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ID: 100

10 Years Follow-Up of CD59 Congenital Deficiency: Neuroinflammation, Hemolysis, Eculizumab, Ravulizumab and Pozelimab

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The MAC inhibitory function of CD59 is related to its capacity to bind C8 within C5b-8 and block recruitment of C9, which is essential for MAC formation. Primary congenital CD59 deficiency in humans is a germline mutation described in 20 individuals and mainly manifested by recurrent Guillain-Barré syndrome (GBS) (19/20), hemolytic anemia (14/16), and recurrent strokes (8/18).

We have recently shown that the frameshift mutants p.Asp24Valfs* and p.Ala16Alafs* generate truncated proteins with a distinct c-terminal sequence, common to both mutants, that are not present at the plasma membrane. In contrast the missense mutants p.Cys64Tyr and p.Asp24Val, do reach the cell surface. Despite this difference, all four mutants produce nonfunctional CD59 and are thus unable to protect the host from MAC attack. As judged by clinical manifestations, another two mutations, the Ser83Ter (Terminal) nonsense8 and Tyr4Asp missense mutations, produce nonfunctional CD59.

How would nonfunctioning mutated CD59 contribute to the appearance of autoinflammatory GBS? In the current study, we use biopsies from healthy and CD59-deficient humans and from WT and CD59-deficient mice. We evaluated cross sections, teased sciatic nerve fibers, and myelinating DRG neuron/Schwann cell cultures for CD59 expression using a variety of antibodies to study the exact anatomical localization of CD59 and other membrane-bound complement with the aim of understanding their physiological and pathophysiological roles.

We were able to show that the nodes of Ranvier have no complement-membrane regulatory proteinsCD59, CD46, CD35, or CD55, rendering this area exposed to complement attack. Furthermore, we showed that myelin in the PNS is protected only by CD59 and CD55, but not by CD46 or CD35, rendering it susceptible to complement attack, conduction block, and demyelination in autoinflammatory situations like CD59 deficiency. Finally we provide clinical follow-up of 12 patients with successful treatment with Eculizumab, Raviluzumab, and Pozelimab

Reference 1:

Molecular pathogenesis of human CD59 deficiency. Karbian N, Eshed-Eisenbach Y, Tabib A, Hoizman H, Morgan BP, Schueler-Furman O, Peles E, Mevorach D. Neurol Genet. 2018 Oct 26;4(6):e280. doi: 10.1212/NXG.0000000000000280. eCollection 2018 Dec

ID: 124

A complement C3-nanobody inhibits the AP convertase stabilization mediated by anti-C3bBb autoantibodies in C3 Glomerulopathy

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- C3 glomerulopathy (C3G) is a clinicopathologic entity secondary to dysregulation of the complement alternative pathway (AP) in plasma and the glomerular microenvironment. This unrestrained complement activation is frequently associated with autoantibodies called C3NeF that enhance the AP C3 convertase half-life. C3NeF specifically binds a neoepitope generated in the C3 convertase but the exact binding site is unknown. Among agents targeting the complement component C3, C3-specific nanobodies (hC3Nb1, hC3Nb2, hC3Nb3) were described as potent complement inhibitors in *in vitro* models. Moreover, hC3Nb3 binding to the C-345C domain of C3/C3b/C3c was demonstrated to prevent the AP proconvertase assembly, but also to compete for C3b with Properdin, an AP positive regulator sharing common functional properties with C3NeF. Here, we explored whether C3-specific nanobodies may interfere with the functional effect of IgG targeting the AP C3 convertase C3bBb in C3G.
- We selected 12 C3G patients tested positive for anti-C3bBb antibodies by ELISA. Using Luminex™, we designed a new functional assay allowing to study the C3bBb stabilization. Briefly, we generated C3bBb convertases on C3b-coated beads, and C3NeF-positive IgGs were detected when an increase in the residual bound-Bb signal in comparison with healthy donors IgG was measured. In both models, each nanobody was incubated with patients IgG on the preformed-C3bBb.
- The IgG binding to C3bBb of 12 /12 patients was inhibited with hC3Nb3 but not hC3Nb1 and hC3Nb2. By Luminex™, 9/12 patients IgG were detected positive for C3NeF. The C3bBb stabilization mediated by the 9/9 C3NeF-positive IgG was specifically inhibited by hC3Nb3.
- We show that hC3Nb3 has a dual action that interfere with AP C3 convertase activity: the inhibition of C3NeF activity on preformed-C3bBb in addition to proconvertase assembly blocking, thus providing a new potential therapeutic application in C3G. Furthermore, our results suggest that C3NeF and hC3Nb3 share a common binding site on the C3bBb convertase.

Reference 1:

Pedersen H, et al. A Complement C3-Specific Nanobody for Modulation of the Alternative Cascade Identifies the C-Terminal Domain of C3b as Functional in C5 Convertase Activity. J Immunol. 2020 Oct 15;205(8):2287-2300.

ID: 164

A high-throughput serum bactericidal assay in evaluating complement-mediated killing of key Gram-negative clinical bacterial isolates

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Several Gram-negative opportunistic and pathogenic bacteria can cause problematic infections. Due to wide-spread and ever-worsening antimicrobial resistance and shortcomings in developing new antimicrobials, new therapeutic methods and novel tools to assess bacterial killing are needed.
We developed a quantitative high-throughput serum bactericidal assay (SBA) from a phage killing assay. We measured IgG titers of several volunteers against two clinical isolates of *E. coli*, *K. pneumoniae* and *E. cloacae* each. Bacterial killing activity of high- and low-titer serum samples, pooled NHS, heat-inactivated serum (HIS) and buffer alone were then evaluated. *E.coli* BL21, a known serum-sensitive strain, acted as positive control.

Our assay uses an environmental incubator-shaker combined with an optical density (OD) reader. For preparing samples for the SBA, bacteria grown overnight are first diluted 1:100 in lysogeny broth (LB). Complement-preserved and heat-inactivated serum are diluted to varying amounts into veronal-buffered saline with 2 mM Ca++ and 0.5 mM Mg++. Then, 100 µL of both the 1:100 bacterial LB stock and NHS or HIS dilution are applied into wells. Bacterial growth at 37°C (with medium shaking) is followed by OD at wavelength 600nm at start and every 30 minutes for a total of 10 hours. This will produce growth curves for each well.

The serum-sensitive *E. coli* BL21 strain showed no growth in any of the 20% NHS-VBS++ conditions, unlike in HIS, indicating complement-mediated killing. For clinical strains, e.g. a wound-isolated *E. coli*-strain grew in HIS, but not in low-titer, high-titer or pooled NHS, indicating serum susceptibility. Small or no differences in growth inhibition were observed for blood-isolates of *E.coli*, *K. pneumoniae* or *E. cloacae* strains.

Our assay is high-throughput and produces easily replicable, robust growth curves for the tested bacteria. It allows analysis of resistance of bacteria to complement-dependent killing and means to overcome this by serum antibodies.

ID: 157

A model to study complement components in human ARPE-19 cells alone or in co-culture with porcine retina

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Background and Aim:
Age-related macular degeneration (AMD) is a complex neuroinflammatory disease which involves the retina and adjacent tissue. Diverse risk factors contribute to the disease such as induced sunlight, oxidative stress, etc. The retina has high metabolic activity, producing a high amount of reactive oxygen species (ROS). The retina and retinal pigmented epithelial (RPE) cells are therefore prone to be exposed to high amounts of ROS and increased oxidative stress and death of RPE cells can activate the complement system. Here, we investigated secreted complement proteins and proinflammatory cytokines and the expression of cell-bound complement components in the spontaneously arising human retinal pigment epithelial cells-19 (ARPE-19) alone or in co-culture with porcine retina.
Methods:
The ARPE-19 were cultured alone or in co-culture with or without porcine retina in Millicellâ-PCF 0.4-mm inserts. Supernatants were taken after 24, 48, 72 and 96 hours. Secreted proteins C5a, factor H (FH) and vascular endothelial growth factor (VEGF), and the proinflammatory cytokines IL-6 and IL-8 were measured by ELISA and Luminex. Cell associated complement proteins were investigated by immunohistochemistry.
Results:
ARPE-19 cells alone or in co-culture showed secretion of C5a, FH, VEGF, IL-6 and IL-8 over time. The secretion of FH was statistically significantly increased after 96 h ($p = <0.0001$).
Conclusions:
Healthy ARPE-19 cells, either alone or in co-culture, secreted different complement components and proinflammatory cytokines. Our model can be a useful tool for future experiments to study pathophysiological mechanisms involved in AMD.

References:

Mohlin, C., Sandholm, K., Kvanta, A., Nilsson Ekdahl, K., Johansson, K. (2018). A model to study complement involvement in experimental retinal degeneration. Upsala Journal of Medical Sciences. 123 (1). 28-42.

Milosz P. Kawa, Anna Machalinska, Dorota Roginska, Boguslaw Machalinski, "Complement System in Pathogenesis of AMD: Dual Player in Degeneration and Protection of Retinal Tissue", Journal of Immunology Research, vol. 2014, Article ID 483960, 12 pages, 2014. https://doi.org/10.1155/2014/483960

ID: 117

A Novel C3 Variant in a Patient with C3 Glomerulopathy

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C3 Glomerulopathy (C3G) is a group of rare kidney disease driven by uncontrolled activity of the presence of acquired and/or genetic drivers of disease. Rare or novel variants in complement genes, including *C3, CFB, CFH, CFI and MCP*, may be involved in the development of C3G. Once a rare/novel genetic variant is

identified, it is important to clarify its functional impact on complement activity. In a 68-year-old male patient with recurrent post-transplant C3G, we identified a novel variant in *C3* (c.2770G>A, p.Gly924Ser). Complement biomarker testing was remarkable for C3 and properdin levels, while sC5b-9 was elevated, consistent with uncontrolled complement activity at both the C3 and C5 convertase levels. The patient was negative for known acquired drivers (autoantibodies) and paraproteins were not detected. To assess functional consequence of this variant, we designed a novel hemolytic-based assay using diluted patient serum as a source of C3b. Results show that FH-mediated DAA of C3 convertase formed with the *C3* variant p.Gly924Ser is slower than DAA with wildtype *C3*. In addition, FI activity with FH was significantly impaired, but activity with soluble complement receptor 1 (CR1) and soluble membrane cofactor protein (MCP or CD46) was normal. In summary, *C3* p.Gly924Ser impacts binding of FH to mutant C3b, reducing DAA and FH-FI mediated C3b degradation thereby leading to greater AP dysregulation in the fluid phase. This case highlights the importance of defining the functional consequence of all genetic rare/novel variants in order to classify their impact in the pathogenesis of C3G and thereby offer patients appropriate clinical care.

ID: 169

A potent, targeted, enzyme-like inhibitor of all three complement activation pathways

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Although few complement mediated diseases can be treated with approved complement inhibitors (CI), many more remain untreated. One of the reasons is the lack of suitable CIs, that can sufficiently prevent complement activation. Based on natural regulators, we designed a novel CI with improved properties to overcome issues such as incomplete inhibition, which occurs under C5 or C3 inhibition, when very strong activation of one or more pathways takes place.

We recombinantly expressed single domain stretches, double or triple fusions of decay accelerating factor complement control protein domains (CCPs)(1-4), Factor H CCP(19-20) and complement receptor 1 CCP(15-17). To assess the activity of these recombinant CIs, affinity towards opsonins and inhibitory potency in haemolysis assays was measured. In preparation of animal studies, we produced a mouse surrogate of the most active construct and assessed complement inhibition in human, rat and mouse serum.

