profiling 21 complement biomarkers, including key components (C1q, FB, C3, C4, C5, and C9), regulators (C1INH, FH, FD, FI, CR1, FHR4, FHR125, properdin, clusterin), and activation products (TCC, iC3b, Ba, C5a, C1INH-C1s, C1INH-MASP), in 166 LC plasma samples and 79 age-matched non-LC controls 2 years post-infection. Data were subjected to ROC analysis to identify predictive biomarkers and select an optimal sub-set for diagnostic use.

Results: Compared to controls, levels of complement activation products (Ba, p<0.001; TCC, p<0.0001; iC3b, p<0.0001; C5a, p=0.017; C1INH-C1s, p< 0.01), components (C3, p=0.0004; C5, p=0.0037; C9, p=0.0103; FD, p=<0.0001) and regulators (FH, p=0.0079; C1INH, p=0.0011; properdin, p=<0.0001) were significantly higher in LC while C1q levels were significantly reduced (p=<0.0001). ROC analysis demonstrated that an activation product marker set was highly predictive for distinguishing LC from controls (AUC >0.8).

Discussion: Our findings show that complement is dysregulated in LC samples two years post SARS-Cov2 infection. Elevated levels of activation markers demonstrate ongoing complement activation particularly in the alternative pathway, progressing through to the terminal pathway. Increased levels of most components likely reflects chronic inflammation; however, low C1q levels may implicate classical pathway as a driver of the dysregulation. The data demonstrate the utility of complement biomarkers for diagnosis and suggest that complement-blocking therapies may be effective in therapy of LC.

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Immune evasion potential associated with infection type of *Staphylococcus aureus*

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Background: Staphylococcus aureus is a major cause of community and healthcare-associated infections resulting in significant morbidity and mortality worldwide. As a successful pathogen, S. aureus utilizes a myriad of immune-evasive tactics to persist within the host, with the complement system a primary target. With antibiotic resistance of major concern, we sought to determine whether infection type or central genotype may be useful indicators for predicting pathogenic potential and highlight possible anti-staphylococcal targets.

Methods: Clinical Isolates: Community associated methicillin sensitive or methicillin resistant S. aureus (CA-MSSA or CA-MRSA) from blood or skin and soft tissue infections (SSTI) were collected from patients of CHKD (Norfolk, Virginia, USA).

Genomics: gDNA was examined using whole genome sequencing, PCR and Multi-Locus Sequence Typing to determine sequence type (ST) and/or clonal complex (CC), capsule type (CP5/CP8, capHIJK) and select virulence-factor gene presence (immune evasion: clfA, sdrE, scn, chp, spA; adhesion: clfA, sdrE; toxin production: hla).

Targeted Transcriptomics: Isolates were incubated ± 20% normal human serum (NHS) and virulence-factor expression was measured (RT-qPCR, normalizing to tpi).

Biofilm Assay/Complement Protection: Select isolates were assessed for biofilm-production capacity in a 96-well polystyrene plate. Protection from C3-fragment deposition was determined by incubating biofilm with 10% NHS followed by methylamine extraction of C3-fragments followed by anti-C3 immunoblotting.

Results: ST8 and CP5 dominated for both CA-MRSA (n=46) and CA-MSSA (n=49). Blood isolates expressed elevated levels of all virulence-factor genes measured compared to SSTI isolates in control conditions, whereas NHS stimulated upregulation of scn and chp for SSTI and blood isolates; clfA was relatively stable across groups. CA-MSSA produced significantly more biofilm resulting in less C3-fragment deposition vs. CA-MRSA. Blood isolates with CP5 were opsonized with significantly less C3b/iC3b than blood isolates with CP8.

Conclusions: These data suggest that S. aureus has adapted to the host environment (blood vs. SSTI) with expression of some virulence factors elevated without external stimuli. This level of preparedness may reflect an evolved state-of-readiness to combat specific host environments. Understanding pathogenic dynamics of S. aureus in the context of infection type may provide valuable information to facilitate the development of better-directed therapies and highlight potential therapeutic targets.

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Placenta specific complement activation in mice produces preeclampsia phenotype and adversely affects fetal liver function

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Background: Elevated complement deposition on placenta has been observed in preeclampsia patients. Preeclampsia is often associated with fetal growth restriction (FGR). Prevalence of non-alcoholic fatty liver disease is about 4 times higher in children after FGR compared with normal birth weight suggesting dysregulated liver metabolism in FGR. We hypothesized that placenta specific complement activation leads to preeclampsia-FGR phenotype affecting fetal liver function.

Method: We used tet-inducible promoter (TRE) to drive the expression of miRE-based shRNA targeting complement receptor 1 related protein y (Crry, Cr1l), induced by reverse tet-transactivator (rtTA3). To avoid leakiness of the TRE promoter loxP-Stop-loxP cassette was used between TRE promoter and miRE. Male Cr1l.634shRNA mice were then crossed with female B6.Cg-Gt (ROSA)26Sortm1(rtTA*M2)Jae/J to obtain Cr1l+/+RosartTA+/+ mice. Male Cr1l+/+RosartTA+/+ mice were mated with Cyp19-Cre female mice to obtain timed pregnancy. The Crry shRNA was induced using doxycycline (75μg/mL) in drinking water from 10.5dpc to 17.5dpc. Fetal livers were collected on D17.5dpc for lipid staining and gene expression analysis using a lipid metabolism panel.

Results: Doxycycline treatment reduced the expression levels of Crry in placentas by 30% resulting in increased complement deposition on placentas. In mice with placental complement activation, systolic (147 v/s 117), diastolic (117 v/s 84), and mean (127 v/s 95) blood pressure (mmHg) were significantly elevated compared to controls. Placental weight (96g v/s 87g), and fetal weight (973g v/s 898g) were significantly reduced in mice with complement activation. Proteinuria as indicated by a significant increase in urine albumin/creatinine ratio (264 v/s138) was observed in mice with complement activation. Steatosis was observed in fetal livers with female showing accumulation of more lipid than male fetuses. Several genes involved in β-oxidation and de novo cholesterol biosynthesis pathways were differentially expressed in the liver from preeclampsia exposed compared to normal fetuses. Male and female fetuses showed difference in the fold change of differentially expressed genes.

Conclusion: Placental complement activation in mice produced preeclampsia-FGR phenotype. Fetal steatosis was present in FGR fetuses. Fetal sex specific differential gene expression of lipid metabolic pathways was observed in the liver from FGR fetuses.

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Involvement of PTX3 and MBL in the clearance of SARS-CoV-2 infected lung airway epithelial cells

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In severe forms of COVID-19, the infection is associated with airway tissue damage and complications due to cellular infection and excessive inflammatory responses. A systematic investigation of the interaction of human humoral fluid-phase pattern recognition molecules (PRM) with SARS-CoV-2 showed that the long pentraxin 3 (PTX3) and mannose-binding lectin (MBL) bound the viral nucleocapsid (N) and spike (S) proteins, respectively. MBL bound trimeric spike protein in a glycan-dependent manner and inhibited SARS-CoV-2 in three in vitro models. After binding to S protein, MBL activated the lectin pathway of complement, potentially contributing to hyperinflammation and tissue damage1. PTX3 interacts with C1q, MBL and ficolins enhancing complement activation, and with factor H regulating complement response, while with Fcγ receptor modulating phagocytosis. In patients, the serum levels of complement proteins and PTX3 significantly increase during the infection period, correlating with severity2.

In this study, we asked the question whether these PRMs influence the interaction between phagocytes and SARS-CoV-2 infected cells, in term of phagocytosis and inflammation activation. We first observed that surface-exposed N proteins on the infected cells colocalize with PTX3 before being internalized by human bone marrow derived macrophages (MDM). By immunofluorescence, we observed the phagocytosis rate by MDMs of SARS-CoV-2 infected cells significantly increases with PTX3 or MBL treatment. In addition, in the MDMs with internalized infected cells, both N protein and dsRNA signals increase over time, suggesting a temporary viral replication activity in those MDMs. In parallel, cellular caspase-1 signal increases over time, showing inflammasome activation in both MDMs and infected epithelial cells. Lastly, we demonstrated complement protein deposition on SARS-CoV-2-infected epithelial cells. In conclusion, our study demonstrated that the interactions of PTX3 with N and MBL with S enhance the phagocytosis of SARS-CoV-2 infected epithelial cells, suggesting their involvement in promotion of antigen presentation and effective specific responses against SARS-CoV-2 infection.

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Increased complement activation in recipients is associated with chronic rejection after lung transplantation

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Background: Excessive activation of the complement system has been associated with fibrosis and other adverse outcomes in multiple organs. However, its role in lung transplantation is unclear. We have previously shown that local complement activation early after lung transplantation is associated with primary graft dysfunction, which increases the risk for chronic lung allograft dysfunction (CLAD), a major impediment to survival and quality of life. We hypothesized that lung transplant recipients with a genetic predisposition to complement activation would have worse CLAD-free survival.

Methods: We conducted a retrospective study of adult primary lung transplant recipients at Barnes-Jewish Hospital (BJH). Saliva was probed using Taqman assays for rs2230199, a known functional C3 polymorphism (C3R102G, minor allele frequency (C) =15%) that results in increased complement activation. Our primary outcome was CLAD-free survival. We subsequently performed mouse orthotopic lung transplantation using recipients deficient in Crry (a complement regulatory protein that inhibits complement activation) and analyzed the occurrence of obliterative bronchiolitis, a pathological feature of CLAD, as well as the development of donor-specific antibodies (DSA).

Results: Of the 176 patients from BJH, 111 (63%) had wild-type C3 (G/G), whereas 64 (36%) had the C3R102G polymorphism (57 - C/G, 7 - C/C). C3R102G was associated with increased risk for CLAD. In a multivariable Cox-proportional hazards model including age, sex, race, transplant type, and pre-transplant diagnosis, C3R102G was associated with increased risk of the composite outcome (CLAD or death). Additionally, recipients with the C3R102G polymorphism had increased risk of developing DSA, a major risk factor for CLAD. In the mouse orthotopic lung transplant model, Crry-deficient recipients (i.e., those mice who had a defect in complement regulation in their circulation and thereby, increased complement activity) developed more severe obliterative bronchiolitis lesions when compared to WT recipients. Crry deficiency also drove the intragraft expansion of IgM+ memory B cells and high levels of DSA.

Conclusion: Recipient C3R102G risk alleles are associated with worse CLAD-free survival. Our experimental data suggest increased complement activation promotes cross-talk between T and B cells, which promotes memory B cell and DSA formation, increasing the risk of CLAD, and worsening survival.

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The dominant form of the C1 inhibitor R444C variant causing type II hereditary angioedema is its covalent adduct with serum albumin explaining elevated levels

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Hereditary angioedema (HAE) with C1 inhibitor (C1-INH) deficiency is caused by heterozygous mutations in the SERPING1 gene. HAE type I is characterized by low antigenic and low functional C1-INH levels, whereas HAE type II patients often have elevated antigenic, but still low functional C1-INH. A common variant causing HAE type II is the one bearing an R444C mutation (or R466C with precursor numbering). We have purified this variant from the

plasma of a patient by sequential chromatographic steps, and found that it co-purifies with a ~66 kDa protein, which proved to be serum albumin. On non-reducing gels a ~170 kDa apparent MW was observed for this variant, suggesting that C1-INH-R444C is covalently bound by a disulfide bridge to albumin, which also contains an unpaired cysteine residue. The a C1-INH-R444C-albumin complex seemed to be the predominant variant in this patient, as in early chromatographic fractions about 80% complex was observed in Coomassie-stained gels. The isolated C1-inh-R444C-albumin complex, used as a calibration standard, enabled us to set up a quantitative Western blot assay in order to determine the concentration of the different C1-INH forms in this patient. Fine tuning of the assay is underway, however our data showed that this patient has ~72% C1-INH-R444C-albumin complex by weight. The remaining uncomplexed fraction comprises ~13% monomeric R444C variant and ~15% wild-type C1-INH by weight. Molar percentages of the three forms are ~57% albumin complex, ~20% monomeric R444C mutant, and ~23% wild-type C1-INH. Testing of further patients is underway, however, we presume that dominance of the C1-INH-R444C-albumin complex is a general phenomenon of this variant. Nevertheless, complex formation with albumin immediately suggests that the elevated level of total antigenic C1-INH in patients carrying this mutation is caused by FcRn receptor mediated recycling.

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Insulin secretion and blood glucose homeostasis in a CD59 double knockout mouse model

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Background: Type 2 diabetes is characterised by an inability to maintain blood glucose homeostasis, caused by insufficient insulin secretion from pancreatic β-cells and insulin resistance in peripheral tissues. The complement inhibitor CD59 and its intracellular splice forms IRIS-1 and IRIS-2 have been found to mediate insulin secretion in pancreatic β-cell lines. Devoid of CD59 and its splice forms, rat and mouse β-cell lines exhibit impaired insulin secretion. Here we wanted to study the effect of CD59 on blood glucose homeostasis in mice.

Methods: In mice the CD59 gene has undergone duplication, resulting in the presence of CD59A and CD59B. To study the role of CD59 in insulin secretion and blood glucose homeostasis we used a CD59 double knockout mouse model that lacks critical exons of each CD59 gene. Through PCR analysis we identified a CD59ba hybrid transcript that was further characterised following exogenous expression in the clonal rat β-cell line INS-1 832/13 cells.

Results: In terms of fasting blood glucose levels and glucose tolerance, no difference could be observed between CD59 double knockout mice and wild type control mice. Additionally, no difference in glucose stimulated insulin secretion from isolated pancreatic islets was found. However, in various tissues of the CD59 double knockout mice, including pancreatic islets, we identified a transcript composed of the remaining exons of both CD59A and CD59B spliced together. When expressed in INS-1 832/13 cells, the identified CD59ba hybrid protein was found to associate with the plasma membrane, similar to canonical CD59. Furthermore, the exogenously expressed CD59ba hybrid was found to be glycosylated in INS-1 832/13 cells, which previously was identified as a prerequisite for CD59's ability to mediate insulin secretion.

Conclusion: Although the CD59ba hybrid is expected to be misfolded and unable to inhibit complement, it may be responsible for rescuing the phenotype pertaining to blood glucose homeostasis in the CD59 double knockout mice. The structural requirements for CD59's ability to mediate insulin secretion is likely located in the N-terminal region, which is present in the CD59ba hybrid, including the N-glycosylation site.

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Roles of complement dysregulation and membrane attack complex formation in developmental synapse loss

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Background: Complement is involved in developmental synaptic pruning and pathological synapse loss in Alzheimer's disease (AD). Our recently published work identified complement dysregulation in AD mice involving the activation (C1q; C3b/iC3b) and terminal membrane attack complex (MAC) pathways. Inhibition or ablation of MAC formation reduced synapse loss in two AD mouse models, demonstrating that MAC formation is a driver of pathological synapse loss (PMID:35794654). However, the precise mechanism of MAC induced synapse loss and its relevance to developmental synaptic pruning remains to be elucidated.

Methods: We explored whether complement dysregulation and MAC formation occurred in the mouse brain during development and whether this contributed to developmental synapse loss. Novel ELISA were used to quantify C1q, C3 fragments and MAC in total brain homogenates from WT and complement C1q deficient (C1q-ko) mouse brains at 8, 15, 28 and 40 days after birth, the peak period of synaptic re-modelling.

Results: C1q and complement activation products were detected in WT brain homogenates across the developmental period examined, suggesting ongoing complement dysregulation. In brains from C1qko mice, C1q was absent and complement activation product levels were decreased compared to WT. Impact of C1q deficiency and deficiencies in MAC components on developmental synapse loss are being investigated using methods established to study pathological loss in AD models.

Conclusion: We show that complement dysregulation occurs in the brain during synaptic remodelling. The absence of C1q in C1qko mice markedly reduced levels of complement activation products in the brain during this period of peak synaptic pruning, demonstrating that complement is predominantly activated via the classical pathway. The impact on synapse loss is under evaluation.

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Factor H-related dimer equilibrium and kinetics revealed through novel specific ELISAs

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Introduction: Factor H-related proteins (FHRs) 1, 2, and 5 possess the unique capacity to form dimers resulting in FHR-1/1, FHR-2/2, FHR-5/5 homodimers, and FHR-1/2 heterodimers. As it is hypothesized that the FHR proteins act as antagonists of complement regulator FH, the dimerization of FHR-1, -2 and -5 is thought to further increase their antagonistic function. As the dimers differ in ligand affinity and specificity, their overall distribution may play a role in (de)regulation of the complement system in various complement-associated diseases. We have previously shown that FHR dimer exchange is dynamic and seems to occur freely and randomly in human plasma, establishing equilibrium with complete dimerization. However, as FHR-2/2 dimers could not be directly measured, it remains unknown if the levels of FHR-1/1, FHR-1/2 and FHR-2/2 indeed follow random distribution laws and solely depend on the ratio between FHR-1 and FHR-2.

Methods: Recently in-house developed FHR-2 specific antibodies were successfully used to develop a FHR-2/2 ELISA, allowing for the first time direct measurement of these dimers in human samples. By measuring human sera, and mixing FHR-1 deficient sera with FHR-2 deficient sera, we determined the kinetics, conditions and distribution of FHR-1/1, FHR-1/2 and FHR-2/2 dimer formation. Furthermore, we studied how temperature, pH, NaCl concentration and addition of L-arginineaffected the dimer kinetics.

Results: Initial findings indicate an equilibrium skewed towards the formation of FHR-1/1 homodimers over FHR-1/2 and FHR-2/2 dimers. This challenges the assumption that FHR-1 and -2 monomer exchange follows random distribution laws and supports the relevance of specifically determining FHR dimer distribution. In addition, the dimer stability was affected by temperature, pH, NaCl and addition of free L-arginine, allowing further biochemical characterisation of these interactions. By combining these approaches, we will further explore the molecular mechanism and functional consequences of FHR dimers within the complement system.

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Transposon mutagenesis: A molecular switch in Acinetobacter baumannii for rapid adaptation to bacteriophages and the complement system

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Multidrug-resistant Acinetobacter baumannii are gram-negative, opportunistic bacteria, which WHO has classified on the critical priority list in need of research and development of new antibiotics [1]. Studies have shown that plasmids, insertion sequence elements, and transposons contribute to horizontal gene transfer and regulate the expression of antibiotic resistance genes in A. baumannii strains [2]. Here, we report transposon mutagenesis as a mechanism for the A. baumannii 5910 strain to adapt and escape from vB_fBen_Aci_003 bacteriophages (phages) by disruption of glucose-6-phosphate isomerase (gpi) within the K-locus that determines capsule expression. The transposon insertion sequence within gpi resulted in the loss of capsule and the expression of shorter lipooligosaccharides on the bacterial surface. Thereby the phages lost their receptors on the bacteria. While A. baumannii became resistant to phages, they became susceptible to complement-mediated killing. Further, upon prolonged incubation of phage-resistant bacteria under sublethal serum conditions, the bacteria reverted to a phenotype that was resistant to complement-killing. This phenotype was again encapsulated, and the insertion sequence was removed from gpi. Furthermore, bacteria that survived complement killing showed similar morphology and features as the wild-type phenotype. This suggested that capsular polysaccharides and longer lipooligosaccharides protect A. baumannii from killing by complement but are essential for the vB_fBen_Aci_003 phage to infect its host. Our study shows that A. baumannii can rapidly undergo phase variation and develop either complement or phage resistance. Preconditioning of the bacteria in serum can mimic the in vivo situation and bring forth the predominant phenotype that is sensitive to phages. Finally, in a heterogenous A. baumannii population that consists of both encapsulated and non-encapsulated forms, the bacteria could be efficiently killed by the combination of normal human serum and phages.