The highest affinity to opsonins was measured with a triple fusion construct owing to its avidity, which correlated with the inhibitory potential in alternative pathway (AP) and classical pathway (CP) haemolysis assays. All fusion constructs that included regulatory active domains, completely inhibited even strong AP or CP activation. Fusions with the regulatory inactive domains Factor H CCP(19-20) substantially increased the activity, but especially for AP regulation of double fusion versions an appropriate spacer was crucial. Although our human CI prevents complement activation in mouse and rat serum, the mouse surrogate is more active in rodent serum, as expected, and therefore more suitable for animal experiments.

Our rationally engineered CI shows very high potency because it combines multiple functions of natural complement regulators in a single molecule. Moreover, it completely blocks all complement activation pathways, despite very strong activation, which is an advantage over existing regulatory approved stoichiometric inhibitors.

ID: 146

Activation of the complement system by polymeric targeted and untargeted nanoparticles: a comparative study between human and mouse serum

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Background

Nanoparticles (NPs) interact with blood proteins constituting a protein corona (PC) which affects targeting, biodistribution, and toxicity[1]. PC characterization allows a more realistic evaluation of the future of NPs after injection. The purpose of this work is to compare human and mouse PCs (HPC, MPC) formed on untargeted NPs (uNPs) and targeted NPs (tNPs: uNPs conjugated with a human-Fc antibody) with particular attention to complement (C) activation.

Material and Methods

HPC and MPC were visualized by TEM and SDS-PAGE. Proteins were identified through mass spectrometry. C proteins deposit was detected through WB; C activation was evaluated through C3 fragmentation and lytic assays. C contribution in NP's interaction with macrophages was evaluated by confocal microscopy and viability test.

RESULTS

Serum increased the dimension of uNPs and tNPs, suggesting the formation of a surrounding PC. HPC differentiated more uNPs from tNPs than MPC suggesting a different fate of NPs when injected in mice or humans. tNPs weakly activated the classical pathway; they strongly activated the lectin one, even in absence of related proteins in the HPC. The alternative pathway was not activated by NPs. tNPs were internalized inside macrophages more than uNPs in absence of C activation, probably due to Fc receptors expressed by cells; after C activation, this difference was flattened. This contrasted with previously data: tNPs accumulated in mouse liver more than uNPs[2].

CONCLUSIONS

Regarding C deposition and activation on NPs, the differences between murine and human were negligible. However, contrasts between *in vitro* experiments on human cells and *in vivo* biodistribution studies on mouse models highlighted the importance of the biological context in which NPs are tested.

References:

Nguyen and Lee. International J Nanomed 2017; 12: 3137–51; **C**apolla et al. Int J Nanomed 2015; 10: 4099-4109.

ID: 165

Activation of the lectin pathway of complement system after ischemic stroke in a humanized mouse model expressing human mannose-binding lectin

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Mannose-binding lectin (MBL) is the central recognition molecule of the lectin pathway (LP) of complement activation driving thromboinflammation after ischemic stroke. MBL genetic or pharmacological targeting is neuroprotective, making it an attractive pharmacological target. There are evolutionary differences, with mice having two (MBL-A and MBL-C) while humans one functional (hMBL) molecule. In view of fostering clinical transferability of MBL inhibitors, we characterized LP activation in mice knock-out for murine MBL isoforms and knock-in (KI) for hMBL
WT or hMBL KI mice (male, 20-30 gr) underwent transient occlusion of the middle cerebral artery (tMCAo) to model ischemic stroke. Two days before (-2d) and 90' and 2d after tMCAo blood was longitudinally obtained. At 2d mice were tested for sensorimotor deficits and sacrificed.
hMBL KI and WT mice had similar deficits (29.9±13.5 vs. 29.6±10.2 of neuroscore±SD, n=10-11) and mortality (4/10 vs. 3/11 of dead or humane endpoint). By in vitro LP residual activation assay on mannan-coated plates using 0.6 to 10 % of diluted EDTA-plasma, C3b deposition decreased early at 90' compared to -2d in hMBL KI (-2d: 6.4, 90': 4.8, area under curve, pools of 3 mice) and WT (-2d: 7.7, 90': 6.6) mice, in line with LP activation in the ischemic mice. By non-reducing Western blot on a Tris-Acetate gradient gel, we identified hMBL bands overlapping to mouse MASP-2, suggestive of complex assembly. At 2d hMBL decreased by 32.5% and MASP-2 by 58.5% than -2d. In line with consumption, at 2d hMBL was found selectively on ischemic vessels by immunofluorescence and confocal microscopy.
Thus in this humanized mouse model hMBL is consumed upon targeting the ischemic vessels and activates the LP as part of ischemic stroke pathogenesis. The model is therefore suitable to test selective MBL inhibitors with therapeutic potential in stroke.

ID: 2

Air emboli trigger a complement C3-dependent thromboinflammation in human whole blood and pigs.

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Background
Air embolism potentially complicates many medical procedures, causing vascular occlusion, organ infarctions, or death. Air also triggers thromboinflammation. In vitro, air triggers the alternative complement pathway, activates platelets, and initiates coagulation. Previous in vitro air studies utilized

serum, plasma, or heparin anticoagulated blood, precluding detailed examination of thromboinflammation. Air-induced thromboinflammation and complement activation has not previously been examined in vitro under near-physiological conditions nor in vivo in pigs. We studied air-induced thromboinflammation in vitro in human whole blood and in vivo in pigs.

Methods and Materials
In vitro, lepirudin anticoagulated human blood from 16 donors was incubated 180 minutes with air and inhibitors of complement C3, C5, and CD14, or without air. Plasma was analyzed for complement activation products C3a and terminal complement complex (TCC), 27 cytokines, tissue factor mRNA (TF), platelet marker β-thromboglobulin (βTG), and coagulation split-product prothrombin fragment 1+2 (PTF1+2). In vivo, air was infused through an ear vein for 300 minutes in 24 pigs receiving minimal heparin infusion to avoid clotting in intravascular canulas. Seven pigs served as shams. Hemostasis was monitored using rotational thromboelastometry, and by measuring plasma thrombin-antithrombin. Plasma and lung tissue homogenates were analyzed for C3a, TCC, and cytokines.

Results
In vitro, air triggered a C3-driven thromboinflammation, with an increase from baseline of C3a 58-fold, TCC 9-fold, 25 cytokines on average 11-fold, TF 26-fold, βTG 13-fold, and PTF1+2 152-fold. The C3a and TCC increase did not correlate. 25 cytokines were reduced on average 2.7-fold by C3 inhibition and 3.9-fold by combined C3- and CD14-inhibition. Only two cytokines were reduced (1.9-fold) by C5 inhibition. PTF1+2 was reduced 66-fold by C3- and 75-fold by C5-inhibition. Neither C3- nor C5-inhibition reduced βTG. In vivo, animals receiving air infusion developed leukocytosis, increased hemostasis, and increased plasma C3a, but not TCC. In the lungs, C3a and interleukins (IL)-1β, IL-6, and IL-8 were increased.

Conclusion
Air triggered a C3-driven thromboinflammation in vitro and in vivo. Complement activation, cytokine release, and coagulation, but not platelet activation, were attenuated by C3 inhibition in vitro.

ID: 30

Alternative and terminal complement pathway activation in patients with carotid atherosclerosis

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Ischemic stroke, caused by carotid atherosclerosis, is one of the leading causes of mortality and loss of productive life years. Complement activation is suggested to be part of the inflammatory arm of plaque progression in carotid atherosclerosis, but data on activation of the alternative complement pathway is scarce. We hypothesized that complement components and activation markers of the alternative and terminal pathway would be altered in patients with carotid atherosclerosis, and related to adverse outcome.

We measured, by ELISA, plasma levels of factor D, factor H, properdin, C3bc, C3bBbP, and soluble terminal C5b-9 complement complex (sC5b-9) in patients with carotid atherosclerosis (n = 343) and healthy controls (n = 70).

Our findings were: 1) compared with controls, the total patient population with carotid atherosclerosis had increased plasma levels of factor D (1.4-fold), properdin (1.2-fold), C3bc (3.5-fold), C3bBbP (1.3-fold)

and sC5b-9 (2.0-fold) (all markers p<0.001), whereas levels of factor H were not altered. 2) The highest levels of sC5b-9 were seen in those with the most recent ischemic event (<2 months ago), while the highest levels of factor D, properdin, C3bc and C3bBbP were seen in those with an ischemic event between 2 and 6 months ago. 3) Circulating levels of alternative pathway markers were associated with measures of dyslipidemia (i.e. triglycerides and HDL) and inflammation (i.e. leukocyte count and CRP). 4) The highest levels (i.e. tertile 3) of factor D and sC5b-9, and lowest levels (i.e. tertile 1) of properdin were associated with all-cause mortality.
We conclude that carotid atherosclerotic patients showed changed levels of several alternative pathway markers, including increased factor D and properdin, and C3 activation reflected by increased C3bc and C3bBbP, implying a dysregulation of the alternative pathway. Subsequently, the terminal pathway was activated with increased sC5b-9. Factor D, properdin and sC5b-9 were associated with adverse outcome.

ID: 82

Alternative splicing encodes intracellular CD59 isoforms which mediate insulin secretion, are downregulated in diabetes, and interact with DNA, suggesting involvement in gene transcription regulation.

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Background: We previously showed that silencing CD59 expression in pancreatic β-cells suppressed insulin secretion. Additionally, the GPI anchor is dispensable for CD59's role in insulin secretion, implying the involvement of intracellular CD59. However, the *in vivo* existence of non-GPI anchored CD59 isoforms was not proven.

Methods: Using available RNA sequencing data from human pancreatic islets, we discovered the presence of two CD59 splice isoforms lacking the GPI anchor and having unique C-terminal domains. Immunolabelling, Western blotting, insulin secretion, and expression studies were performed on primary islets and β-cell lines to assess localization and function of CD59 isoforms.

Results: We named the isoforms IRIS-1 and IRIS-2 (**I**soforms **R**escuing **I**nsulin **S**ecretion **1** and **2**). Both isoforms exist at mRNA and protein levels in human and mouse pancreatic islets. They are found in the cytosol, where they colocalize with insulin granules and SNARE proteins, allowing for insulin secretion. Each IRIS isoform permits different phases of insulin secretion. Antibodies raised against each isoform revealed that both IRIS-1/2 are markedly reduced in islets from diabetic patients compared to healthy controls. Glucotoxicity decreased IRIS-1 expression in healthy human islets. An electropositive patch was found in the C-terminal region of IRIS-1, suggesting potential interaction with DNA. We found that the C-terminal domain of IRIS-1 mediated nuclear localization. IRIS-1 expressing cells displayed significantly higher

expression levels of *Urocortin3* and *Pdx1* (mature β-cell markers, loss of which characterize β-cell dedifferentiation). Similar IRIS-1/2 isoforms were also identified in the mouse *Cd59b* gene.

Conclusions: Our data prove the existence of previously uncharacterized non-GPI anchored CD59 isoforms *in vivo* and indicate that hyperglycemia (raised blood sugar levels) in diabetic patients may be a factor resulting in reduced IRIS-1/2 expression and decreased insulin secretion. IRIS-1 expressing cells displayed higher expression of *Urocortin3* and *Pdx1,* suggesting IRIS-1 requirement for maintaining β-cell identity and function.