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A genome-wide association screening of complement activation from a general population study

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Background: Complement is a fundamental part of the innate immune response. Its alterations are associated with severe systemic diseases.

Methods: To illuminate the complement's genetic underpinnings, we conducted genome-wide association studies of the functional activity of the classical (CP), lectin (LP), and alternative (AP) complement pathways using serum samples, stored at –80°C since the day of collection, analyzed with the WIESLAB® complement system screen kit (SVAR, Malmö, formerly Wieslab AB, Lund, Sweden) according to manufacturers' instructions in the Cooperative Health Research in South Tyrol (CHRIS) study (n=4990).

Results: We identified 7 loci that included 13 independent, pathway-specific variants ($p<5\times10\text{-}8$) located in or near complement (CFHR4, C7, C2, MBL2) and non-complement genes (PDE3A, TNXB, ABO), explaining 12%, 18% and 73% of CP, AP and LP's genetic heritability, respectively. Variants were associated with inflammatory, autoimmune and coagulation disorders and >400 proteins. Transcriptome- and proteome-wide colocalization analyses combined with two-sample Mendelian randomization confirmed known causal pathways, established within-complement feedback loops, and implicated causality of ABO on LP and of CFHR2 and C7 on AP. LP showed causal effects on collectin-11 and KAAG1 levels and mouth ulcers' risk.

Conclusion: These results build a comprehensive resource to investigate the role of complement on human health.

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Factor H-related proteins bind to extracellular matrix components and affect complement activation

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Under pathological conditions, excessive complement activation may occur on both cellular and non-cellular surfaces, including the extracellular matrix (ECM), where factor H (FH) and its splice-variant factor H-like protein 1 (FHL-1) are the major inhibitors of the alternative complement pathway. In contrast to FH, the FH-related (FHR) proteins act as positive regulators of the cascade and enhance complement activation on surfaces. Mutations and polymorphisms in the CFH/CFHR genes can result in variants or hybrid proteins and/or altered serum levels that cause dysfunctions in the regulation of the complement cascade, and are implicated in diseases such as age-related macular degeneration, atypical hemolytic uremic syndrome and C3 glomerulopathy. Since FHR-1 and FHR-5 were reported to bind to certain components of the ECM, such as laminin and fibromodulin, and enhanced alternative pathway activation, our aim was to study the interaction of all FHR proteins with a variety of ECM components, which occur e.g., in Bruch's membrane and glomerular basement membrane, and their effect on complement activation when bound to these ligands.

Binding of FHR proteins to selected ECM components was tested by Western blot using human serum and by ELISA using recombinant proteins. Competition assays, convertase and complement activation assays were performed by ELISA.

Binding of most FHRs to collagen III, collagen IV, nidogen-1, nidogen-2, lumican, tenascin C and vitronectin was detected. FHR-5 bound to the ECM proteins via its CCP3-7 domains and significantly decreased the binding of FH. On ECM-coated wells, when exposed to human serum, FHR-3, FHR-4 and FHR-5 showed variable enhancement of alternative pathway activation as detected by measuring Bb deposition, and also C5-deposition, whereas FHR-1 and FHR-2 had no effect. However, a disease-associated mutant FHR-1 with duplicated dimerization domains significantly enhanced both C3 and FB deposition on ECM-covered surfaces compared to the wild-type protein when added to serum.

Our results identify novel ECM ligands for all FH family proteins and indicate that some FHRs enhance complement activation via the alternative pathway on various ECM proteins. Furthermore, the analysis of mutant FHR-1 suggests that altered FHRs associated with diseases may have an elevated complement activating capacity.

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Complement component C1q can predict patients' response to cisplatin depending on tumour type: A step towards personalized medicine?

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Background: C1q can exert complement-independent pro-tumorigenic functions [1], also by virtue of its binding to hyaluronic acid (HA). HA was previously demonstrated to influence tumor cells' sensitivity to cisplatin [2]. Thus, we attempted to dissect the potential implication of HA-bound C1q in chemoresistance, evaluating its effects on drug sensitivity in both malignant pleural mesothelioma (MPM) and high-grade serous ovarian cancer (HGSOC).

Methods: HA-C1q effect on in vitro response to chemotherapeutic agents was evaluated in MPM (H28, M14K and ZL34) and HGSOC (SKOV-3) cell lines. Its modulation of ABC transporters and detoxification enzymes was evaluated by qPCR, Western blot and functional assays. Isolated primary MPM (n=24) and HGSOC (n = 26) cells were also tested for cisplatin cytotoxicity. Patients' clinical outcomes in terms of radiological response were correlated with in vitrocytotoxicity.

Results: HA-C1q matrix significantly increased MPM cell mortality after cisplatin and PARP inhibitor treatments, restoring chemosensitivity also in a cisplatin-resistant MPM cell line (H28R). HA-C1q downregulated the expression and functionality of drug efflux transporters, comprising MDR1, and promoted cell proliferation. In primary MPM cells, we detected a strong correlation ($R = 0.81$, $p = 0.002$) between the percentage of in vitro cell mortality after cisplatin treatment on HA-C1q matrix and patients' response to chemotherapy. Interestingly, no effect of HA-C1q matrix was observed in HGSOC cells.

Conclusions: C1q can specifically increase MPM cell sensitivity to cisplatin treatment, due to drug transporters' downregulation and increased cell proliferation. Moreover, we demonstrated that C1q-HA matrix is the optimal culture condition to mimic MPM microenvironment and to predict patients' response to chemotherapy. HA-bound C1q exerted no effect on HGSOC cells, highlighting a tumor-type-dependent specificity. Our results confirm the controversial effect of C1q within the tumor microenvironment and allow to set up an easy testing method which may be applied in the future not only as a predictive tool for the individual patient but also as a translational model to test novel therapeutic targets. The prediction of patient response may allow to move a step towards the development of personalized medicine in different solid tumors.

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C1q stimulates hyaluronic acid degradation via gC1qR/HABP1/p32 in malignant pleural mesothelioma

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Background: C1q exerts pro-tumorigenic functions in the tumour microenvironment (TME), independently of complement activation [1]. In malignant pleural mesothelioma (MPM), HA-bound C1q is able to increase tumor cell adhesion, migration and proliferation, but also to enhance the production of pro-inflammatory and pro-metastatic HA fragments due to HAS3 upregulation [2]. An increasingly activated HA metabolism is associated with cancer progression. Here, we investigated HA-bound C1q contribution in HA degradation via modulation of hyaluronidases (HYAL1, HYAL2) and the involvement of globular C1q receptor/HABP1/p32 (gC1qR), as a receptor of both HA and C1q.

Methods: Primary cell isolation, zymography, immunohistochemistry, Real-Time quantitative PCR (RT-qPCR), immunofluorescence, Western blot, surface biotinylation assay, flow cytometry, proximity ligation assay (PLA), RNA interference, bioinformatics analysis.

Results: After initial characterization of HYALs in MPM primary cells, we focused on HYAL2, since GEPIA bioinformatics analysis revealed an unfavorable prognostic index in MPM patients with higher HYAL2 mRNA levels. Upon seeding MPM cells onto HA-bound C1q, HYAL2 upregulation was highlighted by RT-qPCR, flow cytometry and Western blot. In the attempt to determine receptors involved in HA-C1q signaling, a striking membrane and intracellular co-localization between HYAL2 and gC1qR was found by immunofluorescence, surface biotinylation assay and PLA. RNA interference of C1QBP (gene for gC1qR) unveiled a promising regulatory effect of gC1qR on HYAL2 expression, determining an unexpected HYAL2 downregulation. Moreover, functional blockage of gC1qR by a specific antibody hampered HA-C1q signaling and impeded HYAL2 upregulation.

Conclusions: HA-C1q interplay can act as a tumor-promoting signaling complex by enhancing HYAL2 expression, suggesting a consequent higher rate of HA catabolism and the release of pro-inflammatory and pro-tumorigenic HA fragments. Furthermore, we demonstrated a novel regulatory function of gC1qR in the TME due to its involvement in the modulation of HA metabolism. The regulation of HA metabolism is crucial in view of its key role in tumor progression and its connection with most of the hallmarks of cancer.

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Functional characterization of monoclonal antibodies against human complement C4 and C4BP

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Previously, we reported the generation and initial characterization of a panel of complement-specific monoclonal antibodies that could be useful as research and diagnostic tools, to develop specific ELISAs, and as potential therapeutics to inhibit complement activation at various points of the cascade. Inhibitory antibodies targeting C3, C4, C5, C6, C7, C8, factor H and C4b-binding (C4BP) protein were identified within the panel. The complement classical (CP) and lectin (LP) pathways play an important role in the defence against pathogens; they are also involved in the clearance of dead cells and immune complexes. After initiation, both pathways proceed by cleavage of C4. C2 binds to the resulting C4b fragment, and its cleavage leads to the formation of the common CP/LP C3-convertase C4b2b, which amplifies and propagates the cascade by cleaving C3. The main regulatory molecule of CP and LP is C4BP, which facilitates inactivation of C4b and accelerates the decay of C4b2b. In autoimmune diseases with pathological CP/LP activation or immune complex formation, both C4 and C4BP may be promising targets for therapeutic interventions. Here, we characterized 18 anti-human C4 and 26 anti-human C4BP mouse monoclonal antibodies.

ELISA was used to determine which C4 cleavage product the antibodies recognize and whether they inhibit the C4BP-C4b interaction. The anti-C4 antibodies were also used as detection antibodies in Western blot. The ability of the antibodies to influence complement activation was investigated in hemolysis assays using sensitized sheep erythrocytes for the CP, and in ELISA using mannan-coated wells for the LP.

Of the anti-C4 antibodies, 5 recognized both C4b and C4c, 11 only C4b, 2 only C4c. In Western blot with reduced C4b, 10 antibodies recognized the α'-chain, 1 the β-chain, and 1 the γ-chain of C4b. Three anti-C4 and 12 anti-C4BP antibodies inhibited the C4b–C4BP interaction. CP-mediated hemolysis was enhanced by 1 anti-C4 and 1 anti-C4BP antibody, and significantly inhibited by 3 anti-C4 and 1 anti-C4BP antibody. Moreover, 2 anti-C4 antibodies showed >50% inhibition of LP.

Altogether, several anti-C4 and anti-C4BP antibodies that influence CP/LP activation were identified that may be exploited as research tools and/or potential therapeutics.

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Complement C7 and clusterin form a stable complex in circulation

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Background: The complement system is an innate immune system composed of an intertwined network of proteins that play a vital role in host defence and host homeostasis. One distinct role of the complement cascade is the formation of the lytic membrane attack complex (MAC) on target surfaces. The MAC is composed of multiple complement components, and one essential component involved in MAC formation is complement C7. Although the role that C7 plays in MAC assembly is well established, the inherent characteristics of C7 are still understudied.

Methods: To further understand the molecular characteristics of C7, we investigated the properties of C7 purified from serum with polyclonal and newly generated monoclonal antibodies.

Results: Western blot and mass spectrometry analysis of serum-purified C7 demonstrated a strong and direct association between C7 and clusterin, a known complement regulator. This association was also confirmed in clusterin purified from serum. Clusterin inhibits the MAC by binding to polymerized C9, but it has not been shown to associate with native complement proteins in vivo. To further investigate the relationship between C7 and clusterin, we established an enzyme-linked immunosorbent assay (ELISA), which demonstrated a direct interaction between C7 and clusterin, indicating that these proteins form a complex (C7-CLU). Moreover, we detected the C7-CLU complex in healthy serum and plasma donors using the custom ELISA, further confirming the presence of this complex in circulation.

Conclusion: These results showcase that C7 plays a unique role in the complement cascade in association with clusterin, prompting further investigation of the function of the C7-CLU complex and its effects in circulation.

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Complement C7 and clusterin form a stable complex in circulation

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Results: Western blot and mass spectrometry analysis of serum-purified C7 demonstrated a strong and direct association between C7 and clusterin, a known complement regulator. This association was also confirmed in clusterin purified from serum. Clusterin inhibits the MAC by binding to polymerized C9, but it has not been shown to associate with native complement proteins in vivo. To further investigate the relationship between C7 and clusterin, we established an enzyme-linked immunosorbent assay (ELISA), which demonstrated a direct interaction between C7 and clusterin, indicating that these proteins form a complex (C7-CLU). Moreover, we detected the C7-CLU complex in healthy serum and plasma donors using the custom ELISA, further confirming the presence of this complex in circulation.

Conclusion: These results showcase that C7 plays a unique role in the complement cascade in association with clusterin, prompting further investigation of the function of the C7-CLU complex and its effects in circulation.

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Exploring the role of C5aR2 in neutrophil-driven autoimmune diseases: An often overlooked player in the pathogenesis of *epidermolysis bullosa acquisita*

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Background: The anaphylatoxin receptors play a critical role in the pathogenesis of various autoimmune diseases. In this context, the enigmatic second receptor for the anaphylatoxin C5a, C5aR2, is often overlooked. In this study, we aimed to elucidate the impact of C5aR2 on the pathogenesis of epidermolysis bullosa acquisita (EBA), a prototypical autoimmune skin disease characterized by neutrophil-dependent skin blistering in which complement activation is a prerequisite.

Methods: We used mice with a global or a LysM-specific deficiency of C5ar2 in a passive model of EBA. In this model, mice were subcutaneously injected with antibodies against type VII collagen (COL7) to induce neutrophil-dependent blistering of the skin. In addition, we performed in vitro assays to assess how targeted deletion of C5ar2 affects C5a-induced activation and effector functions of neutrophils. In these experiments, neutrophils from wild-type and C5ar1$^{-/-}$ mice served as controls. We employed single-cell RNA-sequencing to identify differentially expressed genes and elucidate the molecular mechanisms underlying C5a-induced activation in neutrophils.

Results: Mice lacking C5aR2 globally or specifically in LysM-positive cells were found to have a significantly ameliorated disease phenotype compared with wild-type mice. The relative C5a-mediated activation and effector functions of neutrophils in these mice were correspondingly decreased. This reduced responsiveness to C5a stimulation was associated with a lower number of differentially expressed genes and a changed transcriptome profile compared with neutrophils from wild-type mice. Notably, neutrophils lacking C5ar1 expression did not respond to C5a stimulation, which was reflected in an unchanged transcriptome profile.

Conclusion: These results suggest that the often-neglected C5aR2 is a critical contributor to neutrophil-driven autoimmune diseases such as EBA. While our in vitro assays clearly indicate a dependence of C5aR2 on C5aR1, our single-cell RNA-sequencing results shed light on how signaling through C5aR2 affects gene expression. However,

to fully understand the signaling cascade downstream of C5aR2 and its dependence on C5aR1, further research is needed. This will also help determine the potential efficacy of targeting C5aR2 to treat neutrophil-driven diseases such as EBA.

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Complement score: A novel prognostic tool in malignant pleural mesothelioma?

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Background: The complement system can exert pro- or anti-tumorigenic functions based on different tumour types and contexts, suggesting novel perspectives for therapeutic targeting in selected subgroups of patients [1]. We aimed at developing an innovative prognostic tool based on the in situ quantification of several complement components for the prediction of malignant pleural mesothelioma (MPM) patient outcome, introducing the definition of "Complement Score".

Methods: Bioinformatics analysis via GEPIA and UALCAN (TCGA-MESO); immunohistochemistry on MPM tissue microarrays (TMAs, n = 88) and whole tissue sections (n =17); clinical information collection and statistics analysis.

Results: Bioinformatics analysis of genes encoding for complement activation components (n = 27) and regulators/receptors (n = 29) allowed the selection of four promising markers in MPM: the mRNA expression of C1S, SERPING1, CFB and CFI resulted to be positively correlated with patient overall survival. C1q was included in further analysis due to its abundance in MPM microenvironment [2]. Protein expression of the selected complement components was evaluated both on TMAs and whole tissue sections of MPM patients, being expressed as percentage of tumour cell positivity, immune cell positivity and deposit. C1s, C1INH, CFB and CFI displayed cytoplasmic positivity of tumour cells, but also of immune cells and deposit, whereas C1q presence was only detected as deposit or positivity of monocytes/macrophages. Univariate analyses were performed in order to correlate the expression of each complement component with MPM histotype, TILs (CD4+, CD8+, CD19+), tumor proliferative activity (Ki-67), PD-L1 expression, overall survival. Survival analysis showed that $C1q^{HIGH}$ (Log-rank test, $\chi 2 = 6.01$; $p = 0.01$) and $C1INH^{HIGH}$ (Log-rank test, $\chi 2 = 5.13$; $p = 0.02$) patients displayed significantly increased survival.

Conclusions: Complement Score could be hopefully used in the future as a tool to stratify MPM patients, directing each patient to a personalized treatment, and its application could be potentially extended to other solid tumours, unveiling the "double-edged sword" role of complement system in cancer.

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CPV-104, an improved recombinant variant of human complement factor H produced in moss, is a prime candidate for clinical application in complement mediated diseases

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Background: Over the last years an increasing amount of complement therapeutics have been developed. Beyond their use in primarily complement driven diseases, complement inhibition has potential benefits in a variety of other disorders. However, most inhibitors aim to completely prevent complement activation, thereby leaving affected individuals susceptible to deleterious side effects. The use of a natural complement regulator like factor H (FH) could constitute a superior treatment option as it physiologically regulates complement activation and actively contributes to the degradation of C3 deposits. Recently, Eleva's moss-based expression system achieved high yields of recombinantly produced FH.[1] Additional improvement of its glycosylation pattern significantly improvement the molecules' pharmacokinetic (PK) profile, which is now comparable to serum-derived human FH (sd-FH) in vivo.

Methods: Moss FH (CPV-101) was produced in Physcomitrium patens as described before.[1] Glyco-optimized variant – CPV-104 was generated via in vitro sialylation of CPV-101 post production. CPV-101 and CPV-104 were both compared to sd-FH. Functional analyses were performed in vitro including assays with patient blood samples. PK profiles of FH variants were determined after radiolabeling in wild type mice. The influence of FH variants on serum C3 concentrations and C3 kidneys deposits was assessed in FH deficient mice. Safety, efficacy and PK profiles of CPV-101 and CPV-104 were further elucidated in cynomolgus monkeys.