ID: 101

Anaphylatoxin receptors regulate the differentiation and function of dendritic cells subsets from bone marrow-derived precursors

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Dendritic cells (DC) comprise plasmacytoid DCs (pDC) and conventional DCs (cDC) originating from bone marrow (BM) derived hematopoietic stem cells. They differentiate into macrophage and DC precursors (MDPs) and later into Common DC Precursors (CDP) eventually giving rise to pDC, cDC1, and cDC2. Here, we determined the impact of C3aR and C5aR1 activation on the differentiation and function of pDCs, cDC1, and cDC2 from BM-derived MDPs and CDPs in response to combined stimulation with GM-CSF and FLT3L.
We found significantly higher frequencies of MDPs and CDPs differentiated from wildtype (WT) as compared to C3ar1$^{-/-}$ mice suggesting that C3aR regulates early BM differentiation of MDPs and CDPs. FACS-sorted MDPs and CDPs from WT, C3ar1$^{-/-}$ and C5ar1$^{-/-}$ mice preferentially induced cDCs and only minor pDC differentiation. The potency to drive cDC differentiation was high using CDPs and modest using MDPs from either WT, C3ar1$^{-/-}$, or C5ar1$^{-/-}$ mice. The frequency of cDCs differentiated from MPDs of C3ar1$^{-/-}$ and C5ar1$^{-/-}$ mice was slightly higher as compared to WT mice suggesting that C3aR/C5aR1 may regulate the differentiation of macrophages/cDCs from MDPs. The frequency of pDCs generated by MDPs from WT mice was significantly higher than that from C5ar1$^{-/-}$ mice pointing toward a role for C5aR1 in MDP-driven pDC differentiation.
CDPs induced a dominant cDC1 differentiation. Interestingly, the ratio of cDC1/cDC2 induced by CDPs from WT mice was 8-fold higher than that induced by CDPs from either C3ar1$^{-/-}$ or C5ar1$^{-/-}$ mice suggesting that both anaphylatoxin receptors regulate the balance of cDC1/cDC2 differentiation. Finally, we found a strong effector T cell response (80-90%) associated with a memory T cell response (10-20%) induced by cDC1/2 in vitro after OVA-stimulation and co-culture with OVA TCR-transgenic T cells. Of note, cDC2 cells promoted a stronger memory T cells response than cDC1, which was highest with cDC2 from C3ar1$^{-/-}$ mice.

ID: 118

Anti SARS-CoV-2 spike and nucleocapsid antibodies may mediate complement driven inflammation in severe COVID-19 patients

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Our previous results showed that patients with SARS-CoV-2 infection are more likely to die if the disease is accompanied by overactivation and consumption of C3. The effects of the antibody response against SARS-CoV-2 are generally considered to be beneficial. However, the developed SARS-CoV-2 antibodies may contribute to enhanced complement driven inflammation exacerbating COVID-19 disease, which has to be further examined.

A total of 128 individuals, 102 hospitalized COVID-19 patients and 26 convalescent outpatients were recruited in the study. Activity of the complement alternative and classical pathways, complement factors, regulators and activation products were measured by hemolytic titration, turbidimetry or enzyme-immunoassays. The levels of IgG and IgG subclasses against anti-spike and anti-nucleocapsid proteins of SARS-CoV-2 were determined by ELISAs.

All IgGs against SARS-CoV-2 were detected in 61% of patients and healthy donors. The 87% and 99% of positive IgG samples contained anti-spike and anti-nucleocapsid IgG antibodies, respectively. IgG1 and IgG3 were the dominant subclasses of SARS-CoV-2 specific IgGs, while IgG2 and IgG4 were slightly detected. Increased anaphylatoxin C3a concentrations were associated with fatal outcme in hospitalized patients, who had also high levels of anti-nucleocapsid IgG1 and IgG3.

The results in Hungarian cohorts corrobrate other studies that SARS-CoV-2 infection leads to the development of IgG1 and IgG3 subclasses against spike and nucleocapsid proteins. Our findings also indicate that antibodies against nucleocapsid protein might trigger harmful inflammatory responses through the activation of C3. Further studies are needed to investigate the direct evidence of antibody-mediated complement activation.

Reference 1:

Frontiers in Immunology, 2021 (Vol12) 663187

ID: 127

Anti-C2 antibody ARGX-117 inhibits complement in a disease model for multifocal motor neuropathy

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Multifocal motor neuropathy (MMN) is a rare, chronic immune-mediated neuropathy characterized by progressive asymmetric weakness mainly affecting hands and forearms and lower legs. MMN is associated with anti-ganglioside IgM antibodies, predominantly directed against GM1. These antibodies bind to the axolemma at nodes of Ranvier, interfering with axon-Schwann cell (SC) interactions, causing widening of the node and axonal damage. The role of complement in the pathogenesis of MMN is supported by correlations between complement activating properties of IgM antibodies and clinical features, including weakness and axonal damage[1].

We investigated complement activation and inhibition in a disease model for MMN using induced pluripotent stem cell-derived motor neurons (MNs) and SCs. We characterized these cells for the expression of complement receptors and membrane-bound regulators, and found that both express CD46, CD55, and CD59, which intrinsically protected these cells against complement-mediated cell lysis, as was demonstrated for Schwann cells. MNs expressed C3aR, C5aR and CR1.

Using a cohort of 103 MMN patients, we observed a strong correlation between anti-GM1 antibody titers, IgM binding, and complement activation on MNs. Anti-GM1 negative patients (as determined via INCAT ELISA) presented with higher IgM binding and C3 fixation compared to healthy controls. Intriguingly, IgM binding and subsequent complement activation was also observed on SCs. The clinical significance of complement activation on SCs on the pathogenesis of MMN is currently being assessed. Finally, ARGX-117, a novel inhibitory monoclonal antibody against C2 that blocks the classical and lectin pathway upstream of C3 convertase formation[2], dose-dependently inhibited complement activation on both MNs and SCs.

By expressing CD59, MNs and SCs are protected against complement-mediated lysis. Since MNs express C3aR, they may be influenced by complement activation upstream of MAC formation. ARGX-117 successfully inhibited complement activation upstream of C3 in this disease model, and therefore represents a potential treatment strategy for MMN.

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ID: 182

Anti-SARS-Cov-2 S-RBD IgG formed after BNT162b2 vaccination can bind C1q and activate complement

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Introduction: Activation of the classical complement pathway through C1q binding to immunoglobulins (Ig) contributes to pathogen neutralization, thus, the ability of Ig produced after vaccination to bind C1q could affect vaccine efficacy. In this study, we investigated C1q binding and subsequent complement activation by anti- spike (S) protein receptor-binding domain (RBD) specific antibodies produced following vaccination with either the mRNA vaccine BNT162b2 or the inactivated vaccine BBIBP-CorV.
Methods: Serum samples were collected in the period July 2021-March 2022. Participants' demographic data, type of vaccine, date of vaccination, as well as adverse effects of the vaccine were recorded. The serum samples were incubated with S protein RBD-coated plates. Levels of human IgG, IgM, and C1q, that were bound to the plate and formed C5b-9, were compared between different groups of participants.
Results: A total of 151 samples were collected from vaccinated (n=116) and non-vaccinated (n=35) participants. Participants who received either one or two doses of BNT162b2 formed higher levels of anti-RBD IgG than participants who received BBIBP-CorV. The anti-RBD IgG formed following either vaccine bound C1q, but significantly more C1q binding was observed in participants who received BNT162b2. Subsequently, C5b-9 formation was significantly higher in participants who received BNT162b2, while no significant difference in C5b-9 formation was found between the non-vaccinated and BBIBP-CorV groups. The formation of C5b-9 was strongly correlated to C1q binding, additionally, the ratio of formed C5b-9/ bound C1q was significantly higher in the BNT162b2 group.
Conclusion: Anti-RBD IgG formed following vaccination can bind C1q with subsequent complement activation, and the degree of terminal complement pathway activation differed between vaccines, which could play a role in the protection offered by COVID-19 vaccines. Further investigation into the correlation between vaccine protection and vaccine-generated antibodies' ability to activate complement is required.

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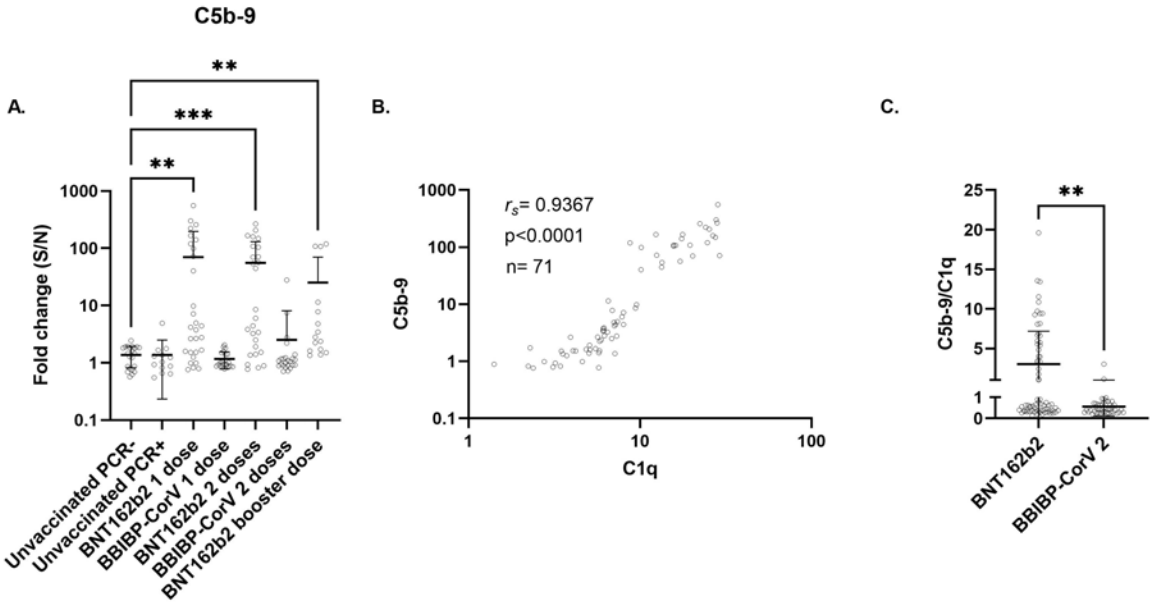

ID: 138

Anti-vector antibodies against ChAdOx1 Covid-19 vaccine activate complement and promote phagocytosis of ChAdOx1 in healthy donors

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The Covid-19 vaccine ChAdOx1 nCoV-19 (AstraZeneca) has been in large-scale use during the Covid-19 outbreak. Because of its limited efficacy and potential side effects we analyzed whether pre-existing anti-vector antibodies or vaccine-induced complement activation could influence the immune response against SARS-CoV2. Complement activation induced by ChAdOx1 *in vitro*, plasma anti-vector IgG titers and phagocytosis of ChAdOx1 by neutrophils were studied in the presence of sera from healthy donors (n=20) with different vaccination status. Plasma IgG against ChAdOx1 or adenovirus 2 hexon were determined by EIA and IgGs from high- and low-titer sera were isolated by MelonGel. Serum complement activation was quantified by measuring generation of C3a and SC5b-9 (EIA). For phagocytosis, neutrophils were isolated as a PMN fraction from blood using density gradient. Isolated neutrophils, pHRodo labeled ChAdOx1, sera and/or IgG were incubated together and analyzed by flow cytometer. ChAdOx1 activated strongly serum complement, increasing C3a and SC5b-9. The SC5b-9 levels in individual sera correlated significantly with anti-hAdV2 and anti-ChAdOx1 IgG levels. Phagocytosis of ChAdOx1 associated significantly to anti-hAdV2 IgG, anti-ChAdOx1 IgG and serum SC5b-9 levels in the sera from individual donors. High titer IgG increased phagocytosis in the presence of sera. These results show that anti-vector antibodies induce *in vitro* complement activation by the ChAdOx1 nCoV-19 vaccine. This is dependent on individual variation in complement activity. In conclusion, our study suggests that vaccine-induced

complement activation is increased by pre-existing anti-vector antibodies. This could be involved in vaccine-induced side effects and influence the efficacy of the ChAdOx1 vaccine against SARS-CoV-2.

ID: 61

Assessment of complement regulation by different structural forms of Shiga toxin 2a

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Enterohemorrhagic *Escherichia coli* (EHEC) associated hemolytic uremic syndrome (eHUS) is one of the foremost causes of acute kidney injury in children. It is proposed that the progression of the disease is directly linked to the interaction and activation of complement by one of EHEC ´s main virulence factors, Shiga toxin 2a (Stx2a). Recently it has been revealed that the biological properties and binding abilities of Stx2a, including its interaction with complement proteins, are determined by the structure of its A subunit (unnicked or nicked). Thus, assessment of complement regulation by Stx2a presenting different A subunit structures (different structural forms of Stx2a) is necessary for better understanding of the subsequent potential impact on eHUS pathogenesis.

The effect of the different structural forms of Stx2a on the regulation of the three complement activating pathways was evaluated by enzyme-linked immunosorbent assays (ELISAs) determining qualitative and/or semi-quantitative functionality of the pathways (Wieslab ®, SVAR).

Preliminary results indicated that the structure of the A subunit of Stx2a dictates the ability of the toxin to regulate the alternative pathway of the complement system. While Stx2a with unnicked A subunit showed to regulate complement by inhibiting the activation of the alternative pathway, Stx2a with artificially nicked A subunit did not have a direct effect on complement regulation.

Elucidation of the impact of different Stx2a structural forms on the regulation of complement system is crucial for better understanding of the biological properties of the different circulating Stx2a structural forms and their effect during EHEC infection. In the present study, Stx2a with unnicked A subunit may inhibit the activation of the complement alternative pathway. It remains to be clarified which roles the different structural forms of Stx2a play for the pathogenicity in the host.

ID: 144

Autoantibodies and complement activation in bronchoalveolar lavage and tissues of COVID-19 patients

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Complement activation and polymorphonuclear leukocytes (PMN) recruitment have been demonstrated in SARS-CoV-2 infection. Here we extended these results analyzing tissues and bronchoalveolar lavage (BAL) fluids from COVID-19 and control patients, showing the production of autoantibodies, the activation of the complement system and the consequent recruitment of PMN.

Lung tissues were obtained from autopsies of 12 SARS-CoV-2 positive and 3 negative patients while BAL were obtained from mechanically ventilated patients (6 SARS-CoV-2 positive and 5 negative). Deposits of IgG and IgA were detected by immunohistochemistry while spike protein, C1q, mannose-binding lectin (MBL), C4, C3, Factor B and C5b-9 were analyzed by immunofluorescence. Proteins were extracted from lung sections and IgG then separated. ELISAs were set up to evaluate IgG and IgA in BAL and their binding to tissue proteins. C5b-9, C5a and IL-8 levels in BAL were measured by ELISA and the presence of PMN was evaluated by peroxidase quantification.

IgG and IgA were detected on lung vessels and pneumocytes of COVID-19 patients, irrespectively from the uneven distribution of the Spike protein of SARS-CoV-2. IgG extracted from SARS-CoV-2 positive tissues and present in SARS-CoV-2 positive BAL were able to target proteins expressed in SARS-CoV-2 positive lungs; these antibodies were also able to bind proteins purified from non-infected lungs, demonstrating the formation of autoantibodies during SARS-CoV-2 infection. Mass-Spect analysis, to characterize cell surface proteins immunoprecipitated by IgG, indicated multiple targets of these autoantibodies.

Autoantibodies and complement activation, but not IL-8 levels, correlate with PMN recruitment in SARS-CoV-2 positive samples, contributing to the development of the inflammatory process in COVID-19.

ID: 85

Autoantibodies targeting C1s in systemic lupus erythematosus: prevalence in patients with active lupus nephritis

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Background
Systemic lupus erythematosus (SLE) is a multi-system inflammatory disease characterized by production of various autoantibodies. Lupus nephritis (LN) is one of the most frequent and serious complications in SLE patients. Conventional biomarkers still lack sensitivity and specificity for detecting ongoing disease activity in lupus kidneys and early relapse of nephritis. Defective clearance of apoptotic bodies is involved in SLE pathogenesis. This study aimed to investigate the presence of anti-C1s antibodies (Abs) in SLE patients, and to evaluate their interest in LN.

Methods
Anti-C1s Abs were measured in 147 SLE patients' sera by ELISA using recombinant pro-enzyme C1s and activated plasma C1s. SLE was quiescent in 68 of these patients, while it was active in 79 patients (40 with active LN and 39 with arthritic flare). Antibody specificity was investigated in sera with positive anti-C1s Abs, using C1s fragments.

Results
Of the 147 SLE patients, 49 presented anti-C1s Abs, the majority of them with active disease (75%). Interestingly, active LN was significantly associated with the presence of anti-C1s Abs ($p<0.0001$), with a prevalence of 67% in these patients. Anti-C1s Abs titers were correlated to anti-dsDNA titers ($p<0.001$) and to the SLEDAI ($p<0.001$). Regarding the specificity of anti-C1s Abs, they recognized different types of C1s: recombinant proenzyme C1s only (31%), activated plasma C1s only (22%), or both (47%). Further differences were observed by testing the anti-C1s Abs reactivities against an activated C1s fragment (catalytic CCP1/2-SP domain) or the activated recombinant C1s. These results suggest that SLE patients can have different types of anti-C1s Abs.

Conclusion
Abs targeting C1s might be interesting new biomarkers for the diagnosis and follow-up of patients with SLE, as they are more frequent in patients with active SLE (especially LN) than in quiescent SLE patients. Additional longitudinal studies are needed to determine their predictive value in SLE.

ID: 177

Autoantibodies to complement components in rheumatoid arthritis

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Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disorder primarily affecting the peripherial joints. Many patients carry anti-citrullinated protein autoantibodies (ACPA). Overactivation of the complement system seems to be part of the pathogenesis of RA, though it is not completely

understood yet. Autoantibodies against the regulator of the alternative pathway, factor H (FH) were previously reported. Our aim was to analyse the presence and role of autoantibodies against FH and additional complement proteins in a RA cohort.

Serum samples of 106 ACPA-positive RA patients and 117 healthy controls were analysed for autoantibodies against FH, factor B (FB), C3b, C3-convertase (C3bBbP), C1q and factor I. Binding site, isotype and titer of the autoantibodies were determined in ELISA. IgG fraction of one patient was isolated by Protein G column and analysed. We detected in vivo formed FB–autoanti-FB complexes in the patient's serum by Western blot that have been removed before further experiments. We analysed the effect of the complex-free autoantibodies on the formation, activity and FH-mediated decay of the C3-convertase in solid phase convertase assays. Haemolysis assays and fluid phase complement activation assays were performed to investigate the effect of the autoantibodies on complement functions.

Two patients had C1q autoantibodies, and five patients had FB autoantibodies. Isotype of the FB autoantibodies in the analysed patient were IgG2, IgG3, IgGκ, IgGλ and their binding site was localized in the Bb part of FB. The autoantibodies partially inhibited the complement-mediated haemolysis of rabbit red blood cells, inhibited the activity of the solid phase C3-convertase and C3 and C5b-9 deposition on complement activating surfaces.

In addition to ACPA, we identified FB autoantibodies, which had inhibitory effect on complement. These results support the involvement of the complement system in the pathomechanism of RA, but further analyses are needed to assess the role of the autoantibodies.

ID: 80

BCX9930, an Oral Potent and Selective Factor D Inhibitor, Suppresses Complement Alternative Pathway (AP) C3 Convertase Activity in vitro and in Patients with Complement 3 Glomerulopathy (C3G)

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INTRODUCTION
C3G is characterized by dysregulation of the AP and is often associated with a prolonged half-life of C3 convertase linked either to autoantibodies that stabilize the AP C3 convertase or mutations in proteins that lead to increased stability of the AP C3 convertase. Factor D is essential for the formation of the AP C3 convertase, C3bBb. This study evaluated the effects of BCX9930, a potent Factor D inhibitor being developed for the treatment of complement-mediated diseases such as C3G, on the formation of C3 convertase.
METHODS
Formation of C3bBb, complexed with properdin (C3bBbP), in normal human serum after zymosan activation was analyzed by C3bBbP Elisa. Effects of oral administration of a single dose of BCX9930 600 mg on the *ex vivo* formation of AP C3 convertase were assessed using serum samples from 16 healthy subjects and 6 subjects with C3G. The formation of C3bBbP was analyzed by measuring C3a generation

after *ex vivo* AP activation using immunofixation electrophoresis of supernatants from the AP Wieslab assays.

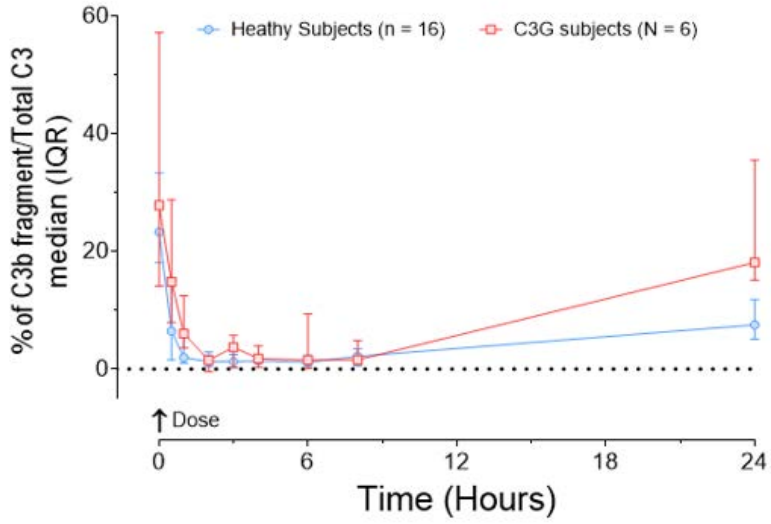
RESULTS

BCX9930 potently inhibited *in vitro* formation of AP C3 convertase in serum after zymosan activation with an IC_{50} of 26 nM. A single oral dose of BCX9930 600 mg resulted in rapid (within □¤1 hour) and maximal (median >99% relative to pre-dose levels) suppression of the formation of C3a, a product of C3 convertase activity, in both healthy subjects and subjects with C3G. BCX9930 potently inhibited the formation of AP C3 convertase by >90% through 8 hours post-dose for both groups (Figure 1).

CONCLUSION

Oral administration of BCX9930 potently suppressed the formation of AP C3 convertase and complement-split products in both healthy subjects and in C3G subjects with dysregulation of complement activity. These results support the further development of BCX9930 for the treatment of C3G and other complement-mediated diseases.