Results: Moss derived CPV-101 and CPV-104 were significantly more potent in inhibiting C3b deposition, TCC formation and hemolysis compared to sd-FH in vitro. Radiolabeling of FH variants showed a significant increase in serum half-life of CPV-104 compared to CPV-

101. In FH deficient mice, peak CPV-104 concentration, half-life as well as its effect on C3 levels and glomerular C3 deposits was comparable to sd-FH. In cynomolgus monkeys, CPV-101 serum concentrations decreased strongly over 24 h and became undetectable thereafter. In contrast, CPV-104 concentrations remained high after 24 h and were still detectable 7 days post injection.

Conclusion: The pronounced improvement in serum half-life and biological efficacy of glyco-optimized CPV-104 in vivo opens new possibilities for systemic treatment, simultaneously reducing the risk of unwanted complications.

Reference

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Assessment of immunogenicity in mice and cross-species activity of an engineered complement regulator

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Background: Approved complement therapeutics have revolutionised the treatment of several severe diseases. However important drawbacks remain including incomplete inhibition upon very strong complement activation and high therapeutic doses due to high target concentrations and re-synthesis rates. To address these issues we developed a regulator that comprises domain stretches of three different regulators (therefore triple fusion protein or TriFu): decay accelerating factor complement control protein domains (CCPs)(1-4), Factor H CCP(19-20) and complement receptor 1 (CR1) CCP(15-17). It completely inhibits even strong activation with high potency. In preparation for animal studies, we assessed the cross-species activity of human and murine versions as well as immunogenicity upon repeated administrations.

Methods: For the murine version of TriFu we replaced the C-terminal CR1 CCP(15-17) domains with CR1-related gene/protein Y CCP(1-5), because mice lack CR1. We produced human and murine TriFu in Pichia pastoris and compared both, the alternative and classical pathway (AP/CP) regulatory potential in standard haemolytic assays (using rabbit or sheep erythrocytes) with mouse or rat sera. To test for immunogenicity, we administered murine TriFu with or without adjuvant i.m. to C57BL/6 mice (one injection per week over five weeks) and evaluated concentration (by ELISA) and functional impact of the occurring antibodies (by haemolysis assays).

Results: In mouse and rat serum both TriFu versions (human and murine) completely protected from AP mediated haemolysis with similar activities. In contrast, in the CP-mediated haemolysis in murine serum murine TriFu was more efficient. Interestingly, in rat serum we observed a higher CP regulatory potential for human over murine TriFu. The immunogenicity study showed that anti-TriFu antibodies were absent after multiple i.m. injections unless the protein was co-administered with an adjuvant. Then low concentrations of anti-TriFu antibodies occurred but those only moderately reduced the regulatory function of TriFu.

Conclusions: Human and murine TriFu shows high activity in mouse and rat sera, enabling animal studies in these species. However, CP activity in rat serum is regulated more efficiently with human TriFu. In addition, anti-drug antibodies were absent after multiple i.m injections proving the feasibility of long-term animal experiments requiring multiple injections.

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C3 deficiency modulates the phenotypic profile of murine macrophages

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C3 is the most abundant protein of the Complement System and plays an important role in the immune response. C3-deficient patients are more susceptible to recurrent and severe infection diseases. Several studies have demonstrated the importance of C3 in controlling infection, but its role in leukocyte differentiation and cellular response is still poorly understood. This study aimed to evaluate several cellular parameters in bone marrow differentiated macrophages (BMDM) and thioglycollated-elicited peritoneal macrophages (TEPM) from C57Black/6 $C3^{-/-}$ ($B6.C3^{-/-}$; C3KO) and C57Black/6 ($B6.C3^{+/+}$; wild type) mice. The total number of cells in the peritoneal exudate was not affected by the absence of C3. TEPM $F4/80^{high}$ cells, resident macrophages that do not depend on the differentiation of new circulating monocytes, are present at higher levels in $B6.C3^{-/-}$ mice than in $B6.C3^{+/+}$ mice. The morphology of the macrophage cytoskeleton plays a crucial role in migration, adhesion and engulfment of particles. We observed an increase in cell roundness and more solid morphology (fewer invaginations) in TEPM $B6.C3^{-/-}$ animals, which may suggest that these cells would be less activated. We did not observe the same phenomenon in BMDM. Microtubules are essential for cell displacement into the inflammatory site. In this study, we observed that in the absence of C3, the presence of F-tubulin filaments increases in both BMDM and TEPM. In addition, a significant increase in CR4 (CD11c/CD18) and CD64 (FcgammaRI) expression was detected in both BMDM and TEPM from $B6.C3^{-/-}$ mice compared to the same cells from $B6.C3^{+/+}$ mice. ROS production and MAPK activation after stimulation with 12-O-tetradecanoylphorbol-13-acetate (TPA) was lower in $B6.C3^{-/-}$ macrophages than their wild type counterparts, suggesting the importance of C3 for cell signaling. As expected, BMDM and TEPM from $B6.C3^{-/-}$ mice phagocytosed less serum-opsonized zymosan particles than the corresponding cells from $B6.C3^{+/+}$ mice. Together our results suggest that C3 plays a role in macrophage phenotype and activation.

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Vemircopan (ALXN2050) monotherapy in paroxysmal nocturnal hemoglobinuria: Interim data from a phase 2 open-label proof-of-concept study

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Background: Paroxysmal nocturnal hemoglobinuria (PNH) is a rare hematologic disorder characterized by hemolysis due uncontrolled complement activation. In vitro, factor D (FD) inhibitors block C3 fragment deposition and lysis of erythrocytes collected from patients with PNH, providing evidence for its potential to inhibit both intravascular and extravascular hemolysis (IVH and EVH). Vemircopan, a second-generation oral FD inhibitor with improved potency against FD and a prolonged T1/2, is being investigated as monotherapy for patients with PNH.

Methods: This ongoing, phase 2, open-label proof-of-concept study (NCT04170023) assesses efficacy, safety, and PK/PD of vemircopan monotherapy in patients with PNH. The study comprises a 60-day screening period, 12-week treatment period, and 96-week long-term extension. Key inclusion criteria for treatment-naïve group: lactate dehydrogenase [LDH] $\geq$1.5 $\times$ upper limit of normal [ULN], absolute reticulocyte count $\geq$100 $\times$ 109/L, and anemia (hemoglobin [Hgb] <10.5 g/dL). Patients receive vemircopan 120 mg BID (can escalate to 180 mg BID per investigator discretion based on protocol). Primary endpoint is change from baseline to week 12 in Hgb. Safety is reported as treatment-emergent adverse events (TEAEs).

Results: At data cutoff, 11 treatment-naïve patients were enrolled (mean [SD] age, 44.4 [17.8] y; 63.6% male; 72.7% Asian, 54.5% received transfusions during screening period). Nine patients completed 12 weeks in the study and were included in this analysis. From baseline to week 12, mean (SD) Hgb increased by 3.9 (1.11) g/dL from 7.9 (1.29) g/dL to 11.8 (1.25) g/dL, LDH decreased by 81% from 7 $\times$ ULN to 1.4 $\times$ ULN, absolute reticulocyte count decreased from 212.4 (86.47) $\times$ 103/uL to 120.0 (51.44) $\times$ 103/uL. No patients needed transfusions except 1 case on day 2 owing to low Hgb (5.1 g/dL). Of 31 TEAEs (n = 9 patients) most (25/31; 80.6%) were considered unrelated to study drug. No serious TEAEs, grade $\geq$3 TEAEs, discontinuations, or deaths were reported. There were no thrombotic events, seizures, or meningococcal infections.

Conclusions: This interim analysis of treatment-naïve patients with PNH suggests that vemircopan monotherapy controlled IVH and prevented EVH. No new safety signals were identified during the 12-week evaluation period. This analysis provides proof-of-concept for FD inhibition in PNH.

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Complement system regulation and inflammation in FHR-E deficient mice

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Complement as an essential part of innate immunity maintains immune homeostasis and plays a key role in several biological processes, such as removal of injured cells and debris, host defense injuries and repair. However, excessive complement activation can lead to diseases. Therefore, this cascade needs to be tightly regulated. Factor H-related protein 1 (FHR1) is a member of Factor H protein family involved in complement control and in the regulation of the innate immune response. In humans, homozygous deletion of FHR1 and FHR3 genes ($\Delta\Delta$FHR3/FHR1) has been reported to be protective against diseases such as immunoglobulin A nephropathy (IgAN) and age-related macular degeneration (AMD), but has also been shown as a risk for atypical hemolytic-uremic syndrome (aHUS) and atherosclerotic cardiovascular diseases (ACVD) (Skerka et al., 2021; Irmscher et al., 2021). Recently, our group also revealed that FHR1 is an inflammatory mediator inducing proinflammatory response of monocytes (Irmscher et al., 2021). Currently, the role of FHR1 has not been fully explored in animal models. Here, we generated a FHR-E-/- (FHR1 murine homolog) knock-out mouse model for investigating further the role of FHR-E in vivo. We first measured plasma levels of complement C3 and the regulator Factor H in FHRE-/- and WT mice. We observed related levels in both type of animals. In addition expression of pro- and anti-inflammatory cytokines in FHR-E-/- and in WT mice was evaluated in several tissues including kidney, liver and heart. Our data revealed that in most of these tissues, cytokines expression such as IL-1β and TNFα are significantly reduced both at RNA and protein levels in FHR-E-/- mice as compared to WT animals. Further analyses also revealed altered levels of reactive oxygen species in FHRE-/- mice. Taken together, the present study suggests that deletion of the FHR-E gene is more protective against inflammation, confirming our previous observations made in humans. Since this study is conducted in vivo, it provides real insights into the mechanisms how FHR-E regulates the complement system and inflammation and lays the groundwork for further studies.

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The activation of the lectin pathway serine proteases by fibrin and it's subsequent effect in clot formation

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Background: The lectin pathway has some canonical activators in polysaccharides such as mannose, glucose, and glucosamine pre-

sent on bacterial membranes. Its role within innate immunity revolves around the generation of a complement response. However, the pathway is not without mystery, the ficolin pattern recognition receptors of the lectin pathway are responsible for activation of serine proteases MASP-1, -2, and -3 have not been limited to foreign surfaces. Their presence has been shown within clots, where they mimic the activity of thrombin and FXIIIa in fibrinogen cleavage and crosslinking. Our goal is to explore the role of MASP-1 and -2 within a clot regarding its activation and activity.

Methods: To determine the significance of MASP-1 and -2 in clotting, MASP specific inhibitors SGMI-1 and -2 were used. A chromogenic assay in combination with wes capillary electrophoresis was used to observe the activation and interaction of MASP-1 and -2 with fibrin and coagulation protein FXII. Thromoboelastography (TEG) analysis was performed to observe the effect of MASP-1 and -2 in conjunction with its inhibitors on the terminal results of coagulation.

Results: The chromogenic substrate and wes analysis both showed less activity and a sequestered generation of catalytic FXIIa when incubated with SGMI-1 and -2. TEG analysis shows an increase in clotting times with inhibitors as well as a lesser degree of crosslinking and an overall weaker clot.

Conclusion: In conclusion, the presence of fibrin leads a positive effect on MASP activity and its interplay with coagulation proteins, evident by the use of MASP specific inhibitors.

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Dual deficiency of C5aR1 and C5aR2 mediates neutrophil dysfunction leading to exacerbated lung injury during streptococcal pneumonia

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Streptococcus pneumoniae (Spn) is one of the leading bacterial pathogens causing mortality among adults. Complement activation plays a pivotal role in the clearance of encapsulated Spn and the pathogenesis of ARDS. The subsequent release of the anaphylatoxin C5a promotes the recruitment and activation of inflammatory cells including neutrophils after C5a recognition by its two homologous receptors, C5aR1 and C5aR2. The blockade of C5a attenuates proinflammatory responses and rescues lethality in various experimental models of sepsis and inflammation. However, there is insufficient evidence whether C5a neutralization exerts protective or harmful effects during Spn infection.

We established a novel homozygous C5aR1/2$^{-/-}$ double-knockout mouse strain using CRISPR/Cas9 guided gene editing to assess the direct role of C5aR1 and C5aR2 during Spn infection. To further profile alveolar cell populations at the single cell level, we performed TOTAL-seq on FACSorted live CD45^{+} BALF cells using ~200 oligonucleotide-conjugated antibodies enabling simultaneous detection of surface protein markers and RNA.

The C5a-induced influx of neutrophils to the airways was completely abrogated in C5aR1/2$^{-/-}$ mice compared to C57BL/6J wild-type (WT) mice. In striking contrast, C5aR1/2$^{-/-}$ mice showed a stronger inflammatory response in the alveolar spaces after Spn infection, as indicated by enhanced neutrophil infiltration, increased cytokine/chemokine secretion, and exacerbated lung vascular permeability. Interestingly, the neutrophils recruited in C5aR1/2$^{-/-}$ mice released more neutrophil extracellular traps (NETs) but exhibited impaired bactericidal function as suggested by higher CFUs in BALF. Single cell proteotranscriptomics revealed 8 distinct subclusters of alveolar macrophages and 7 subclusters of neutrophils after Spn infection - which displayed heterogeneous levels of C5aR1 protein and C5aR2 RNA expression in WT mice. Consistently, neutrophil clusters were more abundant in C5aR1/2$^{-/-}$ mice but macrophage counts were reduced. Furthermore, C5aR1/2$^{-/-}$ neutrophils developed signs of exhaustion resembling human septic neutrophils with elevated expression of PD-L1 and ICAM-1, and decreased expression of CXCR2 and CD62L. These findings would explain the reduced capacity of neutrophils to control bacterial growth in C5aR1/2$^{-/-}$ mice, creating a potent chemotactic local environment for a persistent neutrophil influx and therefore, resulting in greater lung damage.

Concomitant inhibition of the C5a-C5aR1/2 axes could drive neutrophil exhaustion and lung injury after Spn infection.

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Molecular imaging of renal C3d deposition to monitor lupus nephritis

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Lupus nephritis (LN) is a kidney disease that impacts the majority of individuals with systemic lupus erythematosus and necessitates early detection as prompt treatment leads to improved outcomes. The kidney biopsy is critical for the diagnosis of LN. However, due to its invasive nature and the possibility of sampling errors, there is an unmet need for non-invasive tests that can accurately detect the onset of LN before irreversible damage. We, therefore, wanted to determine whether non-invasive detection of renal C3d deposits "in vivo" can serve as an indicator of disease onset and/or activity.

To assess whether renal C3d deposition is a biomarker in LN, we retrospectively analyzed kidney biopsy reports from LN patients that were previously immunostained for C3. In these kidney biopsies, the intensity of C3 deposition correlated with histologic disease activity. Subsequently, we studied renal C3d deposition in MRL/lpr mice, which develop a kidney disease that resembles LN. Glomerular C3d deposits were already seen at 8 weeks when mice had no histological/functional signs of kidney disease. Renal C3d deposition stepwise increased at 12, 16, and 20 weeks, but showed a decrease at 24 weeks. Next, we generated a method to detect C3d in a non-invasive manner, using a monoclonal antibody labeled with a bioluminescence resonance energy transfer (BRET) system. To validate our imaging technique, we examined the BRET-labeled anti-C3d antibody in Cfh-/- mice, a model of C3 glomerulopathy. BRET imaging demonstrated high intensities in their kidneys, whereas low signal or background was detected in wild-type and C3-/- mice. As C3d deposition is an early disease event in MRL/lpr mice, we tested if early C3d imaging can predict future disease severity. Preliminary experiments in MRL/lpr mice suggested that

renal C3d deposition detected by BRET imaging at 8 weeks correlated with increased disease activity at 16 weeks. Lastly, we also tested a PET-based C3d imaging probe in MRL/lpr and control mice, which demonstrated similar findings.

In conclusion, C3 deposition in the kidney is a biomarker of disease activity in LN, and C3d-imaging can be used to non-invasively monitor disease activity in patients with LN.

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Iptacopan (LNP023) inhibits activity of FB mutants identified in patients with atypical hemolytic uremic syndrome (aHUS)

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Background: Iptacopan is a selective low-molecular-weight inhibitor of the alternative complement pathway (AP) that is currently in clinical development for multiple complement-mediated diseases, including aHUS. It inhibits the protease activity of C3bBb by binding to its active site within Bb.

Approximately 3-5% of aHUS patients express one of 18 point-mutations in FB. The aim of this study was to determine if Iptacopan inhibits protease activity of these FB variants.

Methods: Eight FB variants (K533R, E566A, D279G, F286L, K323Q, K323E, K350N and M458I) were selected based on mutations in proximity to the active site of Bb and their capacity to stimulate the AP. We tested activity of iptacopan on these mutants as follows:

1. Binding affinity to FB mutants via displacement of an active site binding probe.
2. Ability to inhibit an activated C3 convertase formed by cobra venom factor (CVF) bound to wtFB or FB mutants.
3. Activity to inhibit lysis of sheep red blood cells (SRBCs) coated with C3 convertases containing wtFB or FB mutants.
4. Activity to inhibit zymosan-induced AP activation in Factor B depleted human serum supplemented with wtFB or FB mutants.

Results: Iptacopan bound to all FB mutants and wtFB with comparable affinity. CVF-Bb C3 convertase activity was increased with two of the mutants (FB(K350N) and FB(D279G)) and reduced in FB (F286L) compared to CVF-Bb containing wtFB, but iptacopan inhibited C3 cleavage by all convertases with similar IC50. Cell surface C3/C5 convertase activity in the hemolytic assay was also increased for FB(K350N) and FB(D279G) and decreased for FB (F286L). Iptacopan inhibited wtFB and all FB variants in this assay, albeit less potently for FB(D279G). While serum containing FB (350N) and FB(279G) caused only limited MAC-formation upon zymosan stimulation, Iptacopan showed similar potency in the zymosan assay for wtFB and all mutants.

Conclusion: This in vitro study demonstrates that Iptacopan binds to and inhibits common FB variants identified from aHUS patients. Therefore, patients with FB variants may be included in clinical aHUS trials with iptacopan.

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Complement receptor 1 length polymorphism impacts microglial functions relevant to Alzheimer's disease

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Background: Genome-wide association studies (GWAS) in Alzheimer's disease (AD) have highlighted the importance of the complement cascade in pathogenesis. Complement receptor 1 (CR1; CD35) is a top AD-associated GWAS hit. The long variant, CR1*2, is associated with increased AD risk. The roles of CR1 in brain and how variants influence AD risk are poorly understood. Our aim was to investigate the impact of the AD-associated CR1 length polymorphism on phagocytic function in induced pluripotent stem cell (iPSC)-derived microglia.