Figure 1. BCX9930 Inhibition the formation of AP C3 convertase in healthy subjects and subjects with C3G



Oral administration of single dose of BCX9930 600 mg resulted in rapid and sustained suppression of ex vivo AP C3 convertase formation

ID: 142

Binding and activation of native C3 on activated platelets without proteolytic cleavage suggesting a new activation mechanism

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Objective: The complement cascade is amplified by the alternative pathway (AP) by formation of C3 convertases triggered by the CP, LP and AP. According to the present dogma the AP is initiated via spontaneous hydrolysis of C3 to C3(H_2O), but we recently have shown that C3(H_2O) is a poor initiator of the AP. Also, C3 can bind and get activated on solid material surfaces without undergoing proteolytic activation. Here, we compared the ability of C3, C3b and C3(H_2O) to bind to and initiate the AP on activated platelets and apoptotic cells.

Methods: Blood was obtained through venipuncture from healthy volunteers and in hirudin or EDTA blood collection tubes. Platelet-rich plasma (PRP) was obtained by centrifugation. Activation of platelets were validated by the expression of P-selectin (CD62 P). The interaction of various forms of C3 and C3 fragments with activated and aggregated platelets was analyzed by capillary immune electrophoreses (WES), flow cytometry and multiplate either in PRP, whole blood or in purified platelet preparations. The ability of the surface bound C3 and C3 fragments to form AP C3 convertases (C3a generation) was analyzed in the presence of factor B, D and properdin.

Results: Native C3, C3b and C3(H_2O), all bound to activated platelets. Properdin increased the binding of the different forms of C3. Unexpectedly, native C3 bound most strongly to the platelets followed by C3b and C3(H_2O). Also, formation of C3a in the presence of B, D and P was most efficient with native C3, followed by C3b and C3(H_2O).

Conclusion: Complement C3 has stronger binding to activated platelets than C3b and C3(H_2O), which was enhanced by properdin. C3 inhibition reduced platelet aggregation and the platelet bound C3 was able to form a C3 convertase. These results propose a novel C3-activation mechanism that leads to platelet-leukocyte interactions during thromboinflammation.

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ID: 154

Biolayer interferometry provides a robust method for the functional characterization of alternative pathway C3-convertase (C3bBb)

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The formation of the alternative pathway (AP) C3-convertase (C3bBb) is the centerpiece of the complement system. The active convertase catalyzes the cleavage of C3 into C3a and C3b. While the small C3a will leave the complex, the nascent C3b can be covalently attached to the activating surface via its exposed thioester group and initiate the assembly of novel C3bBb complexes. Due to this positive feedback loop inherent to the AP, it plays a major role in determining the effector functions of the entire complement system.

In spite of its key importance, developing assays for the continuous and simultaneous monitoring of the formation, activity, and decay of AP C3-convertase is a challenging task. Indeed, previous methods frequently rely on the measurement of C3a production as an indirect readout of convertase activity, use artificial chromogenic substrates, or label C3b and Bb enabling fluorescence resonance energy transfer (FRET) measurements once these proteins form a complex. Surface plasmon resonance (SPR) analysis with chip-bound C3b is suitable for in-depth analysis of the convertase. Nevertheless, the amount of the proteins needed for this technology limits its usability.

Biolayer interferometry (BLI) provides a cost-effective alternative to SPR due to significantly lower amounts of proteins needed. Using this technology, we developed a novel label-free platform that enables the real-time monitoring of the convertase activity. The AP convertase was generated on the surface of the sensor and followed by incubation with C3. The accretion of nascent C3b on the sensor enabled the determination of the kinetic parameters of solid-phase C3bBb complex. The values calculated are in good agreement with historical data[1]. Besides, we used this technology to obtain new insights into convertase regulation to promote the development of innovative drugs.

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ID: 158

Bystander mechanism promoted the formation of membrane attack complexes on melanoma-associated neutrophils

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Previous studies suggested that the complement system and neutrophils are essential parts of the tumor microenvironment. Recently, a large body of evidence indicates that neutrophil extracellular traps (NETs) are involved in cancer progression and metastatic dissemination. However, in the context of tumor progression, knowledge on the crosstalk between the humoral complement system and the cell-mediated neutrophil innate immunity is still scarce. Here, we report that the complement cascade activation is initiated on the tumor endothelium. Further analysis of mouse and human tumor tissue samples indicated that neutrophils become MAC-positive in close proximity to the tumor endothelium, while neutrophils without physical contact to the endothelium remained MAC negative. This result suggests the occurrence of a process, known as bystander effect. The bystander effect describes the transfer of the MAC lipophilic and metastable precursor C5b-7 from a complement activating surface to an adjacent bystander cells. In vitro experiments with human endothelial cells and neutrophils could further confirm this bystander effect mediated MAC formation, indicating a close crosstalk between tumor microenvironment-primed endothelial cells and infiltrating neutrophils. We further analyzed the interplay of MAC and neutrophils in more detail in vitro and found MAC formation on tumor-associated neutrophils is a key step for NETs formation. Additionally, we examined the release of citrullination of histone 3 (CitH3) from MAC-positive neutrophils in murine and human melanoma tissues. Immunofluorescence images show that CitH3 and MAC were formed in close proximity to each other suggesting MAC-induced NETs in melanomas. Taken together, we provided a novel mechanism of the crosstalk between the complement system, endothelial cells and neutrophils in the context of tumor progression. The results reported here extend the role of the complement system as a component of tumor promoting inflammation and indicate the formation of MAC on tumor-associated neutrophils as potent trigger for NETs.

ID: 186

C1-inhibitor is protective against local and remote reperfusion injury of amputated pig limbs after prolonged ischemia

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Background: Revascularization of traumatically injured extremities is hampered by ischemia/reperfusion injury (IRI), characterized by activation of endothelial cells and the plasma cascades. Previous data showed that C1-inhibitor (C1-INH) was protective against IRI in rodents. Here we used an *in vivo* large-animal model to assess the effect of C1-INH in limb IRI.

Methods: Pig forelimbs were amputated and left at 22 °C. After 2h they were either rinsed with C1-INH (3 IU/mL in HAES, n=3) or HAES (n=3) as control, followed by another 7h ischemia. Limbs were then replanted, aiming for 12h reperfusion. Clinical data, blood, and tissue samples were analyzed for muscle/organ damage, complement activation, antibody deposition and cytokine release.

Results: IRI was revealed in the control group, shown as muscle damage (dystrophin staining), tissue deposition of IgM, complement C1q, C3b/c, and C5b-9, and significantly decreased endothelial integrity. C1-INH treated limbs showed significantly less complement deposition and better protection of endothelial integrity compared to control limbs. In addition, C1-INH treated limbs showed significantly lower wet/dry ratios (4.5 ±0.3 vs. 5.0 ±0.2) of muscle tissue and serum CK-MM levels (1071 ±61 vs. 1648 ±106 ng/mL), compared to control limbs. Markers for endothelial integrity, like E-selectin, were restored in reperfused muscle tissues of the C1-INH group compared to those without C1-INH. Moreover, as compared to controls, there was significantly less complement deposition (C4b/c, C3b/c, C5b-9) as well as antibodies (IgM, IgG) in lungs and kidneys from C1-INH treated animals.

Conclusions: While data are still preliminary (n=3) and the study is ongoing, our results suggest that a simple rinse of amputated pig limbs with C1-INH containing solution reduces IRI upon in vivo reperfusion. C1-INH treatment seems to protect both the vascular endothelium and the muscle tissue from deposition of complement as well as immunoglobulins. The protective effect of C1-INH was also seen in the remote organs (lung, kidney).

ID: 167

C4b binding protein protects Group A streptococci from phagocytic killing through internalization in human macrophages and inhibition of inflammasome

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Background. The ß-hemolytic group A streptococcus (GAS) ranks among one of the most dangerous pathogens, causing more than half-million deaths annually. Its membrane virulence factor M-protein triggers inflammasome activation in human macrophages, which leads to secretion of pro-inflammatory cytokines and cell death. Our group has previously shown that the complement inhibitor C4b-binding protein (C4BP) attenuates inflammasome activation by amyloid fibrils, which commonly cause inflammation in type 2 diabetic patients. Surface-bound M-proteins recruit C4BP that in turn reduces complement activation. Therefore, we hypothesized whether C4BP may modulate bacterial uptake, intracellular survival and, finally, the inflammasome response in human macrophages.
Methods. An *ex vivo* sepsis model using fresh human blood was used to investigate bacterial survival. The cytokine IL-1β response was measured by ELISA and a selective reporter cell line, which only detects the mature form of the cytokine. Bacterial uptake in the presence of C4BP, and survival in human cells was investigated using confocal microscopy and western blotting. A set of clinical GAS strains was included in our study.
Results. Recruitment of C4BP to the bacterial surface protects GAS isolates from killing by human blood and hampers IL-1β release in the presence of actin polymerization inhibitors, such as cytochalasin D and latrunculin B. Interestingly, phagocytosis of GAS by human macrophages is not modulated by C4BP, which instead is taken up by cells together with bacteria. Our data also show that cell-internalized C4BP is not present inside low-pH vesicles, but instead localizes within the cytosol and at the inflammasome complex. Thus, we observed that the presence of C4BP prevents the inflammasome response induced by GAS in human macrophages.
Conclusion. We therefore conclude that C4BP recruitment by GAS represents a clever strategy for

bacteria to reduce the immune response triggered by the inflammasome, which may allow intracellular GAS-dependent infections to pass unnoticed.

ID: 44

C4b-binding protein inhibits particulate- and crystal-induced NLRP3 inflammasome activation

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The NLRP3 inflammasome contributes to pathology and inflammation in a large number of human diseases, but endogenous inhibition is poorly characterized. C4b-binding protein (C4BP) is a serum inhibitor of the classical and lectin pathways of complement, but we have also previously identified it as an inhibitor of the NLRP3 inflammasome triggered by amlyoidogenic peptides, in the absence of serum. We now used serum-purified and recombinant C4BP, together with *in vitro* activation of human primary monocyte derived macrophages, to investigate the effect of C4BP on other inflammasome triggers. We found that serum-purified C4BP also inhibits NLRP3 inflammasome activation triggered by crystals (monosodium urate, MSU) and particulates (silica), which cause inflammation in gout and silicosis, respectively. Using a panel of recombinant C4BP mutants, we identified that C4BP binds MSU and silica via alpha-chain domains. C4BP was taken up into macrophages when bound to MSU/Silica, and inhibited inflammasome complex assembly and subsequent IL-1b release. Internalised C4BP had close colocalization to the ASC speck (apoptosis-associated speck-like protein containing a CARD), but did not affect ASC polymerization, as assessed by microscale thermophoresis. However, when internalized with MSU/silica particles, C4BP protected against lysosomal membrane damage, a trigger for NLRP3 activation, as demonstrated by staining for galectin 3, a marker of damaged lysosomal membranes. Furthermore, *C4bp*$^{-/-}$ mice showed increased inflammation after i.p. MSU injection. Addition of WT mouse serum to MSU-stimulated bone marrow derived macrophages also inhibited IL-1b production, compared to *C4bp*$^{-/-}$ serum, even after heat inactivation, demonstrating an inflammasome inhibitory function of C4BP independent of particulate-stimulated complement activation.

ID: 89

C4bp hijacking by the Plasmodium falciparum sporozoite stage

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BACKGROUND: Malaria is a mosquito-borne disease that accounted for 627.000 deaths in 2020. The fact that various stages of the malaria parasites survive in human blood suggests that they escape destruction by complement[1]. Malaria is transmitted by mosquitoes injecting *Plasmodium* sporozoites (SPZ) into human skin, where they migrate and penetrate blood vessels before infecting the liver[2]. We have previously discovered that a region at the N-terminal domain (NTD) of the circumsporozoite protein (CSP) binds the complement regulator C4bp. In this work, we study the CSP-C4bp interaction and its effects on SPZ gliding motility in the presence of complement.

METHODS: For all experiments *P. falciparum* NF54 SPZ were used. Purified C4bp and serum C4bp binding to SPZs was evaluated by radioligand, IFA and ELISA assays. CSP-C4bp interaction was studied by co-immunoprecipitation (Co-Ip), and the interaction with the NTD was evaluated by ELISA. Different anti-CSP antisera specific for various CSP domains combined with IFA were used to determine the exposure of the full-length CSP or its truncated form missing the NTD on SPZ. Finally, the effect of serum on SPZ was studied in a gliding motility trail assay.
RESULTS&DISCUSSION: We observed C4bp binding to *P. falciparum* SPZ following incubation with serum and with purified C4bp. Unexpectedly, not all SPZ were capable of binding C4bp to the same extent. Co-Ip assays combined with ELISA confirmed the interaction between the NTD of CSP and C4bp. Analysis of the CSP domains on SPZ revealed that SPZ exposed different levels of CSP NTD explaining the heterogeneous binding of C4bp. Finally, an anti-C4bp antibody was found to disturb SPZ gliding motility in the presence of complement. We suggest that the CSP-C4bp interaction contributes to the evasion of the human complement by *P. falciparum* SPZ.

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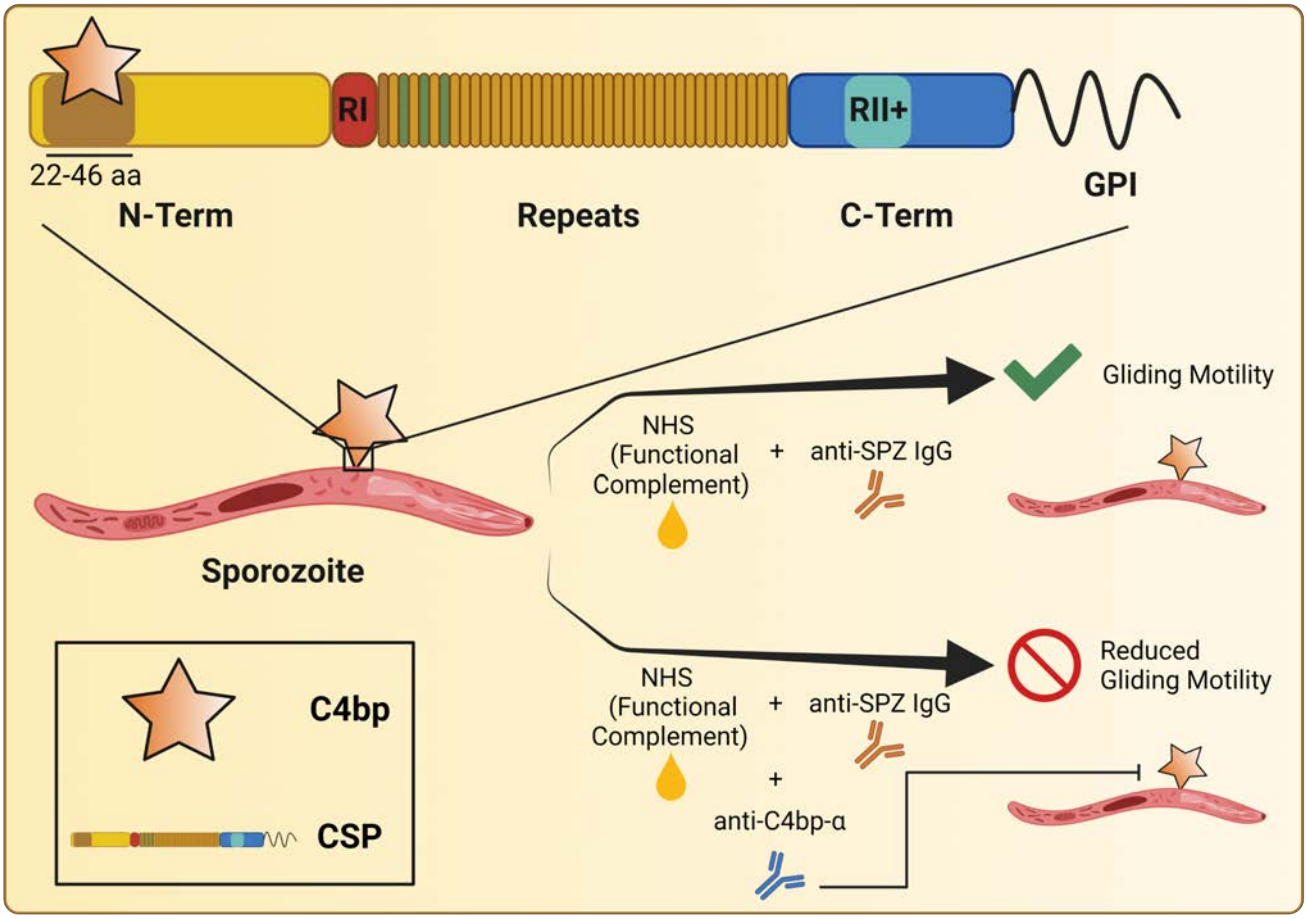

ID: 184

C5a signalling in myeloid cells regulates ILC2 development through unique, cell-dependent mechanisms

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Worldwide, more than 300 million people are affected by allergic asthma, an inflammatory disorder characterized by dysregulated immune response to common airborne allergens. Cellularly, dendritic cells (DCs) and Innate lymphoid cells type 2 (ILC2), play key roles during allergen sensitization, while humoral factors like the anaphylatoxins C3a and C5a, have been recognized as potent regulators of both development and severity of the disease via their binding to their cognate receptors C3aR, C5aR1, and C5aR2. Although C3aR signaling in DCs has been recognized as an important regulator of ILC2 function in allergic rhinitis, the role of C5a in the ILC2 development remains elusive. ILC development is driven by phosphorylation of STAT 3, 4, 6, the expression of GATA3, which participates to the ILC2 commitment, and the secretion of the type 2 cytokine interleukin (IL)-5. Using an *in vitro* developmental assay, we observed that bone marrow (BM)-derived macrophage/dendritic (BM-M-DC) cells promoted ILC2 development. Further, stimulation of BM-M-DC with C5a increased the differentiation of ILC2, while C5a had no direct effect on ILC2. Mechanistically, C5a-activated BM-M-DC enforced the expression of GATA3 in ILC2, while C5a-induced IL-23 secretion by BM-M-DC triggered the phosphorylation of STAT 4 in ILC2. Interestingly, a similar effect was observed using sorted pulmonary MHC class II-expressing cells, consisting mostly in DCs. When using sorted pulmonary myeloid cells stimulated with C5a, we observed that the main drivers of ILC2 development were the alveolar macrophages and the DCs. While the former promoted the production of IL-5 via the C5a-dependent secretion of IL-1a, the latter promoted the ILC2 differentiation via cell-cell contacts. Overall, our data show that C5a contributes to the development of the ILC2 through unique, cell-specific signaling pathways, and suggest that C5a signaling axis may be a potential pharmacological target to regulate ILC2 development in allergic asthma.

ID: 86

C5aR2 deficiency ameliorates inflammation in murine epidermolysis bullosa acquisita by regulating C5a- and FcγR-mediated functions of neutrophils

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Epidermolysis bullosa acquisita (EBA) is a rare blistering skin disease induced by autoantibodies directed against type VII collagen (COL7). Passive transfer of antibodies against murine COL7 (mCOL7) into mice mimics the effector phase of EBA and results in a subepidermal blistering phenotype. The complement system and in particular the C5a/C5aR1 axis, which is important for the recruitment and activation of neutrophil granulocytes, the main effector cell in this model, are important and well-documented players in the pathogenesis of EBA. However, the role of the second C5a receptor, C5aR2, in the pathogenesis of EBA is still elusive. Therefore, we sought to delineate the functional relevance of C5aR2 during the effector phase of EBA. Here, C5aR2-deficient (*C5ar2*$^{-/-}$) mice showed an attenuated disease phenotype, suggesting a pathogenic contribution of C5aR2 to disease progression. *In vitro*, *C5ar2*$^{-/-}$ neutrophils exhibited significantly reduced reactive oxygen species release, as well as $(Ca^{2+})_i$ flux, and migratory capacity when activated with immune complexes or exposed to C5a in a C5aR1-dependent manner. Moreover, C5aR2 deficiency lowered the A/I ratio of FcγRs and thus impeded the sustainment of inflammation. Consistent with these findings suggesting that deficiency of C5aR2 on neutrophils is the driving cause of the amelioration of disease severity in the EBA mouse model, mice with neutrophil/monocyte-specific C5aR2 deficiency (*LyzM*^{cre} *x C5ar2*$^{-/-}$) mimicked the results found in the *in vivo* and *in vitro* experiments with global *C5ar2*$^{-/-}$ mice. Moreover, transcriptome analyses (scRNA-seq) of neutrophils show a lower number of differentially expressed genes in *C5ar2*$^{-/-}$ mice compared with WT mice after C5a stimulation, suggesting that full activation of neutrophils by C5a requires both C5a receptors. Collectively, we demonstrate here a pro-inflammatory contribution of C5aR2 to the pathogenesis of antibody-induced tissue damage in experimental EBA that is dependent on neutrophil activation.

ID: 64

C7 but not C5aR is essential to the development of atypical haemolytic uraemic syndrome in the C3 gain-of-function model.

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Atypical haemolytic uraemic syndrome (aHUS) is rare (incidence 0.53 per million population, PMID:25899302). We previously generated the C3 gain-of-function (GOF, $C3^{D1115N/D1115N}$, $C3^{N/N}$) mouse model of aHUS (PMID:30714990)[1]. Homozygote mice display all the hallmarks of aHUS i.e. acute kidney injury (elevated BUN, proteinuria and haematuria), thrombocytopenia, anaemia and thrombotic microangiopathy (evidenced in renal histology) associated with significant AP dysregulation (systemic changes in C3, C5 turnover as well as significant C3 deposits in the glomerulus).
We aimed to tease apart the molecular pathways that drive disease via backcrossing our model onto C7 and C5aR deficient mice. Renal function, histological appearance, platelets, reticulocytes and C3/C9/fibrin deposits were analysed.
Three of twenty $C5aR^{-/-}.C3^{N/N}$ mice succumbed to disease (thrombotic microangiopathy, TMA) whilst no $C7^{-/-}.C3^{N/N}$ (N = 29) were lost in mice aged up to 12 months, compared with the survival of only 1 of 23 and 4 of 14 $C3^{N/N}$ matched littermates, respectively. BUN, platelet, reticulocytes were normalised in the C7 and the majority of C5aR deficient animals. No histological evidence of renal injury could be identified

in the $C7^{-/-}.C3^{N/N}$ mice up to 12 months of age (similar to that seen in C5 deficiency (PMID:30714990)), suggesting C5b-8 & C5b-9 play a predominant pathogenic role in renal TMA.
These data suggest that both C5aR and C7 do play a role in the development of the conditions required for TMA in the kidney. However, removal of the membrane attack complex is critical to prevent the inducement of a thrombotic phenotype in the C3 GOF model. While clearly distinct, our findings share some similarities with those noted with the renal TMA based on W1206R change in FH (PMID: 29858280). Overall, these data confirm the importance of C5 blockade in aHUS but also provide some rational for targeting C7 for complement inhibition in patients with complement mediated aHUS.