Methods: Donors were screened for erythrocyte expression of common CR1 variants, CR1*1 and CR1*2, using western blotting and junction PCR genotyping. Homozygote donors (CR1*1/CR1*1, CR*2/CR1*2) were identified, bled, peripheral blood mononuclear cells (PBMCs) isolated and reprogrammed to iPSCs using the CytoTune-iPS 2.0 Sendai Reprogramming kit. Clones were tested for virus clearance and expression of iPSC markers using immunofluorescence and qPCR prior to differentiation into microglia via embryoid bodies. Microglia precursors and iPSC-microglia were tested for marker expression using immunofluorescence, qPCR and flow-cytometry. Microglia expressing CR1*1 and CR1*2 variants were tested for phagocytosis of diverse targets (pHrodo-E.coli bioparticles, pHrodo-human synaptoneurosomes, Alexa-488-amyloid β fibrils), either unopsonised or opsonised with human serum or Factor I (FI)-depleted human serum to mediate C3 fragment deposition, verified by flow cytometry. Synaptoneurosomes were extracted from control or AD brain. Uptake of fluorescent targets was measured in real-time using live imaging in the OperaPhenix high content screening system with inbuilt Harmony software for analysis.

Results: iPSC lines were established by reprogramming CR1*1/CR1*2 homozygote donor PBMCs. Pluripotency was confirmed by demonstrating expression of pluripotency markers. iPSCs were differentiated into microglia and characterised for microglia-specific marker expression. CR1 was expressed in all iPSC-microglia lines for both CR1*1 and CR1*2 variants. iPSC-microglia were phagocytic and phagocytosis was enhanced by opsonisation of targets with human serum; target opsonisation with FI-depleted serum reduced this enhancing effect. Comparison of CR1*1 and CR1*2 expressing iPSC-microglia cells showed that expression of CR1*2, the AD risk variant, enhanced phagocytosis of opsonised targets.

Conclusions: CR1 is an important component of microglial phagocytic activity; expression of CR1*2 increased phagocytosis of opsonised targets, perhaps explaining its association with AD risk.

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Measurements of complement regulators in peritoneal dialysis patients

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Background: Peritoneal dialysis (PD) is a treatment for kidney failure. However, fibrosis is a frequent complication in PD, thereby impacting the ability of the peritoneal membrane to function, leading to technique failure. Membrane failure and other clinical outcomes in PD patients may be related to inflammation and complement. PD has been suggested to modify the expression of membrane-bound complement regulators (CRegs) on the peritoneal mesothelium and thereby trigger complement activation. We aimed to evaluate the importance of soluble CRegs levels in PD patients.

Methods: In a cohort of PD patients, plasma and dialysate levels of sCD46, sCD55, sCD59, and sC5b-9 were measured. Additionally, IL-6 and MMP-2 were determined in the dialysate. Complement measurements were also performed in plasma samples of hemodialysis (HD) patients, non-dialysis chronic kidney disease (CKD) patients, and healthy controls.

Results: Plasma levels of sCD46, sCD55, and sCD59 were significantly higher in PD patients than in CKD patients and healthy controls. However, plasma levels of sCD55 and sCD59 were similar among HD and PD, while sCD46 levels were higher in HD than in PD. Furthermore, sCD46, sCD55, and sCD59 were detectable in the peritoneal dialysate but did not correlate with their respective plasma levels or with peritoneal protein loss. In the dialysate, levels of sCD55, but not sCD46 and sCD59, correlated with local sC5b-9 formation. In accordance, dialysate sCD55 levels also correlated with IL-6 and MMP-2 levels. Next, we constructed a CRegs score by combining sCD46, sCD55, and sCD59 levels. Both the systemic CRegs score as well as the dialysate CRegs score were associated with peritoneal membrane failure in PD patients during follow-up.

Conclusions: Soluble forms of CRegs are present in the circulation and dialysate of PD patients, indicating altered expression could be due to shedding. The soluble forms of CRegs seem to originate from different sources, as plasma and dialysate levels didn't correlate. Furthermore, the shedding of CD55 might trigger local complement activation, inflammation, and tissue remodeling in PD. Lastly, CRegs were associated with the risk of adverse clinical outcomes in PD, making them potential biomarkers for clinical practice.

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Oxidative stress and anaphylatoxin receptor activation control mitochondrial calcium uptake, ATP production and fission-fusion status in RPE cells

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Complement component 3 and C5 fragments C3a and C5a are anaphylatoxins involved in promoting cellular responses important in immune response and host defense. Its receptors (C3a/C5a receptors, C3aR and C5aR) are distributed on the plasma membrane; however, intracellular localization in immune cells has been reported. Oxidative stress increases intracellular reactive oxygen species (ROS), and ROS activate complement signaling in immune cells and metabolic reprogramming. Here we tested oxidative stress and complement activation in mitochondrial dysfunction in retinal pigment epithelial (RPE) cells using high resolution live-cell imaging, and metabolism analysis in isolated mitochondria using Seahorse technology. Focusing on plasma membrane C5aR, C5a stimulation was found to control fission/fusion state of mitochondria, leading to fusion when presented to control cells, and excess fission when presented to oxidatively stressed cells. While overall C3aR levels were unaffected by oxidative stress, its cell membrane levels decreased and mitochondrial (mt) localization increased. Trafficking was dependent on endocytosis, utilizing endosomal-to-mitochondrial cargo transfer. In isolated mitochondria from H_2O_2-treated cells C3a increased mitochondrial calcium uptake, and inhibited mitochondrial respiration; mitochondria from control cells did not respond to C3a. Our findings suggest that oxidative stress increases C5aR-dependent fission as well as mtC3aR transfer, leading to altered mitochondrial calcium uptake and ATP production. These studies will have important implication in our understanding on the balance of extra- and intracellular complement signaling in controlling cellular health and dysfunction.

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Keeping properdin in check: Serum component(s) inhibit(s) the binding of properdin

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Properdin is the only known positive regulator of the complement system. It has a dual function, acting both as a stabilizer of the alternative pathway (AP) convertase and as a pattern recognition molecule (PRM). We have demonstrated that even within cell-mixtures properdin forms a platform for AP activation, where exposure to serum results in an increased C3 deposition on cells opsonized with properdin. These activities of properdin are potentially deleterious and needs proper regulation, which is maintained at several levels. Properdin concentrations in serum are relatively low and properdin is mainly produced by myeloid cells, which implies a local rather than systemic role. In addition, we and others have demonstrated that serum contains unidentified factor(s) that inhibit the activity of properdin. In the current study we have further characterized this inhibitory factor.

We used ELISA-based systems to study the interaction of properdin with different ligands, and to characterize the inhibitory activity. ELISA plates were coated with C3b and LPS and in both cases binding of properdin was demonstrated, representing AP and PRM activity respectively. To circumvent activation of complement, heat inactivated serum was tested for inhibitory activity. Normal human serum (NHS) showed dose-dependent inhibition of properdin binding to either C3b or LPS. The same inhibitory activity was observed with fetal calf serum (FCS). Size fractionation of both types of sera showed that the inhibitory activity was observed in the high MW fractions. The inhibitory factor was immunoprecipitated using properdin-coated epoxy beads. Supernatant, harvested after incubation of the fractions with properdin-coated beads, did not inhibit properdin binding to LPS and C3b, indicating that the inhibitory factors were captured. In contrast, supernatant harvested from fractions incubated with HSA and BSA-coated beads still contained the inhibitory capacity, showing reduced properdin binding to LPS and C3b.

In conclusion, a factor in serum regulates the function of properdin systemically. We have developed a method to immunoprecipitate the inhibitory factor from serum after size exclusion chromatography. The eluted factor will be subjected to mass spectrometry to further elucidate the inhibitory factor.

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Novel natural antibodies targeted against cardiolipin are associated with higher risk of rejection in kidney transplant recipients

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Background: Long-term kidney allograft survival remains poor, largely due to chronic allograft rejection. Allorecognition may be influenced by natural antibodies' ability to activate complement system. We aimed to assess the role of natural antibodies against cardiolipin in kidney transplant recipients (KTR).

Methods: We used human samples from CTOT-1/17 trial that were drawn at the time of transplantation and 3 months after. Enzyme-linked immunosorbent assay (ELISA) was used to assess levels of IgM and IgG anti-cardiolipin antibodies. 5-years follow-up was available.

Results: Samples and clinical data from 56 kidney transplant recipients were available. We measured IgM and IgG levels at both time points. Anti-cardiolipin IgM was positive in 83.9% and 73.2% of KTR pretransplant and 3 months after transplant, respectively. Anti-cardiolipin IgG was positive in 87.5% and 67.9% of KTR pretransplant and 3 months after transplant, respectively. We found that IgG anti-cardiolipin was significantly higher in KTR who subsequently developed T-cell mediated rejection (pretransplant IgG - median (IQR): 77 (48–125) vs. 149 (118 – 755), p = 0.075; 3 months posttransplant: 23 (10–68) vs. 135 (71–297), p = 0.028), no such association was found for IgM neither pretransplant, nor 3 months posttransplant. We also found an association between IgG anti-cardiolipin antibodies at transplantation and allograft function in 5 years (Pearson's correlation coefficient −0.24, p = 0.084), no such association was found for IgM anti-cardiolipin antibodies.

Conclusions: We report on a novel non-HLA natural antibody system potentially involved in allograft rejection and survival. Further research in large cohort of KTR and with mechanistic studies is therefore warranted.

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A role for complement in sickle cell disease

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Background: Sickle cell disease (SCD) is a life-threatening red blood cell (RBC) disorder involving a mutation in β-globin which causes sickled RBCs that obstruct blood vessels, initiating painful vaso-occlusive crises (VOC), endothelial cell (EC) damage, and ischemia/reperfusion injury. These complications often lead to organ damage/failure. Treatment options for SCD remain extremely limited. Recent research indicates that heme-mediated activation of complement may be involved and presents a possible treatment target, however further work to understand this mechanism is required.

Methods: SCD patient serum/plasma was collected during VOC and steady state. Pooled normal human serum (NHS) and plasma from healthy donors was used as a control. Patient hemolytic parameters were gleaned from patient records. Patient heme concentration was quantified using a commercial colorimetric assay. C3b and C5b-9 deposition on the surface of ECs exposed to (1) 50% patient serum or (2) 50% NHS or (3) heme plus 50% NHS was quantified using immunofluorescence imaging. Complement protein concentrations and pathway activity were quantified using established immunoassays.

Results: Crisis patients had significantly lower hemoglobin levels compared to steady state, indicative of increased hemolysis. Heme concentration was significantly elevated in both crisis and steady state plasma. Treating ECs with increasing heme doses, followed by 50% NHS, led to a dose-dependent increase in complement deposition. Interestingly, ECs treated with patient serum from early crisis (1–3 days in pain) had significantly greater C3b and C5b-9 deposition compared to later crisis (4+ days in pain) and steady state patients, indicating complement activation during early crisis. Assessment of the three complement pathways revealed a trend for increased lectin pathway activity during crisis suggesting this may be contributing to complement activity. Although patients with pathologies which may confound results were excluded, unknown confounding variables may have impacted this data.

Conclusion: Complement activity may be elevated in SCD early crisis, potentially driven by a combination of hemolysis and lectin pathway activation, suggesting a role for complement in SCD pathology. Ongoing work is focused on establishing and rescuing

the functional consequences of this activation using human samples and complement blockers to test complement as a therapeutic target.

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Complement drives Parkinson's disease neuropathology through activation of microglial C5a receptors

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Parkinson's disease (PD) is the most common neurodegenerative movement disorder and imposes a severe social and economic burden on ageing populations. PD results from the progressive loss of dopaminergic neurons which is accompanied by a chronic neuroinflammatory response that propagates disease progression. Despite clear evidence for complement involvement in Alzheimer's disease, the contribution of complement to PD neuropathology remains poorly defined. Using publically available data, we demonstrate that complement is widely upregulated in PD patient brains, at sites of dopaminergic neuron loss, and in peripheral blood. Similarly, activation of complement is observed in multiple preclinical PD models. Genetic deletion of key complement effectors at the level of C3, C5, and MAC highlighted a critical role for complement C5a receptors (C5aR1) in driving neurodegeneration in vivo in response to dopaminergic toxins. Fibrillar α-synuclein aggregates (PFF-synuclein), the predominant protein found in PD brain Lewy bodies, directly activated complement to generate C5a and markedly increased C5aR1 expression in human and mouse microglia. Oral administration of a C5aR1 antagonist significantly protected against behavioral motor deficits and nigrostriatal dopaminergic degeneration in acute (28-day 6-OHDA) and chronic (12-month PFF-synuclein) mouse models of PD. Notably, delaying drug administration until symptom onset prevented further motor functional decline and remained neuroprotective. Live visualisation of neuroinflammation using [18F]DPA-714 PET/CT-imaging, demonstrated that both prophylactic and therapeutic inhibition of C5aR1 blunted microglial activation in living mice. Mechanistically, cell-intrinsic microglial NLRP3 inflammasome activation by multiple stimuli was impaired in the genetic absence of C5aR1. Furthermore primary human microglia were unable to secrete IL-1β in response to α-synuclein fibrils in the presence of C5aR1 inhibitors. Targeting this complement-microglia-inflammasome axis with brain-permeable inhibitors could be a feasible approach to tame neuroinflammation, and slow neuronal death in people living with PD.

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Leptospira interrogans leptolysin displays proteolytic activity against complement proteins

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Pathogenic Leptospira species are extremely efficient in disseminating in the host, a fact attributed to their ability to escape complement system activation, and to degrade extracellular matrix and other components of the human plasma. Recently, our group evaluated the proteolytic activity of secreted proteins by leptospires, and exoproteome analyzes of these bacteria allowed the identification of some proteases, including the metalloprotease pappalysin-1 domain protein, which we named leptolysin. In this work we produced and functionally characterized leptolysin from L. interrogans to expand our knowledge on this metalloprotease from Leptospira in the processes of invasion and immune evasion. According to in silico analyzes this protease belongs to the category of short pappalysins, also found in other bacteria. Leptolysin is present in all Leptospira species but is more conserved among pathogenic species of the P1 subclade. A preliminary biochemical characterization of its proteolytic activity was performed using FRET (Free Resonance Energy Transfer) peptides. The enzyme exhibited maximum activity at pH 8.0 and 37°C, was active in the presence of different salts and was strongly inhibited by EDTA and 1,10-phenanthroline. It showed a marked preference for arginine residues in the P1 position. The proteolytic activity of recombinant leptolysin on host molecules was also evaluated in vitro and in vivo. The metalloprotease was active against extracellular matrix proteins (proteoglycans and fibronectin), coagulation cascade molecules (fibrinogen and thrombin) and effector proteins of the human complement system (C2 to C9). A leptolysin knockout strain (Δlic13434) was produced and characterized. This strain showed lower survival in normal human serum (SHN) compared to the wild-type strain. However, in a model of epicutaneous infection in hamsters, no attenuation of virulence was observed with the knockout strain, although the bacterial load in the kidneys of these animals was lower than that observed in animals inoculated with the wild-type strain. Finally, data with sera from leptospirosis patients suggest that leptolysin is produced during natural infections by pathogenic leptospires. The characterization of toxins, their targets and mechanisms of action can help in the development of strategies to combat leptospirosis.

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Development of ex-vivo models to study complement activation in bio-incompatibility reactions induced by extracorporeal devices

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Although remarkable progress has been made in extracorporeal circulation systems (such as hemodialysis, organ perfusion, and extracorporeal membrane oxygenation), bio-incompatibility reactions continue to pose a persistent problem in these systems. The recurrent contact of blood with these biomaterials incited and/or amplifies the inflammatory response in patients, which in turn contributes to unfavorable clinical outcomes. Moreover, complement activation is a key mediator of these bio-incompatibility reactions. However, the lack of appropriate models for translation research is a major hindrance to study the effects of complement therapeutics. Our objective was to create ex-vivo models of hemodialysis and extracorporeal oxygenation to better investigate complement activation in bio-incompatibility reactions associated with these systems.

For both models, a closed circuit was set-up with bloodlines whilst flow-controlled perfusion was achieved by using a peristaltic pump. The temperature was maintained at 37 °C and regulated through an external heater. For our hemodialysis model, a polysulfone hollow-fiber dialyzer was incorporated into the circuit, whereas an oxygenator was included for the extracorporeal oxygenation model. The circuit was primed with NaCl 0.9% before the addition of whole blood, which was then perfused for 4 h. At the start of perfusion and at 30, 60, 120, 180, and 240 min, samples were collected.

In the ex-vivo hemodialysis model, there was a progressive increase in sC5b-9 levels and C3d/C3-ratios, which was significantly reduced when the circuit was operated without a dialysis membrane. Moreover, there was a substantial decrease in levels of components of the lectin pathway during perfusion, which was directly proportional to the increase in complement activation. Consistent with the complement activation results, inflammatory and coagulation markers also increased during perfusion. Similarly, in the extracorporeal oxygenation model, there was a gradual increase during perfusion in C3d/C3-ratios and sC5b-9 levels, indicating significant complement activation. In this model, a reduction was seen for components of the lectin and alternative pathway, whilst components of the classical pathway seem largely unaffected.

To conclude, both our ex-vivo perfusion systems appear to be suitable translational models to study bio-incompatibility reactions and may serve as a valuable tool for preliminary testing of complement therapeutics in the future.

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Annexin A2: A positive regulator of the alternative complement pathway in the kidney

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Annexin A2 (A2) is a recently discovered dysregulator of the Alternative pathway protein complement Factor H (FH) has not yet been fully characterized in the complement system and no reagents to mitigate this interaction have been developed. We have previously demonstrated that Annexin A2 binds to and is endogenously expressed on certain kidney cells. Additionally we demonstrated that A2 blocks the functionality of complement fH and increases complement C3 deposition on these cells. (Renner, J. Immunol. 2016 Feb 1;196(3):1355–1365.) To better characterize the A2:FH interactions we created recombinant protein fragments of the 4 regions of A2 and used in house ELISAs to determine where FH binds to A2. We then created novel monoclonal A2 specific antibodies and we showed they blocked this interaction of the A2:FH interaction site. Additionally these antibodies were used to detect expression changes of A2 on multiple kidney cells in vitro, to detect increases in A2 of mouse models of kidney injury associated with complement , as well as A2 localization and levels in human kidney diseases. These tools will allow us to further investigate A2 disease association in models of SLE and in situations similar to how the FHRs alter the critical FH functional availability to further understand the role of A2.

In conclusion we have manipulated the protein-protein interaction site of A2 and FH, we have shown expression level changes associated with diseases in kidney cells and have developed antibodies to assist in further characterization of the A2:FH interaction.

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Novel functions of complement factor H in macrophages

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Background: Complement factor H (FH) is a critical complement regulator and its absence results in kidney disease. FH deficiency renders C57BL6 mice susceptible to immune complex mediated glomerulonephritis. Significant macrophage infiltration occurs in this setting and understandably, depletion of macrophages reduced the disease pathology. Macrophages participate in defense, inflammation, restoration and fibrosis of the kidney.

Gap in knowledge: Macrophages express intracellular FH, but its role in these cells remains incompletely understood. The present study steps into this gap in our understanding and investigates the role of FH in macrophages.