Reference 1:

Smith-Jackson, K., Y. Yang, H. Denton, I. Y. Pappworth, K. Cooke, P. N. Barlow, J. P. Atkinson, M. K. Liszewski, M. C. Pickering, D. Kavanagh, H. T. Cook, and K. J. Marchbank. 2019. Hyperfunctional complement C3 promotes C5-dependent atypical hemolytic uremic syndrome in mice. J Clin Invest 129: 1061-1075.

ID: 43

Cell-intrinsic C3 mediates pancreatic beta-cell survival in inflammatory and cell stress conditions

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C3 is highly expressed in human islets and this expression is further upregulated during diabetes. We used CRISPR/Cas9 to create gene-edited INS-1 beta-cell clones to investigate the role of C3 in beta-cell biology. The loss of C3 expression rendered INS-1 cells more susceptible to apoptosis induction by the presence of IL-1beta, a cytokine involved in both diabetes type 1 and type 2 development, as measured by annexin-V binding, viability assays, and caspase 3 cleavage. This was not due to our previously reported role for C3 in autophagy. We have shown that C3 can be expressed within the cytosol from an alternative downstream translational start site; in gene-edited cells lacking the canonical C3 AUG start site (ΔAUG1), C3 is expressed within the cytosol in an unglycosylated and unprocessed pro-C3-like form, but is not secreted. ΔAUG1 INS-1 cells had rescued viability when challenged with IL-1beta, which was reversed by C3 siRNA treatment, demonstrating a pro-survival function of cytosolic C3 in beta-cells. The reduction in viability of C3 KO cells was not reversed by addition of exogenous serum, purified C3, or C3a, showing that cell-intrinsic C3 is required. To verify the cell-intrinsic role of C3 in viability, we used C3-flox/RIP-Cre mice, which have normal amounts of C3 in serum, but lack beta-cell C3 expression. These mice were treated with streptozotocin, a beta-cell specific toxin. C3-flox/RIP-Cre mice developed diabetes at a faster rate then control C3-flox mice, indicating a more rapid loss of beta-cells in the absence of beta-cell C3 expression. Together, these results support a role of cell-intrinsic cytosolic C3 as a protective factor in beta-cells.

ID: 173

Characterization of a Novel Small Interfering RNA for Complement Regulation Through Targeted Silencing of C3

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Background: Dysregulated complement activation plays a key role in the pathophysiology of many diseases, making complement an attractive therapeutic target. The third component, C3, represents a clinically validated target for broad control of complement activity because of its central position within the cascade. However, high abundance of circulating C3 and other complement proteins often necessitates therapeutic intervention with frequent dosing. We describe a novel C3-targeting small interfering RNA (siRNA) with potential for a favorable dosing regimen over other therapeutic approaches.
Methods: >5000 siRNA sequences potentially targeting the C3-mRNA were screened *in silico* designed 177 were selected for synthesis. *In vitro* potency was assessed by C3-mRNA silencing post transfection in HepG2 cells and primary human hepatocytes (PHH). Six siRNAs were selected for further chemical optimization to enhance silencing activity. For selective delivery to hepatocytes, siRNAs were conjugated to an N-acetylgalactosamine cluster. A lead candidate (APL-3007) was identified based on potency and tolerability. *In-vivo* pharmacology of APL-3007 was further characterized in cynomolgus monkeys at doses of 0.3, 3, and 30 mg/kg administered subcutaneously (N=3).
Results: From the *in silico* screen, 177 siRNAs were identified for synthesis and *in vitro* potency testing. In HepG2 and PHH, several compounds showed subnanomolar and submicromolar 50% IC_{50} values, respectively. Introducing chemical modifications at selected positions enhanced siRNA potency in a sequence-specific manner. APL-3007 was stable over 48 hours in human and monkey biological matrices. Additionally, transfection of human peripheral blood mononuclear cells did not stimulate cytokine release. APL-3007 was well tolerated and selected as the lead candidate for nonclinical development. Following a single administration of APL-3007 in monkeys, robust, persistent, and dose-dependent silencing of serum C3 protein and complement-mediated hemolytic activity was observed.
Conclusions: APL-3007, a novel C3-targeting siRNA, demonstrated robust and durable silencing of C3 and complement inhibition in cynomolgus monkeys in a dose-dependent manner.

ID: 152

Characterization of C5aR1-blocking monoclonal antibodies

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Background: The evolutionarily conserved complement system protects against infections and maintains tissue homeostasis. Activation of the complement system leads to various effector functions, including immune cell activation via anaphylatoxins C3a and C5a. We aim to characterize the role of anaphylatoxins

and their respective receptors (C5aR1, C5aR2, C3aR) in the pathogenesis of *Aspergillus fumigatus* by the generation of receptor-modulating monoclonal antibodies (mAbs). This is thought to contribute to the establishment of new antifungal treatment strategies.

Methods: Mouse-mAbs were developed by utilizing hybridoma technology using defined synthetic peptides, resembling exposed sections of all three human anaphylatoxin receptors as antigens. Screening of hybridoma clones was carried out on blood cells. aC5aR1 mAb blocking properties were initially determined via a granulocyte activation assay. The standardized C5aR1 iLite system was used to investigate commercial and in-house aC5aR1 mAb blocking efficacies while testing commonly used non-peptidic (W-54011, Avacopan) and peptidic (PMX-53) C5aR1 antagonists.

Results: Several promising mAbs targeting C5aR1 were identified. Blocking of C5aR1 with isolated mAbs prior to stimulation with native human C5a displayed a fundamental reduction of the selected activation marker signals (CD11b and CD66b) on isolated neutrophils for a fraction of mAbs. Several in-house aC5aR1 mAbs exceeded commercially available C5aR1-blocking mAbs within the C5aR1 iLite system, displaying low nanomolar half-maximal inhibitory concentration values.

Conclusions: Several potential mAb candidates for C5aR1 detection and blocking were identified.

ID: 94

CipA of Acinetobacter baumannii terminates complement-mediated killing by the formation of a factor I-mediated, quadripartite-ordered assemblage

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Multidrug-resistant *Acinetobacter (A.) baumannii* is known to be one of the leading pathogens that cause severe nosocomial infections. To overcome eradication by the innate immune system, *A. baumannii* developed a number of sophisticated strategies. Previously, we identified CipA as a plasminogen-binding and complement-inhibitory protein. In order to further elucidate the molecular mechanisms by which CipA impairs complement, different functional assays were employed. Here we show that CipA terminates all three complement activation pathways by binding of Factor I. Utilization of different CipA variants suggested that the Factor I-interacting region was localized within the C-terminus of the protein. Moreover, CipA by binding factor I promotes the formation of a quadripartite-ordered complex to degrade C3b and C4b in the presence of complement regulators Factor H and C4BP, respectively. We also show that loss of Factor I binding directly correlates with a higher susceptibility to human serum when a CipA knockout *A. baumannii* strain or a strain producing a CipA variant lacking Factor I-binding capability were investigated. This finding indicates that recruitment of serum-derived Factor I is crucial for *A. baumannii* to resist complement-mediated killing. Moreover, structure predictions of CipA and a Factor I-negative CipA variant using AlphaFold2 revealed that the orientation of the C-terminal beat sheet domain is necessary for the interaction with Factor I. In conclusion, CipA is a multifunctional, inhibitory molecule that affects complement activation by the formation of a factor I-mediated quadripartite complex.

ID: 32

Circulating Extracellular Vesicles - A Potential Biomarker for Viral but Not Bacterial Sepsis?

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Bloodstream infections (BSIs) and their manifestation, sepsis, are linked to severe patient conditions. Currently, the diagnosis of BSI is based on blood culture, supported by molecular-based methods and common pro-inflammatory biomarkers. Yet, biomarkers are lacking the ability to discriminate the presence of a pathogen in the bloodstream over systemic, non-infectious inflammatory response, both presenting similar symptoms. Here, we investigated circulating extracellular vesicles (EVs) as a potential novel biomarker for BSI.

In total, 81 septic plasma samples (73 septic patients) and 25 plasma samples from healthy individuals were investigated for established and novel sepsis biomarkers, including C-reactive protein (CRP), procalcitonin (PCT), and EVs. The amount of EVs and their association with CRP were analyzed using flow cytometry. Furthermore, the presence of complement proteins C3 and terminal complement complex (TCC) was detected by Western blotting.

The 81 septic plasma samples were collected from patients with (n = 16) or without (n = 65) confirmed BSI. Additionally, 14 of these patients suffered from COVID-19. CRP and PCT levels were significantly elevated in BSI compared to non-BSI patients. The amount of EVs and CRP-bound EVs was significantly increased in plasma of patients compared to healthy controls. While non of the experimental parameters showed the potential to serve as a BSI biomarker, the number of circulating EVs was significantly increased in septic COVID-19 patients compared to septic non-COVID-19 patients. Moreover, immunoblotting demonstrated the presence of C3 and TCC on all EVs with a tendency of increased band intensities for EVs derived from BSI patients.

In conclusion, the results of our study proposed the number of circulating EVs as a potential biomarker of COVID-19 and therefore viral, but not bacterial, sepsis. In addition, the presence of complement proteins was demonstrated on EVs from septic patients and healthy controls. Further studies in a larger setup need to confirm these results.

ID: 87

Circulating levels of endogenous complement inhibitors correlate inversely with complement consumption in Systemic Lupus Erythematosus

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Background:
Complement proteins execute a dual function in the pathogenicity of systemic lupus erythematosus (SLE). They play a key role in preventing the disease, but exacerbate the disease after onset. Nevertheless, there is limited knowledge about the regulation of the complement system by endogenous soluble complement inhibitors. Neuropsychiatric systemic lupus erythematosus (NPSLE) is one of the poorest understood manifestations of SLE. Currently, there is no biomarker for NPSLE. Here, we investigate the levels of complement inhibitors in SLE, their relation to disease activity, and their value to discriminate between SLE and NPSLE.
Methods:
Complement inhibitor levels were determined in serum of 356 SLE patients and 108 healthy controls. Four in-house sandwich ELISAs were developed and standardized for C1-inhibitor (C1-INH), C4b-binding protein (C4BP), Factor I, and Factor H. An additional 53 samples were measured in sera obtained at two time points.
Results:
The C1-INH and C4BP levels were increased in SLE sera compared to the healthy controls (median 148.8 AU/ml vs 98.4 AU/ml, $p<0.0001$ and median 124.2 AU/ml vs 116.1 AU/ml, $p<0.0288$), while Factor I and Factor H levels were both decreased (median 74.1 AU/ml vs 85.5 AU/ml, $p=0.0004$ and median 164.0 µg/ml vs 200.0 µg/ml, $p<0.0001$).
Comparing samples obtained during two time points revealed a normalization of the levels of C1-INH and Factor H over time as C1 -INH levels significant decreased (median 156.0 AU/ml vs 101.2 AU/ml, $p< 0.0001$) and Factor H levels increased (median 188.0 µg/ml vs 234.0 µg/ml, $p=0.004$).
Conclusion:
The disease activity of SLE associate differentially with the levels of the endogenous complement inhibitors, with an increase in C1-INH and C4BP probably as part of the acute phase response and a decrease in Factor I and Factor H, probably as a result of complement consumption. The levels of complement inhibitors have no discriminative value for the presence of NPSLE.