Methods and results: Wild type (WT) and FH knockout (KO) bone marrow derived macrophages were isolated, cultured and analyzed to understand the impact of FH deficiency. In FH deficient

macrophages, nanostring gene expression analysis using mouse myeloid panel and stringent analysis revealed that of the 754 genes on the array, 73 genes were increased and 20 genes were decreased with at least 1.5 fold change and $p<0.05$. The significantly increased genes include the inflammatory genes TNF, Nlrp3, IL1B and IL6. In line with the increased mRNA expression of VEGF, ATF3, Egr3, Myc, Csf1R genes, macrophage trafficking and migration were increased compared to WT macrophages. Phagocytosis was reduced in FH deficient macrophages, and oxidative metabolism was decreased by 40%. Since transient receptor potential (TRP) channels modulate macrophage functions such as phagocytosis and cytokine generation, we studied the effect of FH on these channels. FH modulated the mRNA expression of the transient receptor potential cation channel, subfamily M, member 7 (TRPM7) in macrophages. In line with increase in mRNA expression of TRPM7, patch clamp assessment showed that TRPM7 current was significantly higher in FH deficient macrophages compared to wildtype macrophages. The TRPM7 current was blocked by FTY720, a specific TRPM7 inhibitor. FTY720 also significantly attenuated the FH regulated proliferation of macrophages.

Conclusion: For the first time our results show that FH is of functional relevance in macrophages, and establishes a critical link between intrinsic FH and macrophage effector functions through TRPM7, which could serve as a potential therapeutic target in FH modulated disease settings.

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The complex role of complement in hepatocellular carcinoma

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Modern advances in cancer detection, treatment, and screening protocols have dramatically increased survival and decreased death associated with most cancers. Hepatocellular carcinoma (HCC) is one of the few cancers that continues to rise in prevalence and mortality. Worldwide, it is currently the sixth most common cancer and the fourth leading cause of cancer-related death. By 2025, it is estimated that new HCC cases around the world will surpass 1 million annually. In collaboration with transplant hepatologists and medical oncologists at the Universities of Colorado and Iowa, we procured plasma (n=30) and tissue samples (n=32) from HCC patients at various stages of disease (curative to advanced HCC). We first evaluated the levels of plasma complement proteins and fragments using nephelometry and ELISA, respectively from HCC and normal control subjects (30 per group). Plasma C4d and iC3b were significantly lower in the HCC group and Ba was significantly higher, suggesting altered complement activity. There was no difference in sC5b-9 nor in total plasma C3, C4 or factor B. We assessed the transcriptomic profile of liver tissues from HCC and normal subjects (4 in each group) using the NanoString PanCancer Immune panel. Between the two groups, we found significant differential expression in mRNA from 124 genes, including 5 complement genes. mRNA of CRP (C-Reactive Protein), CD59, ITGAX (integrin subunit alpha X), and C7 was upregulated in HCC, and properdin was downregulated. To interrogate the nature of the inflammatory milieu within the tumor and peritumor regions, we stained and imaged formalin fixed paraffin embedded tissues using the Polaris multiplex IHC platform. HCC patients had significantly higher myeloid and lymphocyte cell populations compared to normal controls, with the majority of patients exhibiting more immune activity in the peritumor region compared to the tumor. C3d+ cells were higher in the peritumor region of patients with curative HCC, but in the tumor region of those with intermediate or advanced HCC. Our findings suggest a role for complement in the development and/or progression of human HCC. A better understanding of the inflammatory mechanisms underlying HCC etiology could lead to faster detection and more effective therapies.

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Expression of complement proteins by M1 and M2 macrophages: Complement factor B induces inflammatory macrophage phenotype

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Background: Macrophages and the complement system are the two key components of innate immunity that perform diverse functions including immune homeostasis and modulation of inflammatory responses. Macrophages can respond to the stimuli in their surrounding environment and acquire specialized functions in a process known as macrophage polarization. They exist between two extremes known as “M1” – the inflammatory macrophages and “M2” – the anti-inflammatory macrophages. These cells are also the second largest producers of complement and display various complement receptors on their surface. Herein we examined whether macrophage polarization affects the synthesis of complement proteins and whether this in turn can influence their inflammatory status.

Methods and results: We analysed the complement gene expression in the bone marrow-derived macrophages (BMDMs; M0) and their polarized phenotypes M1 and M2 by employing qRT-PCR. Quantitation of transcript expression of 26 complement genes demonstrated that multiple complement proteins are differentially expressed in M1 and M2 cells. Amongst those that are highly expressed, factor B (FB) expression was remarkably increased (>800 fold) in the M1 macrophages (M1-LPS and M1-IFNg) with negligible change in its expression in the M2 macrophages (M2-IL4 or M2-IL4+IL-13). Furthermore, when assessing the influence of FB supplementation on the polarization of FB-/- macrophages, we observed a significant selective increase in M1 markers in both M1 as well as M2 macrophages.

Conclusion: Overall, our data indicate that complement FB which is highly expressed in M1 macrophages can alter their plasticity and contribute to their polarization towards the M1 state. These findings may have implications for the development of therapies targeting complement-mediated macrophage polarization in inflammatory diseases.

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Crystal structure of the Factor H domains 8-14 in complex with the Factor H-recruiting peptide 5C6 validates the unprecedented domain arrangement in the central segment of Factor H

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Factor H (FH) is an abundant plasma glycoprotein that restricts alternative pathway activation in the fluid phase and on host surfaces. FH is also exploited for therapeutic purposes, e.g., the cyclic peptide 5C6 was developed to recruit FH via its central domains to biomaterial and cell surfaces that require protection from complement attack. The central CCP domain segment of FH is thought to orient the regulator's functional termini to facilitate self vs. nonself discrimination. However, structural details about the centre of FH have been scarce until recently when a crystal structure of FH CCPs 8-14 had been presented at a resolution of 2.2 Å. Whereas CCPs 8-9 are elongated and show few inter-domain contacts, CCPs 10–14 form a complete loop that is induced by intricate contacts of CCP14, which is wedged into a cleft formed by CCPs 10 and 11.

Intriguingly, structure-activity relationship studies (SAR) of 5C6 identified FH CCPs 10-14 as the essential binding region for the peptide, suggesting that 5C6 binds to a discontinuous domain platform. To cross-validate the unprecedented central domain arrangement of FH and target interaction mode of 5C6, we solved the co-crystal structure of 5C6 peptide and FH CCPs 8-14 by crystallography at a resolution of 2.87 Å.

In agreement with SAR studies, 5C6 is moulded into a small niche that arises at the ternary contact interface between FH CCP domains 10, 11 and 14 and interacts with all three domains. The peptide is in closest contact with CCP10, followed by CCP11 and CCP14. All 5C6 residues that were experimentally shown to be required for FH binding indeed form contacts in the 5C6:FH8-14 structure, predominantly via the cyclic core and exocyclic C-terminus of 5C6.

The structural complex of FH8-14 with 5C6 validates the unprecedented domain arrangement of CCP domain 10, 11 and 14 in FH. At the same time, the structure provides a rationale for the highly specific interaction of a small peptide with a presumably elongated and flexible target such as FH and an important basis for additional SAR studies to facilitate the rational design of enhanced 5C6 analogues for biomedical applications.

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Pregnancy-related Thrombotic Microangiopathy has a spectrum of underlying causes that affect vascular endothelium

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Background: Pregnancy-related Thrombotic Microangiopathy (TMA) is a heterogeneous group of disorders that can lead to significant morbidity and mortality in the mother. The complement system is believed to play a role in the pathogenesis of TMA. The timely diagnosis and management of these conditions remain challenging due to overlapping clinical features with other pregnancy-related complications, as well as the rarity of these conditions. In this study we wanted to explore the spectrum of underlying reasons in pregnant women with severe TMA.

Methods: Clinical and molecular data were analyzed from 15 women who experienced Pregnancy-related TMA complications between 2012-2022. All patients provided blood samples that were taken during the acute stages of TMA. Clinical data was retrospectively characterized from patient archives. Comprehensive candidate gene genotyping for TMA was done for all patients in the cohort by next generation exome sequencing and for available samples, also by Multiplex Ligation-dependent Probe Amplification (MLPA) for the CFH/CFHR gene region. Patient sera were tested by Elisa and Western blot techniques for anti-FH antibodies and complement FH and factor H-related proteins.

Results: By examining the clinical pre- and post-pregnancy records, we classified our cohort into two main categories: preeclampsia and non-preeclampsia -associated TMA. We recognized risk factors and categorized them into subgroups: metabolic, immune, and genetic, as well as followed the long-term effects on maternal health. Mutations in C3(n=3), ADAMTS13(n=2) and thrombomodulin (n=1) genes were detected in 33% (n=5) of the genotyped individuals. FHR1-3 deletion frequency did not differ from that in healthy individuals. No anti-Factor H autoantibodies were detected.

Conclusion: Complement mutations are involved in only a proportion of the cases of pregnancy-related TMA. Characterization of risk factors such as thrombocytopenia, coagulation disorders, postpartum hemorrhage, and endothelial damage may help in earlier detection of patients at risk. Long-term maternal effects include kidney damage, cardiovascular disease, and psychological effects. Understanding the involvement of the complement system has led to the development of targeted therapies that have improved the prognosis for affected individuals. Early recognition, appropriate management, and close follow-up are crucial to minimize adverse outcomes and improve the overall health of the mother.

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Complement activation in patients with ANCA-associated vasculitis – focusing on complement activation markers in remission

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Background: Involvement of the complement system is of great importance in the pathogenesis of antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV). Increased levels of complement activation products have been reported both during remission and active disease, and the reason for the high levels even without visible symptoms has not yet been fully explored. Our primary aim is to investigate if complement levels in remission are correlated to number of flares, with potential to be used as biomarkers detecting patients prone to recurrent relapses.

Methods: The study included 76 AAV-patients, with a total of 179 samples (1-6 samples per patient). 147 samples were taken during remission (BVAS 0) and 32 during active disease (BVAS $\geq$ 1). Plasma complement component levels were measured by ELISA: C4d for classical pathway activation, C3bBbP for alternative pathway activation and soluble terminal complement complex (sTCC) for general activation.

Results: Preliminary results show that complement levels in patients with multiple flares were not higher than in patients without any relapse. Patients with the diagnosis microscopic polyangiitis had higher levels of sTCC than patients with granulomatosis with polyangiitis (p=0.023). Interestingly, the sTCC levels in remission were also significantly higher in patients that had renal involvement at time of diagnosis (p=0.028), and were positively correlated to creatinine levels in remission (p=0.003). In active disease, C4d levels showed significant correlation to symptoms scored using BVAS (p=0.002).

Conclusion: Our results show no correlation between complement levels in remission and number flares in this cohort. The sTCC levels, reflecting general complement activation, correlated to creatinine indicating organ damage. Correlation between C4d and BVAS score suggests involvement of the classical pathway, and should be further investigated. Previous studies focused on the alternative pathway of complement activation, but evaluation of the classical pathway have often been made using a C4d ELISA kit known to be sensitive to sample handling. Both sTCC and C4d can easily be measured with commercial kits, and C4d preferably with a novel assay using an antibody against a cleavage neoepitope, less prone to artifacts.

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Platelet supernatants modulate the inflammatory response in thrombocytopenic blood ex vivo

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Background: Platelets contain more than 300 intracellularly stored mediators, which are released in response to platelet activation. Many of these mediators have immunomodulatory functions which may shape the inflammatory response in various conditions, including platelet transfusions to thrombocytopenic patients. Here, we investigated the impact of the platelet release reaction on the inflammatory response, including complement activation, in thrombocytopenic human blood incubated ex vivo.

Methods: Platelet concentrates destined for transfusion (n=6) were stored at 22°C for up to seven days. At days 1, 3, 5, and 7 at its shelf-life, samples were activated with TRAP-6 for 15 minutes at 37°C. The platelet supernatants were isolated and stored at -80°C. Human lepirudin-anticoagulated whole blood was isolated from healthy volunteers (n=8) and depleted from endogenous platelets by repeated centrifugations (>95% depletion). The platelet supernatants were added to the thrombocytopenic blood and incubated with or without Escherichia coli (10^7/mL) for four hours at 37°C. The plasma supernatant from the whole blood incubations was analyzed for a large set of biomarkers.

Results: Complement activation increased in response to E. coli-incubation in platelet-depleted blood; the terminal sC5b-9 complement complex increased from 8.2 +/- 2.4 CAU/mL (mean +/- SD) to 67.6 +/- 16.9 CAU/mL. Adding platelet supernatants to platelet-depleted blood increased sC5b-9 significantly ($p<0.05$) in blood without E. coli (13.9 +/- 5.1 CAU/mL), but not in blood with E. coli (66.0 +/- 14.4 CAU/mL). The pro-inflammatory cytokines IL-1β, IL-6, IL-8, and TNF were not detected in platelet-depleted blood without E. coli but highly elevated in response to E. coli. The addition of platelet supernatants modulated the inflammatory response for all these cytokines ($p<0.01$); IL-1β, IL-6, and TNF decreased significantly ($p<0.01$) by 1.7, 2.0, and 2.0 times respectively, and IL-8 increased significantly ($p<0.01$) by 1.7-fold. The platelet concentrates storage time had only a marginal effect on the inflammatory response, and all data reported here refers to storage for one day.

Conclusion: Platelet transfusions to thrombocytopenic patients may modulate the inflammatory response, both regarding complement activation and cytokine release. Our data indicate that the inflammatory response varies depending on the degree of underlying inflammation.

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Determinants resulting in high residual C5 activity in presence of Eculizumab or Coversin

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Therapeutic C5 inhibition has revolutionised the treatment of several diseases with a primary or secondary pathophysiological involvement of the complement cascade. Despite a very good clinical response, continuous residual C5 activity at a low level has been described clinically and mechanistically. Importantly during complement amplifying conditions like infections or surgery this residual, lytic C5 activity can exacerbate and lead to critical haemolytic conditions of patients who suffer from paroxysmal nocturnal haemoglobinuria (PNH) and are treated with therapeutic C5 inhibitors. To uncover mechanistic determinants that lead to the exacerbation of residual lytic C5 activity during complement amplifying conditions in presence of C5 inhibitors, we have performed several in vitro assays.

Screening the residual haemolytic activity of serum from healthy donors revealed that C5 inhibition by Eculizumab or Coversin exhibited different levels of residual C5 activity. One donor showed very untypical high residual haemolytic activity. To investigate why serum from this donor shows higher complement mediated haemolytic activity upon C5 inhibition, a novel surface plasmon resonance (SPR) based assay using plasma from individual subjects was established to be able to conveniently detect differences in the C3b deposition rate of different donor sera. This novel SPR assay can indeed detect increased activity of convertases by the mean of determining the C3b deposition rate of different plasma or sera. As hypothesised, the donor serum that exhibited higher than usual residual lytic C5 activity, when C5 was inhibited, did show a higher than usual C3b deposition rate in our novel SPR assay.

In conclusion, increased activity of convertases as measured by our novel SPR based assay correlates with higher than usual residual, lytic C5 activity when C5 is inhibited in vitro. With this new SPR-based assay a convenient tool was established that can directly measure the C3b deposition rate in serum or plasma specimen of donors or patients.

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Complement C3 deficiency enhances the effector function of CD8+ T cells and inhibits tumour growth

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Introduction: The complement system is important in modulating adaptive immunity, maintaining cellular homeostasis and protecting from infection. Additionally, it also promotes tumour growth in several cancers. The anti-tumour activity of CD8+ T cells

is very well established. However, how the complement system modulates the effector function of CD8+ T cells in anti-tumour immunity is unclear.

Methods: Mouse melanoma cells B16F10 were subcutaneously injected in C57BL/6 wild-type, complement C3-/-, C5aR1-/-, or factor B-/- mice, and tumour growth was monitored. The immune cells in the tumour and secondary lymphoid tissues were analyzed using flow cytometry.

Results: Under homeostatic conditions, CD4+ and CD8+ T cells in the inguinal lymph nodes of C3-/- mice showed reduced secretion of IFN-γ and increased production of IL-10 compared to wild-type mice. In C3-/- mice, subcutaneously implanted melanoma tumours show significantly decreased growth compared to wild-type mice. Further, antagonising the C3aR or C5aR in wild-type mice or deficiency of C5aR1 in mice showed a considerably reduced tumour growth compared to wild-type mice. Factor B-/- mice showed no significant change in tumour growth. Cellular analysis revealed increased intratumoral effector T-bet+CD8+ T cells and produced significantly high TNF-a, IFN-g and IL-10 cytokines in C3-/- mice compared to wild-type mice. High-dimensional spectral flow cytometric analysis showed increased intratumoral CD8+PD-1lowT-bet+GITR+Granzyme B+ cells in C3-/- mice compared to wild-type mice. Depletion of CD8 T cells with anti-CD8 antibody in C3-/- promoted tumour growth.

Conclusions: We show that the absence of complement C3 or antagonising its receptor enhances the effector function of intratumoral CD8+ T cells and inhibits tumour growth. Our results suggest that targeting C3 holds great therapeutic potential and may help in designing a better strategy to control tumor growth.

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Platelets balance revascularization by anaphylatoxin C5a receptor-dependent release of paracrine factors

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In ischemic tissue, platelets can modulate angiogenesis. The specific factors influencing this function, however, are poorly understood. Here, we characterized the complement anaphylatoxin C5a-mediated activation of C5a receptor 1 (C5aR1) expressed on platelets as a potent regulator of ischemia-driven revascularization and assessed its role if diseases featuring tissue ischemia in patients.

We have employed genetic mouse models to substantiate this. Indeed, the presence of C5aR1-expressing platelets was increased in the hindlimb ischemia model, a mouse model of ischemia-induced revascularization. Ischemia-driven angiogenesis was significantly improved in C5aR1-/- mice, but not in C5-/- mice suggesting a specific role of C5aR1. Experiments using supernatant of C5a-stimulated platelets suggested a paracrine mechanism of angiogenesis inhibition by platelets by means of antiangiogenic CXC chemokine ligand 4 (CXCL4, PF4). Lineage-specific C5aR1 deletion verified that the secretion of CXCL4 depends on C5aR1 ligation on platelets. WE characterized this secretion ot be a specific sub-alpha-granule specific event featuring a specific signaling cascade. Finally, we applied the C5aR1 inhibitor PMX-205 in vivo in the hindlimb ischemia model. This drug induced a phenotype of improved revascularization in mice.

Furthermore, we assessed the relevance of the anaphylatoxin receptor C5aR1 on platelets in coronary artery disease and found a correlation between specific but not all markers of platelet activation and C5aR1 expression on platelets. Furthermore, we have assessed the role of C5aR1 in peripheral artery disease (PAD) patients. We have matched a cohort with proven high-grade arterial stenoses of the leg or groin arteries but no ischemic walking pain with patients with PAD and typical symptoms. We hypothesized that lack of symptoms might be due to a better capacity for collateral artery growth in asymptomatic PAD patients. Strikingly, the C5aR1 expression on platelets and CXCL4 correlated with pain-free walking distance. Thus, the C5aR1-CXCL4 axis seems to play a role in adaption to diseases featuring tissue ischemia also in cardiovascular patients.

We identified a novel mechanism for inhibition of neovascularization via platelet C5aR1, which was mediated by release of antiangiogenic CXCL4. Importantly, we can present evidence for the significance of this mechanism in cardiovascular patients suffering from diseases featuring tissue ischemia.

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Association of a new variant of complement regulator FHR2 with C3 glomerulopathy

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C3 glomerulopathy (C3G) is caused by a dysregulation of the complement system leading to C3 deposition and formation of glomerular deposits. Several C3G patients harbor mutations or copy number variations in the human Factor H (FH) and/or Factor H-Related (FHRs) genes. Therefore, FH and FHRs are emerging immune targets for inhibition of the complement cascade, as well as markers to monitor patients on complement regulatory drugs to test their efficiency. Here, we focused our study on FHR2, known to inhibit in vitro formation of the terminal complement complex. We identified new variants for the FHR2 gene in a cohort of C3G patients and performed detailed functional studies on the novel variant FHR2L46, which has the Pro at position 46 replaced by Leu. Patients with FHR2L46 variant presented increased FHR2 plasma level, as compared to controls and displayed FHR2 deposits in glomeruli. We generated a recombinant FHR2L46mutant protein to gain insight into the effect of this novel FHR2 variant on complement regulation. As the amino acid exchange occurred in the first short consensus repeat (SCR1), we first tested if the Leu at position 46 altered FHR2 homodimerization and heterodimerization of FHR2 with FHR1 and FHR5. We observed that FHR2L46 binds significantly less to FHR2 and FHR1 but more to FHR5. Furthermore, FHR246L acquired the capacity to bind to cell surfaces by interacting with glycosaminoglycans heparin and malondialdehyde (MDA)-modified amino group (MAA) epitopes. FHR2L46 also bound substantially more to necrotic cells compared to wild-type FHR2 (FHR2WT). In contrast, no difference was observed between FHR246L and FHR2 WT binding to C3 and C5. Taken together, the

present study identified a novel FHR246L variant in a C3G patient and suggests that the FHR2L46 mutant forms stable oligomers with FHR5 and enhances complement activation.

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An ecotin ortholog turns out to be a potent classical pathway inhibitor and might provide information on the relative efficacy of C1r versus C1s inhibition

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Ecotin was first isolated from the periplasm of E. coli 40 years ago and was shown to inhibit all types of pancreatic serine proteases, i.e. trypsin, chymotrypsin and elastase. The role of ecotin was believed to protect the microbe in the gut from these host enzymes. Later ecotin was shown to inhibit leukocyte elastase as well suggesting some immune evading capacities. Recently, we have discovered that most ecotin orthologs from pathogen species potently inhibit MASP-3 and MASP-2, and through the latter activity, block lectin pathway activation. We also showed that most ecotin orthologs are considerably good alternative pathway inhibitors as well. However, it was also apparent that classical pathway inhibiting ecotin orthologs are either scarce or might not even exist. This suggested that the major targets of ecotin orthologs are those antimicrobial host systems that do not depend on the adaptive immune system and can unleash an immediate attack. Nevertheless, we have very recently identified the first ecotin ortholog that is a potent classical pathway inhibitor. Our preliminary results might shed some light on the relative efficacy of C1r versus C1s-inhibition in blocking classical pathway activation.

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Roles of complement C3 in non-alcoholic fatty liver disease

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Background: Non-alcoholic fatty liver disease (NAFLD) is a growing threat to global public health, affecting approximately 30% of adults and becoming the leading reason for liver transplantation. Nevertheless, no approved specific treatment is currently available for NAFLD. Accumulating evidence suggests an important role of the complement system in causation of NAFLD. As the axial component of the complement system, C3 is predominantly produced in the liver, the metabolic 'engine' of the body; however, its role in metabolism remains little known.

Methods: In this study, we have carried out a comprehensive characterisation of C3 spatial expression in normal and NAFLD human liver using single cell and bulk RNA sequencing data. These findings were validated in free fatty acid-induced steatotic human hepatocytes and a high fat diet-induced mouse NAFLD model.

Results: We found that C3 was expressed in a zonal manner, with abundant expression in both periportal and pericentral hepatocytes. Cellular deconvolution of NAFLD liver showed a disruption of hepatic zonal structure, with a depletion of pericentral hepato-

cytes while periportal hepatocytes were expanded. This alteration of hepatic zonal distribution was disease severity dependent with involvement of liver progenitor activation. RNA sequencing data from 408 liver biopsies confirmed significant alterations of complement C3 expression in response to NAFLD development. C3 was upregulated in all forms of NAFLD and positively correlated with liver fat load in patients with steatohepatitis. C3 expression was increased in steatotic human hepatocytes and hepatic C3 expression was higher in a mouse NAFLD model. C3 knockout protected against high fat diet induced hepatic lipid droplet accumulation. In the hepatic response to a high fat diet, C3 knockout repressed hepatic de novo lipogenesis and triglyceride synthesis through downregulating liver lipogenic regulators.

Conclusion: We demonstrate the impact of lipid accumulation on C3 expression in hepatocytes and show that C3 knockout impacts hepatic lipid handling. Overall, our study provides new insights into the role of C3 in NAFLD pathogenesis and suggests its potential as a therapeutic target for the treatment of NAFLD.

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Therapy induced senescence in cancer cell lines affects complement activation and complement regulatory proteins expression

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Background: Cancer therapy can result in the formation of viable, metabolically active, yet growth arrested cancer cells, in a phenomenon known as therapy induced senescence (TIS). Those senescent cells are thought to affect the cancer's inflammatory microenvironment. The interaction of complement and senescent cells is not well understood but could yield valuable insights into complement's role in cancer development and treatment response.

Methods: As a model for senescence in cancer, four cancer cell lines (A549, MCF7, PANC-1, and HEPG2) were treated with sublethal doses of the chemotherapeutic agents etoposide and doxorubicin. Following validation of TIS by several senescence markers, complement activation as well as C1q and MBL binding were investigated in senescent and control cells, and the expression of complement regulatory proteins (CRP) and C3 was measured using qPCR, while double staining of C3 and the senescence marker p21Cip1 was used to verify C3 levels in A549 senescent cells.

Results: The percent of cancer cells that displayed a senescent phenotype following treatment varied in the tested cell lines. Activation of complement as evident by the formation of C5b-9 was found only in A549 and MCF7, but no significant increase in C1q or MBL binding was found in any of the tested cell lines. CRP expression following TIS was generally increased, with increased Factor H (FH) in all the tested cell lines. In MCF7 cells, the expression of CD46, CD55, CD59, FH, and C4BP was increased by over 10 folds. Additionally, expression of C3 in A549 cells was increased by around 19-fold as observed by qPCR. The increase in C3 levels following TIS was confirmed by immunofluorescence microscopy and was associated with an increase in the senescence marker p21Cip1.

Conclusion: Various changes in complement expression and activation following TIS were observed and indicated activation of complement and a general increase in CRP in the some of the tested cell lines, which is in line with the inflammatory phenotype of senescent cancer cells. Further studies in each cancer type are required to investigate whether TIS is associated with unique changes in complement expression and activation.

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CD59-/- is protected from glucose intolerance, insulin resistance and fatty liver disease

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Objectives: CD59 is known as a membrane regulator of the complement system and inhibits the final step of membrane attack complex (MAC) formation to protect host cells from MAC-mediated injury. Noncanonical function was suggested for CD59 in relation to insulin secretion. Here we investigate its possible noncanonical function in developing insulin resistance and non-alcoholic fatty liver disease (NAFLD).

Methods: The study population was comprised of male C57BL/6 mice, aged 2 months, divided into 4 groups: CD59+/+ high fat diet (HFD), CD59-/- HFD, CD59+/+ standard chow diet (SD) and CD59-/- SD. Mice had unrestricted access for 16 weeks to a pelleted HFD [45%kcal Fat Diet (21% MF, 2% SBO)] or a SD. Intraperitoneal glucose tolerance test (IPGTT), insulin tolerance test (ITT), pyruvate assays, and insulin, glucagon, and blood chemistry measurements were performed every 4 weeks during the HFD. Western blots of the insulin signaling pathway were performed. PET-MRI was performed. Tissues of all mice were extracted at the end of the experiment for further investigations.

Results: CD59-/- HFD mice gained significantly less weight than CD59+/+ HFD mice. In metabolic cages, we found no significant differences in food intake, activity, or spirometry between the 4 groups, suggesting that weight gain did not result from these factors. IPGTT, ITT, and Western Blot of the insulin signaling pathway showed that absence of CD59 improved glucose intolerance and significantly reduced insulin resistance. Pyruvate assay indicated that CD59+/+ HFD mice had augmented gluconeogenesis compared to CD59-/- HFD mice. Moreover, the HFD resulted in fatty liver in the CD59+/+ HFD group but not the CD59-/- HFD group. In CD59 +/+ HFD mice we observed weight increase, lipid droplets in the liver, elevated cholesterol, lipids and SGPT in blood serum, and more infiltration of macrophages in liver and muscle tissues.

Conclusion: A novel unpredicted tole for CD59 was found in insulin resistance, glucose intolerance and NAFLD.

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SYNERGY-1: A Phase 1, first-in-human, randomized, double-blind, placebo-controlled safety, tolerability, immunogenicity, PK and PD study of KP104 in escalating single and multiple doses

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Background: Aberrant complement activation is linked to various human diseases. Currently approved and in-pipeline anti-complement drugs are all single pathway inhibitors that may not completely address disease pathology whereby multiple complement pathways are implicated. KP104 is a bifunctional complement inhibitor composed of an anti-C5 mAb fused with the N-terminal SCR1-5 of factor H. It inhibits both the terminal pathway (TP) and alternative pathway (AP) complement activation.

Methods: SYNERGY-1 is a first-in-human, randomized, double-blind, placebo-controlled, single center study of KP104 in healthy volunteers. Safety, tolerability, anti–drug antibodies (ADA) development, PK, and PD were assessed in single ascending dose (SAD) and multiple ascending dose (MAD) cohorts. A total of 66 healthy subjects (50 for SAD, and 16 for MAD) completed the study. SAD dose levels were 60-1200 mg; MAD was 600mg IV QW for 5 weeks or 720 mg SC QW after 1200 mg IV loading for 5 weeks.

Results: Majority of subjects were Caucasian (81.3% SAD and 62.5% MAD), male (26 SAD and 6 MAD), and mean age was 30.5 and 33.6 years for SAD and MAD, respectively.

No deaths, dose limiting toxicities, or severe TEAEs were reported. SAD (n=38/48, 79.2%) reported 126 TEAEs in all cohorts. No dose related trend was observed. MAD (n=8/8, 100%) reported 24 TEAEs. Majority of TEAEs were Grade 1 and resolved without treatment.

In SAD cohorts, all 3 pharmacodynamics (PD) markers, rabbit RBC hemolysis (AP + TP), C3b (AP), and free C5 (TP), showed dose-dependent inhibition. Maximum hemolysis, C3b, and free C5 inhibition reached >85%, >90%, and >99.5%, respectively, at $\geq$360 mg and inhibition duration were dose dependent. At 1200 mg, all 3 PD markers achieved $\geq$99% inhibition. In the MAD cohort, all PD markers achieved sustained inhibition during entire dosing period.

ADAs were detected in 3 SAD and 4 MAD but did not appear to impact subject safety.

Conclusion: KP104 was safe, well tolerated, and showed proof of mechanism with potent TP and AP inhibition in the SYNERGY-1 study. The data supports further clinical studies of KP104 in complement-mediated diseases.

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Complement Activation in Cytokine Storms Associated with COVID-19, Sepsis, and Chimeric Antigen Receptor (CAR)-T Therapy

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Background: Cytokine storms, an exaggerated immune response, are associated with a variety of infectious and non-infectious diseases. Recent reports have suggested cellular and molecular mechanisms contributing to cytokine storms may be present in viral disease, including COVID-19, where some publications have indicated that they play an important part in medical complications. Here we sought to characterize complement activation in patients with cytokine storm associated with COVID-19, sepsis, and CAR-T therapy.

Methods: Patients were included following a clinical definition of cytokine storm associated with COVID-19, CAR T cell therapy, or mild sepsis. Consent was obtained (IRB HMO-456-20). C4a, Bb, C3a, C4d, C5a, and SC5b-9 complement factors were measured by sandwich ELISA kits (Quidel). All reagents were provided with the kits and prepared per manufacturers' protocols. Assays were performed in 96-well plates per the protocols provided. OD plate reading was performed with the Infinite F50 (Tecan) and analyzed using Magellan software (Tecan). Raw data was measured as 450nm optical density (OD). Concentration was calculated using a linear standard curve generated from 6–7 standards.

Results: 122 patients participated in this study100 patients with Covid 19, 12 with CAR T cell therapy, and 10 with mild sepsis.. Complement activation was more prominent in COVID-19 patients than in sepsis or CRS patients, with clear correlation between terminal C5b-9 complex and COVID-19 severity. There was mild elevation of C5a and C4d in patients with mild sepsis (average SOFA score 3.4), and mild C5a and moderate C4d in patients with CRS due to CAR-T therapy. However, in COVID-19 patients there was marked elevation in C4a, Bb, C3a, C4d, C5a, and SC5b-9. SC5b-9 mostly correlated with COVID-19 severity.

Conclusion: Complement activation in COVID-19 was clearly seen. COVID-19 severity clearly correlated with SC5b-9 level. Mild sepsis and CAR T cytokine storm had milder activation of the complement cascade.

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Beta cell-derived C3 is protective in a mouse model of diet-induced obesity and diabetes

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Type 2 diabetes (T2D) and obesity are associated with increased inflammatory reactions. C3, is a central player of the complement system, and contributes to inflammatory processes as observed in T2D and obesity. Serum C3 levels correlate with body fat mass, BMI, and insulin resistance. The cleavage product of C3, C3a has

also been linked to inflammation and subsequent insulin resistance in adipose tissue.

Recently we discovered that C3 is highly expressed in human pancreatic islets, and upregulated in human diabetic islet donors as well as in islets of diabetic animal models. We showed that cytosolic C3 contributes to cytoprotective autophagy in pancreatic beta-cells and protects them against IL-1β-induced cell death. C3 therefore seems to play a dual role in pancreatic islets: a cyto-protective intracellular role in beta cells, but a proinflammatory role in the extracellular environment.

To understand this dual role of C3 in islets, we investigated a global knock out (KO) of C3 on a high fat diet. After six months on high fat feeding , there was no difference in weight gain, glucose homeostasis or tissue inflammation between total C3 knockout and WT mice. This was most likely due to the contrasting effects of serum C3 and beta cell-derived C3. Therefore, we created a beta cell-specific C3KO mouse (C3 flox/RIPCre) to assess the physiological role of beta cell-derived C3 under diabetogenic conditions. Interestingly, beta cell-specific C3KO mice displayed an increased weight gain, adipose tissue inflammation and impaired glucose homeostasis when fed a high fat diet. This confirms a protective role for beta cell-derived C3.

The study suggests that a better understanding of the mechanisms controlling and alleviating beta-cell stress could lead to the development of novel strategies to extend beta-cell survival and function.

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Specificity vs. versatility: Exploring the selectivity profile of the leech inhibitor gigastasin for serine proteases of the complement system and other pathways

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Trypsin-like serine proteases are the driving factors behind host defense cascades such as the complement and coagulation system. Interestingly, the enzymatic domain of this family is structurally well-preserved, yet substrates or regulators can exert very narrow or broad specificities. Understanding how structurally similar but functionally distinct proteases can be targeted for inhibition is of great medical importance for the development of innovative therapeutics. To investigate such specificity principles, we explored the target binding modes of gigastasin, a small protein extracted from the saliva of the giant Amazon leech that inhibits both C1s and MASP-2 and therefore blocks the classical and lectin pathway.

In this study, we used in silico analyses and simulations to identify selectivity determinants and validated some of the observations and predictions using experimental substrate cleavage assays. Starting from a published crystal structure of gigastasin in complex with C1s, we established a series of predicted binding complexes of gigastasin with other serine proteases by structurally aligning them to C1s and optimizing the binding surface using rotamer optimization algorithms. Subsequently, molecular dynamics (MD) simulations were employed to characterize the quality and stability of the gigastasin-protease interactions.

Using this 'protease fingerprinting', we could predict potential targets for gigastasin while excluding others. For example, MD simulations revealed that the N-terminus of the inhibitor readily detaches from the predicted binding surface between plasma kallikrein and gigastasin. Indeed, we did not observe any inhibition of this protease in vitro. In the case of FXa, in-depth analysis of the interaction fingerprint with gigastasin identified several key contacts, especially in the C-terminus, that did not optimally form when compared to C1s, which explains the notable yet weak inhibitory activity for FXa observed in the cleavage assay. In addition to target-specific interactions, we identified contact points that may confer specificity between protease subfamilies, e.g., between the complement and coagulation cascades.

The insight provided structural hypotheses about the target selectivity profile of gigastasin and suggest that the inhibitor's activity, selectivity and specificity can be tuned by mutagenesis. The combination of computational methods and experimental validation thereby improved our understanding of the ligand-binding profiles of complement-associated serine proteases.

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Single cell analysis (SCA) of PBMCs for a patient with congenital non-functional CD59

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Background: Homozygous CD59-deficient patients manifest with recurrent peripheral neuropathy resembling Guillain-Barré syndrome (GBS), hemolytic anemia and recurrent strokes. Variable mutations in CD59, leading to loss of function, have been described in 18 patients. Here we investigated single cell RNA sequencing of PBMCs from a patient deficient in CD59.

Methods: Single Cell RNA Sequencing. PBMCs from a patient with CD59 homozygous mutation and an age- and gender-matched control were diluted with FACS buffer to a concentration of 0.6–0.7×106 cells/ml and viability above 90% was confirmed. RNA from the barcoded cells from each sample was subsequently reverse-transcribed and sequencing libraries were constructed with reagents from a chromium single cell 3′ v2 reagent kit (10× Genomics) according to the manufacturer's instructions. Sequencing was performed with the Illumina NextSeq 500 high-output kit (Illumina, San Diego, CA, USA) for 75 cycles with 26bp read from read1 and 58bp from read2. Single-Cell RNA-Seq Analysis. Single-cell RNA-seq analyses were performed used Seurat 4.3.0. Uniform Manifold Approximation and Projection (UMAP) was then performed. Clusters were determined using the shared nearest neighbor (SNN) modularity optimization-based clustering algorithm. Differentially expressed markers were identified.

Results: PBMCs from the patient with homozygous CD59 deficiency showed marked depletion of NK cells (36% of normal) and marked elevation in CD4:CD8 ratio; 6.14 compared to 3.75 in normal control. Tregs were twice in number in the patient and both CD4 and CD8 cells expressed differentially gene clusters. B cells were a little bit elevated in the patient, but naïve B cells were elevated 2.5 folds. In one cluster SNHG5, RPS28, ARL4C, HLA-DPB1, AES, ABRACL, CSTB, BAX, CAP1, were elevated along with others that were differentially negatively expressed. Monocytes were relatively depleted in the patient. These gene changes fitted to known patterns in antigen presentation, TH17 signaling pathway, type I

diabetes and other autoimmunity, GvHD, and some viral and bacterial infections.

Conclusion: SCA reveals expected and unexpected changes in PBMC subpopulations from membrane CD59 deficient patient. These finding may elucidate unknown functions of both canonic and non-canonic CD59 functions.

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A high dimensional analysis of the injured brain unravels the contribution of complement C3 to the immune landscape and the neurologic outcomes following traumatic brain injury

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Background: Traumatic brain injury (TBI) gives rise to a secondary neuroinflammation that can last years after injury and is associated with the development of progressive cognitive impairment and chronic disabilities. We and others have shown that activation of an immune response, including activation of complement, plays a central role in this process by mediating the aberrant phagocytosis of perilesional neurons and synapses. However, since the immune system can promote both brain repair and brain damage, identifying the cellular components and their temporal distribution would contribute to our understanding of the neuroinflammatory process and help identify TBI treatment strategies.

Methods: Using a controlled cortical impact TBI model, we leveraged mass cytometry to evaluate how complement and complement inhibition affects the immune landscape after TBI. We designed a 35-antibody panel to identify resident and peripheral immune cells and their functional receptors involved in phagocytosis, chemotaxis, and complement signaling at various time points after TBI. We used the injury-site targeted complement inhibitor, CR2-Crry, consisting of the complement inhibitor (Crry) linked to a fragment of complement receptor 2 (CR2), which increases complement inhibition bioavailability and efficacy without exerting systemic immunosuppressive effects.

Results: We found an increase in the percentage of infiltrating peripheral immune cells at acute time points and identified distinct microglia sub-populations, among which one subpopulation expressing complement receptor 4 (CR4) showed progressive increase through at least 28 days after injury. CR2-Crry treatment after TBI improved cognitive functions, impacted the expression of functional receptors on infiltrating cells, and reduced the abundance of brain resident immune cells. Conversely, inhibition of C5aR effectively reduced the infiltration of immune cells, but did not improve histological or behavioral outcomes, which suggests the neuroprotective effect of CR2-Crry is not mediated via inhibition of C5a generation.

Conclusions: CR2-Crry improved post-TBI outcomes and altered immune cell infiltration, as well as complement receptor and phagocytic receptor expression. Together with data from previous studies and the current C5aR inhibition data, the results indicate that the neuroprotective effect of CR2-Crry is likely via modulating C3 opsonization and complement receptor expression.

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Nomacopan as an anti-shock drug for pre-hospital treatment of traumatic hemorrhage through switching the injury phenotype to survival phenotype

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Background: Traumatic hemorrhage (TH) is the leading cause of potentially preventable deaths that occur during the prehospital phase of care. No effective pharmacological therapeutics are available for critical TH patients yet. Here, we identify terminal complement activation (TCA) as a therapeutic target in combat casualties and evaluate the efficacy of TCA inhibitor (nomacopan) on organ damage and survival in vivo.

Methods: Complement activation products and cytokines were analyzed in plasma from 54 combat casualties. The correlations between activated complement pathway(s) and the clinical outcomes in trauma patients were assessed. Nomacopan was administrated to rats subjected to lethal TH (blast injury and hemorrhagic shock). Effects of nomacopan on TH were determined using survival rate, organ damage, physiologic parameters, and laboratory profiles.

Results: Early TCA was found to be associated with systemic inflammatory responses and clinical outcomes in this trauma cohort. Lethal TH in the untreated rats induced early TCA that correlated with the severity of tissue damage and mortality. The addition of nomacopan to a damage control resuscitation (DCR) protocol significantly inhibited TCA, decreased local and systemic inflammatory responses, improved hemodynamics and metabolism, attenuated tissue and organ damage, and increased survival.

Conclusion and Implications: Previous findings of our and other groups revealed that early TCA represents a rational therapeutic target for trauma patients. Nomacopan as a pro-survival and organ-protective drug, could emerge as a promising immunological DCR that may significantly reduce the morbidity and mortality in severe TH patients while awaiting transport to critical care facilities.

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The multi-faceted role of complement C3aR in the development and treatment of colorectal cancer

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Background: Inflammation plays a pivotal role in the development and progression of colorectal cancer (CRC). Emerging evidence suggests that complement anaphylatoxin C3a produced upon complement activation and acting via its receptor (C3aR) may play a role in intestinal homeostasis. However, its role in CRC is currently unknown.

Methods: We used a comprehensive approach encompassing analysis of publicly available human CRC datasets, inflammation-driven and newly generated spontaneous mouse models of CRC, and multi-platform high dimensional analysis of immune responses using microbiota sequencing, RNASeq, and mass cytometry.

Results: By mining publicly available datasets, we found that CpG island methylation of c3ar1 occurs in CRC patients and is associated with significant downregulation of C3aR. By reverse-translating this finding, we were able to shift in $APC^{Min/+}$ mice the tumorigenesis from the small intestine to the colon, therefore, generating a novel mouse model which more closely mirrors CRC in humans. RNAseq analysis on the polyps from our newly developed mouse model ($APC^{Min/+}$/C3aR-/-) revealed a significant increase in immune signatures. Interestingly, loss of C3aR significantly impacted the fecal and tumor-associated microbiota, which promoted enhanced immune infiltration in typically "cold" tumors. In line with our findings in the mouse model, human CRC with C3aR downregulation showed increased innate and adaptive immune cells. Since immune infiltration is often a favorable prognostic factor in CRC and predisposes to response to immune checkpoint blockade (ICB) therapy, we assessed whether the enhanced immune infiltrate could be exploited to treat the tumors of $APC^{Min/+}$/C3aR-/- mice. We found that using anti-PD1 in $APC^{Min/+}$/C3aR-/- but not $APC^{Min/+}$ mice resulted in significant tumor reduction. Therefore, the lack of C3a in the colon activates a microbiota-mediated pro-inflammatory program, promoting tumors' development with an immune signature that renders them responsive to ICB therapy.

Conclusions: The complement system in the gastrointestinal tract is essential to avoid overt inflammation in health. However, this regulatory mechanism may restrain the activation of immune responses during tumor development. Our findings reveal that C3aR may act as a previously unrecognized checkpoint to enhance anti-tumor immunity in CRC. C3aR can thus be exploited to overcome ICB resistance in a larger group of CRC patients.

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Effects of combined complement complement and CD14 inhibition on Escherichia coli-induced thromboinflammation in human whole blood in the presence and absence of antibiotics

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Background: Sepsis is a serious condition causing inflammation and organ damage and the incidence is increasing. A common complication of sepsis is coagulation disorders, such as disseminated intravascular coagulation (DIC). This study examined the effects of selective and combined inhibition of complement C3 and the TLR coreceptor CD14 on Escherichia coli (E. coli)-induced thromboinflammation in human whole blood in the presence and absence of antibiotics.

Methods: Fresh venous human whole blood was obtained from six healthy blood donors using lepirudin (50 μg/mL) as anticoagulant. Live E.coli bacteria (10^7/mL) was added to the blood in the presence or absence of the antibiotics Cefotaxime or Tigecycline (both 2,5 mg/L) and incubated 120 min at 37°C. The effects of a complement inhibitor and a CD14 inhibitor , alone and in combination were examined. A control peptide and isotype control served as negative controls. Bacterial growth was determined by colony-forming units (CFU). The terminal complement complex (TCC), tissue factor (TF) on microparticles (MP-TF), prothrombin fragment 1+2 (PTF1+2), and β-thromboglobulin (BTG) in plasma were analyzed by ELISA. Cytokines in plasma (27 different) were analyzed using multiplex technology. The surface expression of TF on whole blood monocytes was analyzed by flow cytometry.

Results: E.coli significantly increased plasma TCC, PTF 1+2 and TF surface expression on monocytes, and 24 of the 27 cytokines ($P<0.05$) in plasma after 120 min. Selective inhibition of complement and combined complement and CD14 inhibition showed similar results in the absence and presence of antibiotics. TCC was significantly reduced ($P<0.05$) by complement inhibition. Inhibition of complement and CD14 significantly reduced the release of interleukin (IL)-6 and IL-8, while no effects on BTG release was observed. Interestingly, combined inhibition of complement and CD14 significantly reduced the E. coli-induced PTF 1+2, MP-TF, and TF surface expression on monocytes (all $P<0.05$).

Conclusion: Combined inhibition of complement and CD14, both in the presence or absence of antibiotics, appears to be a promising adjuvant treatment for sepsis-induced thromboinflammation.

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Interaction of factor H and factor H-related proteins with S and N proteins of SARS-CoV-2

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COVID-19-related morbidity and mortality is largely due to overactivation of the immune system, including innate immunity. Multiple studies reported the activation of the complement system in COVID-19. Particularly, the SARS-CoV-2 nucleocapsid (N) protein was shown to activate the lectin pathway, but the alternative pathway is also activated in COVID-19, and altered serum levels of factor H (FH) family proteins were reported in comparison with healthy controls. Since FH is an important soluble regulator of the alternative pathway, which inhibits the activation of the complement system at the level of the central C3 molecule, our goal was to investigate whether FH and the factor H-related (FHR) proteins interact with the N and spike (S) proteins of SARS-CoV-2 and influence complement activation.

To this end, FH was purified from serum, and FHRs as well as mini-FH were produced as recombinant proteins in insect cells. Wild-type recombinant S and N proteins of the SARS-CoV-2 virus were obtained from commercial source. Binding and complement activation assays were performed by ELISA, cofactor activity was analyzed by Western blot.

In ELISA, FH and FHR-1 bound to immobilized N protein, but not to the S protein. FHR-5 showed weaker binding to the virus proteins in these experiments. Using blocking antibody, we found that the C-terminal domains of FH and FHR-1 were responsible for binding to the N protein; this was corroborated by the binding of mini-FH. FH and mini-FH when bound to N-protein retained their cofactor activity for C3b-cleavage by factor I. When exposed to human serum, exogenously added FHR-1 and FHR-5 enhanced C3 deposition on S protein and on N protein in a dose-dependent manner; on N protein, C3 deposition was also slightly enhanced by FHR-4. However, activation of the alternative and terminal pathways, measured by factor B and C5 deposition, respectively, was only induced by FHR-5.

Altogether, these results demonstrate interaction of SARS-CoV-2 S and N proteins with several FH family proteins, including the complement inhibitor FH and complement activator FHRs. Further investigation is required to understand in more detail their complex interaction with SARS-CoV-2 and their role in the virus-induced complement activation.

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Expression and Function of Complement Components in Head and Neck Cancer: Analysis Across Various Model Systems

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Background: Head and neck squamous cell carcinoma (HNSCC) is a complex and heterogeneous malignancy with an intricate relationship to the complement system. Recent evidence suggests a crucial role for the complement system in the pathogenesis of HNSCC. However, the expression and function of complement components in HNSCC remain to be fully elucidated.

Methods: We analyzed the gene expression and protein secretion of complement components in normal human oral keratinocytes (HOKs), 2D cell cultures, spheroids, chorioallantoic membrane (CAM) models, and primary tumor samples using Luminex® multiplex-ELISA and quantitative PCR (qPCR). Additionally, we investigated the effects of modulating CD46 and inhibiting CFD using Danicopan on tumor cell migration and invasion.

Results: HOKs exhibited high gene expression of C3 and notable expression of CFB, CFD, C5, CFI, and C3AR. We observed consistently high protein expression and secretion of C3, as well as increased expression levels of complement regulatory components of the alternative pathway, particularly CFD, FH, and FI, in spheroids and patient samples. Modulation of CD46 impacted the migration behavior of tumor cells, and inhibition of CFD using Danicopan resulted in a significant reduction in spheroid invasion, as well as a concentration-dependent increase in apoptosis.

Conclusion: Our findings indicate that, depending on the model system, HNSCC tumor cells predominantly express specific regulatory complement components. This study serves as a preliminary investigation into the cellular effects of these components, with future research aiming to extend these insights to more complex cellular environments. Overall, our work highlights the potential role of the complement system in HNSCC pathogenesis and provides a foundation for further exploration of targeted therapeutic strategies.

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Effect of IL-1ß and C3a on complement expression and secretion on human osteoarthritic chondrocytes

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Background: Chronic, low-grade inflammation and complement activation play a crucial role in the pathogenesis of osteoarthritis (OA), a degenerative joint disease. The alternative

complement pathway seems to have an impact already in early stages of OA, with high levels of C3 and activation products being detected in joint tissues, associated with both OA pain and disease progression. The underlying molecular mechanisms are mostly unknown yet. Therefore, this study aims to investigate the effect of inflammation and complement activation in OA by stimulating chondrocytes with either IL-1ß or C3a.

Methods: Chondrocytes were isolated from human OA tissue explants and stimulated with either IL-1ß (0.1 – 10 ng/ml), or C3a (250 – 1000 ng/ml). RNA expression of complement and chondrocyte markers was analyzed using quantitative PCR (qPCR). Complement Protein expression and secretion was analyzed using immunofluorescence and Luminex® Mulitplex-ELISA.

Results: Human OA-chondrocytes showed a concentration-dependent decrease in mRNA expression of chondrocyte markers COMP, SOX9, COL1A1, and COL2A1 when stimulated with IL-1ß, whereas time and concentration affected chondrocyte marker transcripts when stimulated with C3a. Elevated C3, CFB, C3AR and C5AR1 expression and C3 and C3b secretion were detected by IL1-ß stimulation, concentration-dependently. Expression of CFH and CFI mRNA was decreased in OA-chondrocytes pre-incubated with increasing concentrations of IL-1ß, while protein secretion showed opposite results. After C3a stimulation, a concentration-dependent opposing effect on C3 transcripts was detected, while consistently reduced protein secretion levels were observed. CFB and CFD expression decreased after C3a incubation, while a time-dependent increase in CFB secretion was observed. CFH, CFI and CD46 transcripts and FH and FI secretion was significantly decreased after C3a stimulation. RNA expression of C3AR was increased, while expression of C5AR1 and C5AR2 was reduced in OA-chondrocytes after C3a stimulation.

Conclusion: These results demonstrate the complex interplay between inflammation and complement activation in OA-pathogenesis and contribute to the idea that C3a may act in both directions: pro-inflammatory for chondrocytes and anti-inflammatory in the context of OA. Local, cell-associated complement activation may play a more complex role in OA-progression, and further studies are needed to understand the molecular mechanisms underlying complement-mediated joint inflammation and destruction in OA.

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Activated C5ar1 signaling induces fibrogenic development in Mycobacterium avium (Mav)-infected mouse lungs via the proinflammatory T helper-type I cell response

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Mycobacterium avium (Mav) is one of the most successful pathogens infecting billions of people with daily exposure to water and soil. Mav belongs to the group of non-tuberculous mycobacteria (NTM) which affects the elderly and immunocompromised individuals. With the evasion and alteration of host inflammatory response, Mav persistence leads to severe pathogenicity resulting in interstitial chronic lung disease. Given that the C5a peptide is pivotal in chronic inflammatory and infectious diseases, we studied the role of C5a receptor 1 (C5ar1) signaling response in the in vivo Mav pathogenicity. We infected C5ar1-deficient mice ($C5ar1KO^{C57B6/J}$), and the wildtype littermates ($WT^{C57B6/J}$) with the aerosol exposure of a clinical strain of Mav ($5x10^8$ CFU/ml) and examined the immunopathologic changes up to 90 days post-infection (dpi). The Ashcroft scores obtained using Masson's trichrome staining revealed significant fibrogenic burden with Mav granulomas in the 30-dpi Mav-WT lung tissues that increased through 60 and 90-dpi than their C5ar1-deficient counterparts. Significantly increased inflammatory cytokines expressions including Il-1β and Tnfα were observed in the Mav- WT lungs at 60 and 90-dpi, which also corroborated with significantly increased proinflammatory helper T cell (Th1) specific marker Ifnγ, and its regulator T-Box transcription factor 21, Tbx21 expressions in the BAL fluid cells. These markers were significantly low in the Mav-C5ar1KO mice correlating with low Ifnγ levels measured by ELISA. Importantly, marked Tbx21/T-bet immunofluorescent staining indicated Th1-specific immune cell subset in the Mav-WT lungs. Finally, we confirmed the role of C5ar1-dependent Th1 response in Mav-induced fibrogenic changes using splenocytes isolated from WT and C5ar1KO mice. In the absence of C5ar1 signaling, Mav-infected splenocytes demonstrated an impaired helper T cell response with a significant decrease in the Ifnγ and Tbx21 transcript levels, and decreased $CD4^+$ /$CCR5^+$ -proinflammatory Th1 cell subsets were measured flow-cytometrically. Therefore, our data indicated a C5ar1-dependent proinflammatory Th1 response during the fibrotic tissue remodeling in Mav-infected mouse lungs.

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Crystallographic and SAXS structure of the immune evasive factor GAPDH from Leptospira interrogans and interaction with human C5a

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Background: Moonlighting proteins from pathogenic bacteria can perform immune evasive functions. D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been shown to bind human C5a when located on the surface of gram-positive bacteria like Streptococcus pyogenes. GAPDH from Atopobium vaginae and Clostridium perfringens can also interact with C5a. Interestingly, the secretome of gram-negative bacteria contains GAPDH. This raises the question of whether GAPDH from gram-negative pathogens can also bind C5a. Here, we present the structure of GAPDH from a gram-negative pathogen, Leptospira interrogans, and show that it can bind C5a.

Methods: The gene for Leptospira interrogans (Li)GAPDH was amplified from gDNA and subcloned into a bacterial expression vector. Expression and purification of LiGAPDH were performed following established procedures. The structure of LiGAPDH was determined by X-ray crystallography and the shape in solution was calculated from small-angle X-ray diffraction. A crosslinking assay with BMOE helped to demonstrate the association between LiGAPDH and human C5a. GAPDH activity was measured with standard enzymatic assays and inhibition by two natural products.

Results: We cloned, expressed, and purified LiGAPDH, and crystallized and solved its structure by X-ray crystallography to 2.3 Å resolution. The shape restored in solution from SAXS closely matches the crystal structure. LiGAPDH can bind human C5a in a weak and transient manner, which could be trapped by a highly specific crosslinking assay that requires a distance < 6-8 Å between thiol groups in free Cys residues. We have modeled the interaction between LiGAPDH and C5a with docking with crosslinking-derived distance restraints. The inferred interacting surfaces explain why GAPDH from evolutionarily distant bacteria can bind C5a, albeit with low affinity. Finally, we have characterized two natural products with antimicrobial activity as potential LiGAPDH inhibitors.

Conclusions: The structure of LiGAPDH is available for experimental and computational drug discovery. LiGAPDH can interact with human C5a in a dose-dependent manner. The interaction with C5a would occur on the bacterium's surface or the extracellular milieu, where LiGAPDH concentration and the oxidative environment could promote C5a sequestration and delay the development of the anaphylatoxin gradient. Our studies have also highlighted curcumin and anacardic acid as micromolar noncompetitive inhibitors.

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Despite altered immune compartments and increased complement consumption, heterozygous C3 gain-of-function mice do not develop atypical haemolytic syndrome following exposure to various inflammatory stimuli

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Atypical haemolytic uraemic syndrome(aHUS) is rare kidney disease, caused by thrombotic microangiopathy (TMA). Mutations in complement regulators and activators can 'trigger' TMA but exact mechanisms remain unclear. Recently, we showed that a C3 gain-of-function point mutation ($C3^{D1115N}$) when in homozygous (or $C3^{N/N}$) led to TMA and aHUS in mice. However, as patients seldom have homozygous mutations, we sought to investigate whether aHUS could be 'precipitated' in $C3^{+/N}$ (heterozygous) mice following various stimuli, that would mimic infections long suspected to be linked to aHUS in patients.

$C3^{+/N}$ mice were injected intraperitoneally with O111:B4 lipopolysaccharide (LPS) at various concentrations (0.1, 0.2 or 0.5 mg/kg) and were monitored (weight and urine analysis) until day (D)3. In additional studies, mice received 0.2 mg/kg LPS on D0 and D7, mice were tracked until D10. Alternatively, $C3^{+/N}$ and wild type mice received an intra nasal dose of influenza strain X31 ($7.8x10^5$ plaque forming units) and monitored until D14. In our final approach, a Freund's adjuvant free method of nephrotoxic serum (NTS) nephritis was performed. Briefly, mice received two priming doses of sheep IgG on D0 and D7, before receiving 25, 50, 100 or 200µl of NTS on D14, mice were monitored to D17. In all cases, serum was analysed at cull for evidence of renal disease and complement activation split products. Kidneys were assessed for TMA by histological approaches.

While there was evidence that $C3^{+/N}$ mice where more susceptible to these agents, with increased proteinuria/weight loss being common, no mice succumbed to TMA or exhibited significant haematuria. Furthermore, while altered B and T cell populations were found and greater levels of C3 breakdown products detectable in the serum and kidney of the $C3^{+/N}$ mice, this did not translate into aHUS.

These data confirm that excess complement activation systemically, through viral infection or immune complex is not sufficient to precipitate aHUS in $C3^{+/N}$ mice. These data indicate the mouse is potentially more robust than man with respect to development of aHUS and CRRY may play a role in this situation. Although further investigation is needed to clarify this finding.

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Characterizations of structural and functional diversities of human complement C4A and C4B allotypes

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Complement component C4 is crucial for the classical and lectin complement activation pathways with great structural diversities and differential immune functions. Deciphering the polymorphisms and biochemical properties for various allotypes of C4A and C4B help understand their immune functions and roles in diseases. We purified through protein elution after resolution by agarose gel electrophoresis of C4 variants from EDTA-plasma of different human subjects for eight slow migrating C4B allotypes including B96, B93, B92, B1, B2, B3, B5 and B7; and six fast migrating C4A allotypes A91, A92, A3, A4, A5 and A6. Copy-number variations of C4A and C4B genes were characterized by Southern blot analyses and realtime PCR, and the structural bases of selected C4 variants were determined by cloning and sequencing. We performed flow cytometry of activated C4 bound to erythrocytes using monoclonal antibodies against human C4d for both C4A and C4B, and monoclonals specific for the Rogers blood group, generally associated with C4A. Among human subjects with equal numbers of C4A and C4B genes, the levels of C4d protein from C4B deposited on red cells are consistently 3-10 times higher than that from C4A. We performed biochemical kinetics studies of activated C4A and C4B by measuring the time course of bindings and cell lysis to antibody-sensitized sheep erythrocytes (EA) using human plasma samples. We used heat-aggregates of gamma-globulins for inhibition of EA lysis. Our results showed that both C4A and C4B from plasma lysed sensitized red cells, but C4A exhibited hysteresis properties by lagging C4B. At dilutions to <2 µg/mL, the efficiency of C4B on lysis EA remained significantly higher than that of C4A. Addition of gamma-globulin aggregates inhibited the time courses of C4A and C4B on lyses of EA. Specifically, 1.17 µg/mL of immune aggregates inhibited 50% EA lysis by 1.20 µg/mL C4A; 5.32 µg/mL immune aggregates was required to inhibit 50% EA lysis by 1.01

μg/mL of C4B. In summary, activated C4B binds quickly to sensitized erythrocytes, which is consistent with our observations through flow cytometry that cell-bound C4d on red cells are mostly derived from activated C4B.

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Novel alternative polyadenylation as a post-transcriptional mechanism in C5ar1 signaling-dependent pleural hypertrophy and fibrosis in collagen-induced arthritic mice

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Rheumatoid arthritis-associated interstitial lung disease (RA-ILD) is a major cause of death in RA patients with 40% mortality rate within 72 months from the time of clinical diagnosis. RA-ILD is manifested with usual and non-specific interstitial pneumonia and lung fibrosis. The role of complement activation is poorly understood in the pathogenesis of RA-ILD. We hypothesized that complement anaphylatoxin C5a via its receptor C5ar1, may contribute to the pathogenesis of RA-ILD. To unravel the molecular mechanisms of C5ar1-signaling, we induced collagen-induced arthritis in C5ar1 deficient-C57B6/J (C5ar1KO) mice and their wildtype littermates (WT). A set of arthritic mice were challenged with intradermal C5a (2.5mg/50μl) at 24-hour intervals for 27 days. All mice were euthanized on day 28 and lung tissues were collected for histologic and molecular analyses. Masson's trichrome staining revealed pleural hypertrophy and fibrogenic foci in the WT arthritic lungs. C5a-challenged WT arthritic mice revealed enhanced hyaluronan production and presence of myofibroblast activation marker, α-SMA as compared to the C5a-challenged C5ar1KO group. We further approached our hypothesis with Poly (A)-ClickSeq RNA sequencing analysis and the PolyA-miner algorithms to study alternative polyadenylation (APA) as a post-transcriptional mechanism of gene regulation involving 3'-UTR sequences. Distinct 3'-UTR landscapes in proinflammatory genes result in transcript isoforms lacking regulatory miRNA binding sites during chronic inflammation. Our data indicated increased 3'-UTR lengthening profile with 477 transcripts in arthritic C5ar1KO mice compared to their WT counterparts. This included the presence of cleavage and polyadenylation specificity subunit 5 (Cpsf5/Nudt21), a key regulator of pulmonary fibrosis. C5a-pulsed WT mice showed 1240 3'-UTR-shortened genes including Notch4 and RNA-binding protein Zfp871, compared to their C5ar1KO counterparts. C5a-pulsed arthritic WT mice demonstrated 972 disease-specific 3'-UTR-shortened genes relating C5ar1 signaling with endosomal trafficking, histone modifications, cellular senescence, and fibrogenesis. Intriguingly, significant upregulation of miRNA-29a and miRNA-30 was observed in C5a-arthritic mice, given that miRNA-29a is involved in inhibiting histone modification and fibrogenesis; and miRNA-30 targets cellular senescence. Thus, our data offer a novel mechanism of C5ar1-dependent 3'-UTR shortening in profibrogenic modulators that attenuates regulatory targeting by miR29a and miR-30 during arthritic lung remodeling.

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MASP3 deficiency ameliorates but does not completely prevent alternative pathway complement-mediated tissue injury in a factor H mutant mouse model

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Background: Mannan-binding lectin-associated serine protease 3 (MASP3) converts pro-factor D (pro-FD) to mature FD. It was reported earlier that only mature FD was catalytically active, making MASP3 a key component of the alternative pathway (AP) complement and an attractive drug target. However, the role of MASP3 in AP complement has been controversial as various levels of AP activity were detected in the absence of MASP3, both in human and in mice. We have recently demonstrated significant AP complement activity in a newly generated Masp3$^{-/-}$ mouse strain at high (20-50%) but not low (5-10%) plasma concentrations and attributed this activity to intrinsic catalytic activity of pro-FD [1]. The aim of this study is to assess the functional significance of this partial activity in AP complement-mediated tissue injury.

Methods: We crossed the Masp3$^{-/-}$ mouse with a factor H mutant mouse (FH$^{R/R}$) that develops AP complement-dependent atypical hemolytic uremic syndrome and systemic thrombophilia [2] and assessed the effect of MASP3 deficiency on disease development by evaluating FH$^{R/R}$ and FH$^{R/R}$Masp3$^{-/-}$ littermates.

Results: We found that MASP3 deficiency significantly reduced mortality, and ameliorated thrombocytopenia, anemia, and renal injury in FH$^{R/R}$ mice. It also prevented systemic thrombophilia and macro-vessel thrombosis. However, about 30% of FH$^{R/R}$-Masp3$^{-/-}$ mice (5/17) still died prematurely because of extra-renal organ injury including intestinal and pulmonary hemorrhage and cerebral ischemia. These findings contrasted with the phenotype of other AP complement and FH$^{R/R}$ double knockout mice such as FH$^{R/R}$C3$^{-/-}$ or FH$^{R/R}$FD$^{-/-}$ mice which showed complete disease rescue. AP complement activity in FH$^{R/R}$MASP3$^{-/-}$ mice, as measured in 20% serum using an LPS-based ELISA assay, was significantly lower than that of FH$^{R/R}$ mice but significantly higher than that of FH$^{R/R}$FD$^{-/-}$ mice.

Conclusion: Partial AP complement activity afforded by pro-FD in the absence of MASP3$^{-/-}$ was sufficient to cause tissue injury and therapeutic targeting of MASP3 may not be completely efficacious in all settings of AP complement-mediated diseases.

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The Dynamics of Neutrophil Intracellular C3 in Homeostasis and Inflammation

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The dynamics and functions of intracellular complement proteins have been increasingly described in immune and non-immune cells in recent years. However, studies investigating the presence and role of intracellular complement in neutrophils remain scarce. Here, we investigated the dynamics of complement protein uptake and production in neutrophils, focusing specifically on Complement 3 (C3). We utilized an in vitro time course culture model and used freshly-isolated human neutrophils from whole blood to examine the presence and dynamics of intracellular C3 protein levels by western blot and immunofluorescent imaging. Our analysis demonstrated that neutrophils contain large stores of C3 present throughout their cytoplasm immediately upon isolation from whole blood. Further, neutrophil C3 gene expression, measured by qPCR and RNA-seq, immediately following isolation was very low, indicating that the majority of protein C3 found at the time of isolation was taken up from the fluid phase while in the circulation. We next cultured neutrophils in either C3-sufficient or C3-deficient sera and found that neutrophils maintain a consistent level of intracellular C3 when cultured in C3-sufficient sera only. This, along with reports of T cells and macrophages increasing C3 gene transcription upon tissue diapedesis, led us to hypothesize that neutrophils may rely on uptake and shuttling of exogenous C3 until they extravasate into tissues at sites of inflammation. To test this hypothesis, we utilized an in vivo mouse model of LPS-induced acute lung injury. Using an RNAscope Assay probing for C3, we detected active C3 gene transcription in neutrophils throughout the inflamed lung, while neutrophils in the intravascular space and those attached to the luminal side of endothelium lacked significant C3 gene transcription. We further confirmed these findings in an in vivo mouse orthotopic lung transplant model and observed an identical phenomenon. Taken together, our results indicate that neutrophils rely on exogenous sources of complement C3 to supply their intracellular stores until such time that they extravasate into sites of inflammation, upon which neutrophils increase endogenous C3 production.

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Does C1q modulate myelin sheath development in zebrafish?

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Myelination is a key process in the development of the central nervous system. Defects in myelination lead to neurobehavioral disorders, cognitive, and psychiatric disorders. Developing myelin is plastic and an adaptive process that can be modulated by activity. Activity-led refinement of synapses and neuron elimination are classic hallmarks of plasticity in the developing nervous system. New data have shown that developing myelin also undergo a refinement process. The myelin refinement process can be modulated by activity similar to synapse elimination and microglia play a crucial role in this process during development. What remains unknown is the identity of signals that mark myelin sheaths for microglia phagocytosis. One possibility is the complement protein c1q, which targets synapses for elimination. Prior research showed that complement proteins associate with and can be activated by myelin. Based on these observations we hypothesize that complement c1q marks myelin sheaths for microglia phagocytosis in development. Utilizing the genetic and transparent nature of developing zebrafish we can capture in vivo the relationship between neuronal activity and complement protein manipulation. To determine the function of c1q on myelination we utilized CRISPR/Cas9 gene editing to target the c1qc zebrafish gene and have identified four different loss-of-function alleles. Current experiments measuring myelin sheath characteristics have shown that c1qc mutant larvae do not have significant differences in myelin specific measurements. Future experiments will determine the effects neuron activity and immune challenges have on myelination utilizing c1qc mutants. This work will define if myelin sheaths are marked by complement proteins during myelin refinement, as well as give insights onto how myelin disorders can become dysregulated when complement proteins are not deposited.

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Anti-GPC1 IgM activating the complement system is an effective treatment in a pancreatic ductal adenocarcinoma mouse model

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Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive malignancies with a survival rate at 5 years very low. Chemotherapy agents causes only a modest enhancement in the survival and is usually associated to toxic effects. The proteoglycan GPC1 showed a cell surface localization in PDAC tissues but limited or absent expression in the most of adult tissues and in chronic pancreatitis. Exploiting this promising tumor-associated antigen, an anti-GPC1 monoclonal IgM was produced with the aim to induce a strong complement activation on PDAC cells and in tumor microenvironment.

The antibody was characterized both in vitro and in vivo. Biodistribution studies, using near-infrared optical imaging technology, confirmed the capacity of anti-GPC1 IgM to selectively bind its target in a localized PDAC model developed in Nude mice, with a pick after 4 days; this antibody was mainly eliminated by the liver. Moreover, a single injection of anti-GPC1 IgM induced a strong activation of the classical pathway of the mouse complement system; as expected, C1q, C3 and C9 deposits were documented in tumor microenvironment by immunofluorescence analysis, causing extended necrotic areas as well as the recruitment of CD14+ macrophages and CD56+ NK cells. Repeated injection of anti-GPC1 IgM (twice a week) controlled tumor growth in all tumor-bearing mice. All saline-treated animals died in 19 days after the first treatment while 60% of anti-GPC1 IgM treated mice survived at the end of the study (50 days after the first treatment).

Collectively, these findings showed the capacity of anti-GPC1 IgM to strongly activate the complement system on PDAC cells, causing cancer cell death, modifying tumor microenvironment and finally increasing mice survival.

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The role of the complement system in DCD liver transplantation

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Background: The complement system is an integral part of the pathophysiology in ischaemia-reperfusion injury in transplantation. There has been increasing interest in its role specifically with regards to a possible therapy target to ameliorate the effects of IRI. Ischaemic cholangiopathy is a significant complication after DCD liver transplantation, however there has not been a consensus on the pathophysiology of this complication. There have not been any studies looking at the role of the complement system within liver transplantation.

Methods: Liver grafts that have been declined for transplantation were placed on a machine perfusion circuit that perfused the graft with oxygenated packed red blood cells at 38 degrees Celsius. Perfusion dynamics were noted, along with bile production and biochemistry. Perfusate samples were taken in EDTA and frozen. Core biopsies of the livers were taken and fixed in formalin for 24 hours before embedding in paraffin.

Results: A total of 9 livers were perfused (3 DBD, 3 DCD, 3 DCD treated with eculizumab). There were no significant differences in perfusion dynamics including weight-adjusted hepatic artery and portal vein flow rates. Treated DCD livers produced more bile than untreated DCD livers (8ml/hour vs 3ml/hour, p=0.04). The blood-bile bicarbonate gradient percentage was greater in the treated DCD livers, not reaching significance (2g/ml vs 1.5g/ml, p = 0.08). There was greater deposition of C5b-9 within the arterial endothelium of the portal tracts of the liver seen in DCD livers when compared to DBD livers (mean 1.8 relative units vs mean 1.5 relative units, p = 0.07).

Discussion: Ischaemic cholangiopathy is thought to be mediated by the damage to the peribiliary vascular plexus, thereby affecting the regenerative capacity of the cholangiocytes. This study has suggested that complement mediated damage of the arterial endothelium might play an important role in the damage to the peribiliary plexus. This study has also shown that complement inhibition has a possible role in ameliorating the damage to the endothelium, thereby improving cholangiocyte function. Further studies are required to appropriately study the long term effects of complement inhibition in a DCD liver graft, specifically looking at the incidence of ischaemic cholangiopathy.

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Sez6L2 is a brain-resident complement inhibitor that is necessary for proper brain development

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Background: Sez6, Sez6L, and Sez6L2 are novel complement regulatory proteins that are primarily expressed by neurons. The Sez6 proteins can inhibit complement deposition on cell surfaces by accelerating the dissociation of C3 convertases or through cofactor activity that facilitates the cleavage of C3b. The purpose of this study is to further investigate this regulatory mechanism by determining which protein domains are required for the binding and function of Sez6L2, and by investigating whether Sez6L2 function impacts brain development.

Methods: To detect the inhibition of complement deposition, cells expressing variants of Sez6L2 were stimulated to induce complement activation, and the amount of C3b/iC3b deposited on the surface of cells was quantified using flow cytometry. Co-immunoprecipitation binding assays were performed to identify the ability of Sez6 proteins to bind to different complement proteins. To examine complement levels in vivo, total C3 levels were quantified from the brains of mice with a genetic knockout of Sez6L2 (Sez6L2 KO) using western blot. Sez6L2 KO mice were also analyzed using behavioral assays.

Results: By deleting individual domains from the Sez6L2 gene, we found that the tandem CCP repeat – a motif found in all C3 convertase inhibitors – is responsible for only a small portion of Sez6L2's inhibition of C3b deposition. Instead, the N-terminal region of Sez6L2, conaining the CCP-CUB-CCP-CUB domains, is responsible for the majority of the complement inhibitory activity. This suggests that Sez6L2 interacts with additional complement factors beyond C3 to exert its regulatory activity. We found that Sez6L2 binds to complement-initiating proteins C1q and FCN1. In addition, the inhibitory activity of Sez6L2 has consequences for complement levels in the brain. We identified elevated C3 expression in the cortex of juvenile Sez6L2 KO mice. Sez6L2's function is critical to proper brain development, as Sez6L2 KO mice also show impairments in open field mobility, nesting, pre-pulse inhibition, 3-chamber social interaction tests, marble burying, and contextual fear conditioning.

Conclusions: Sez6L2 is a brain-specific complement inhibitor that regulates complement activity in part by targeting the initiation of the classical and lectin arms of the complement pathway. This Sez6L2-mediated complement inhibition is required for proper brain development.

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