ID: 114

Classical pathway complement activation hinders interaction of the IgG Fc-tail with Fc-gamma receptor CD32

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Fc-gamma receptors (FcγRs) influence the activity of IgG antibodies by their ability to activate immune processes in FcγR-positive cells such as NK cells and neutrophils. Besides interacting with FcγRs, target bound IgG can also activate the classical pathway of the complement system. This requires direct binding of C1 to hexamerized IgG and results in deposition of other complement factors on the antibody coated target surface. It is currently unknown how the complement reaction affects the IgG-Fc:FcγR interaction. In this project, we studied the contribution of IgG-Fc:FcγR interaction to phagocytosis of antibody-coated *Staphylococcus aureus* by neutrophils in the presence of complement. We show that, while classical pathway complement deposition enhances bacterial uptake by neutrophils, it significantly reduces the contribution of FcγRs to the phagocytosis process. Furthermore, we use CD32 expressing cell lines and show that C1 itself can hinder IgG-Fc:CD32 binding, depending on the ability of IgGs to form hexamers. The subsequent surface deposition of C4b and C3b further reduces efficient IgG-Fc:CD32 interactions. Using natural IgG and monoclonal IgG antibodies, this study provides fundamental insights into antibody mediated effector functions in natural immune response and therapy.

ID: 17

Combined inhibition of complement (C3/C5) and the Toll-like receptors co-factor CD14 in inflammation induced by Candida spp.

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Candida albicans is one of the foremost fungi causing opportunistic infections in immunocompromised patients. Complement and the Toll-like receptors (TLRs) are two main components of our innate immune system, reacting upstream by being initial recognizers of pathogens. The complement system is often evaded by *Candida albicans*. It has been shown before that double inhibition of complement, directly targeting C3 or C5, and of CD14, a key co-receptor for TLR4, TLR2, and others, in Gram-negative *Escherichia coli* and *Neisseria meningitides*, as well as several Gram-positive *Staphylococci* species, significantly decreases the inflammatory response in the whole blood. Now, we for the first time describe the effect of this double inhibition in fungi, using an *ex vivo* human whole-blood model of *Candida albicans* induced inflammation.
Complement activation products (terminal C5b-9 complement complex [TCC] and C3 activation [C3bc]), granulocyte (myeloperoxidase) and platelet activation (β-thromboglobulin), cytokines (tumor necrosis factor [TNF], IL-1β, IL6, and IL-8), and leukocyte activation (CD11b) were quantified, by enzyme-linked immunosorbent assays, multiplex technology, flow cytometry, and *iLite® Cell-Based Solutions*.
In *Candida albicans*-incubated whole blood, preliminary results of the double inhibition of both C5 and CD14 showed a decrease of most inflammatory markers. As seen before for bacteria, when compared to the single inhibition, the double inhibition strategy presented a synergistic effect, with CD14 inhibition potentiating the blockade of C5.
In conclusion, despite the limitations of our *ex vivo* model, the double inhibition of complement and

CD14 shows potential as a future therapeutic approach for immunocompromised patients suffering *Candida albicans* infections and sepsis with adverse innate immunity-induced inflammatory responses.

ID: 48

Common variants in C3 and CR1 enhance complement attack on PNH erythrocytes and increase risk for C3-mediated extravascular haemolysis

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Background
Paroxysmal nocturnal haemoglobinuria (PNH) is a rare blood disorder characterised by complement-mediated destruction of PNH erythrocytes (E) due to the loss of CD59. The anti-C5 antibody, eculizumab, inhibits this; however, 30% of patients still require blood transfusions. This is associated with C3 fragment accumulation on PNH-E due to the loss of CD55, causing extravascular haemolysis (EVH) by erythrophagocytosis. C3b deposition and breakdown to iC3b/C3dg fragments is controlled by binding of complement receptor type 1 (CR1) to C3b with factor I (FI), forming a trimolecular complex (TMC). We hypothesise that C3 and CR1 polymorphisms influence the efficacy of C3b inactivation and can dictate EVH risk.
Methods
Eculizumab-treated PNH patients were genotyped for their C3 (rs2230199,S/F) and CR1 (rs11118133,H/L) polymorphisms and C3 loading on PNH-E were measured by flow cytometry. Interaction of CR1 to C3b variants and TMC formation were analysed using surface plasmon resonance. Effect of CR1 density polymorphism on C3b inactivation was assessed by incubating C3b-coated streptavidin beads with solubilised E from either CR1-H/H or -H/L donors and iC3b/C3dg formation was measured by flow cytometry.
Results
Patients who have the C3-S/S allele and intermediate (H/L) CR1 expression showed a trend for higher mean percentage of C3 loading. We observed higher affinity of CR1 to C3b-F than C3b-S leading to higher levels of TMC formation with FI. Conversion of C3b/iC3b to C3dg was more effective in CR1-H/H donors compared to CR1-H/L.
Conclusions
Our data indicate that C3 and CR1 density polymorphisms influence C3b loading and inactivation on PNH-E, influencing EVH risk. Weaker binding of C3b-S to CR1 led to a decreased regulatory TMC formation with FI and individuals with lower CR1 expression convert iC3b to C3dg less effectively. Overall, our data demonstrate mechanisms that enhance erythrophagocytosis in PNH by an increased C3 loading and a decreased in C3b inactivation.

ID: 67

Comparing the classical haemolytic setup to Functional WIESLAB® Complement Screen

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The complement system is a key component of the innate immune system and acts through three different pathways called classical, lectin, and alternative pathways, respectively. The activity of each pathway is triggered by different mechanisms; however, they merge quite early at the level of C3, after which all pathways follow the same route towards the formation of the Membrane Attack Complex. The complement system has gained a lot of focus from both a clinical and a drug development point of view, as increased understanding of the complement cascade has revealed how the dysregulation of the system can make the human body susceptible to infections on one end and cause inflammatory conditions on the other.

Many labs today continue to use the classical hemolytic setup utilizing sheep erythrocytes. This method carries certain challenges. The classical setup is labor-intensive and often difficult to standardize and is also prone to have stability and quality issues. An alternative to this can be functional assays of the complement cascade, such as Svar's WIESLAB ® Complement System Screen.

WIESLAB® Complement Screen (Svar Life Science) is based on ELISA, where the complement cascade in a patient sample is triggered by various activators specific to each pathway. Each pathway is allowed to run until the endpoint when the terminal complement complex is formed, and this complex is then detected to show the level of activation. The sum of all pathways also allows for narrowing down to a few selected "problem" components, based on the combination of how each pathway responds.

The conclusion drawn from the presented validation study is that the WIESLAB® Complement Screen closely correlates with the haemolytic assay, while adding to it with the benefits of an ELISA, such as robustness, reproducibility, and ease of performance.

ID: 150

Comparison of ex vivo and in vivo models to assess reperfusion injury in amputated pig limbs after prolonged ischemia

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Background: Complement activation plays a major role in ischemia/reperfusion injury (IRI) of amputated and replanted extremities. We aim to develop a clinically relevant large animal model to study limb IRI and pharmaceutical interventions with complement inhibitors in pigs. From a 3R point of view, an ex vivo setting would clearly be desirable. We therefore compared ex vivo with in vivo reperfusion of amputated pig limbs after prolonged ischemia.

Methods: Amputated porcine forelimbs exposed to 1h (control) or 9h of ischemia at 22 °C were reperfused for up to 12 hours. Extracorporeal machine perfusion using heparin-anticoagulated whole blood at 37 °C (ex vivo) was compared with replantation to the donor pig (in vivo). Muscle damage was evaluated systemically by CK-MM ELISA and locally by dystrophin staining. Complement activation was assessed as C3b/c and C5b-9 in tissue and C3a in plasma.
Results: After 9h of ischemia, limbs of both the ex vivo and in vivo groups had an increased muscle damage compared to 1h of ischemia. However, when extracorporeal perfusion was used, muscle damage was more pronounced. Tissue complement deposition was significantly increased in ex vivo perfused limbs and a 3-fold increase of plasma C3a at endpoint was observed in ex vivo vs. in vivo reperfusion after 9h of ischemia ($p=0.0056$). This correlated with higher plasm levels of CK-MM (30'400 vs. 1'400 ng/ml, respectively, $p<0.0001$), and increased tissue damage as observed by dystrophin staining.
Conclusion: Our preliminary findings show that both ex vivo and in vivo models mimic an IRI scenario. The more pronounced damage seen with extracorporeal perfusion may be an artifact caused by additional complement activation on tubings and filters of the heart-lung-machine. Thus, although an ex-vivo system can be used for screening of therapeutic drugs, an in vivo setting may still be required for a final confirmation and assessment of systemic consequences of IRI.

ID: 191

Compartmentalized early cascade complement activation predicts disease severity in Multiple Sclerosis

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Introduction: Complement (C) in combination with immunoglobulins (Ig) might play an important pathophysiological role in Multiple Sclerosis (MS).

Objectives: To investigate if intrathecal C-activation is increased a)in MS patients, b)especially in those with intrathecal IgM synthesis; and to determine associations with c)MS Severity Score (MSSS) and d)neurofilament-light-chain (NfL) levels in cerebrospinal fluid (CSF).

Methods: C and their activation components (Factor H,I,C1q,C3,C4,C5,Ba,Bb,C3a,C4a,C5a,sC5b-9) were measured in CSF and plasma of 112 clinically-isolated-syndrome (CIS) and 127 MS patients (90 relapsing-

remitting (RRMS), 14 primary-progressive (PPMS), 23 secondary-progressive (SPMS)) and 44 healthy controls (HC).
Patients were categorised in ascending order for presence of oligoclonal IgG bands (OCGB), intrathecal $IgG_{(IF=IntrathecalFraction)}$ and IgM_{IF}:
1)$OCGB^-/IgG_{IF}^-/IgM_{IF}^-$,
2)$OCGB^+/IgG_{IF}^-/IgM_{IF}^-$,
3)$OCGB^+/IgG_{IF}^+/IgM_{IF}^-$,
4)$OCGB^+/IgG_{IF}^+/IgM_{IF}^+$.
MSSS was determined at last follow-up visit.
Linear regression, adjusted for age, sex and albumin-ratio, was used to investigate associations of a)MS subgroups (vs HC) and b)CSF-Ig-categories 2-4 (vs 1) with C-levels in CSF and plasma. Associations of the 12 C_{CSF}-levels with c)MSSS and d)NfL-levels were analysed.
Results: a) In CIS/MS, CSF (but not plasma) levels of C3a,C4a,Ba,Bb were increased, e.g. PPMS had 85% and 53% higher C3a- and C4a-levels than HC (both $p<0.01$).
b)CSF-levels of C3a,C4a,Ba,Bb,C1q were increased along CSF-Ig-categories in CIS/MS, i.e. were highest in IgM_{IF}^+-patients: increase for C3a:128%; C4a:50%; Ba:37%; Bb:55%; C1q:21% (all $p<0.01$).
c)Doubling of $C3a_{CSF}$ was associated with a MSSS increase of 0.69 units ($p<0.01$) in CIS, and of 0.61 units ($p<0.01$) in CIS/MS overall.
d)In CIS/MS CSF levels of C3a,C4a,Ba and Bb correlated with NfL: e.g. doubling of C3a increased NfL-levels by 34%, C4a:30%, Ba:29%, and Bb:17% (all $p<0.01$).

Conclusions: Compartmentalized early cascade complement-activation is increased in MS, especially in presence of $IgM_{IF,}$ and correlates with future MSSS and NfL-levels, pointing towards an important pathophysiological role. Complement inhibition might be a novel therapeutic option for attenuating disease severity in MS.

ID: 88

Complement 5a Receptor 2 (C5aR2) expression regulates NKp46 levels on NK cells and influences formation of pulmonary metastases

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Although no longer considered a mere decoy receptor, the functional properties of C5aR2 still remain enigmatic. Recently, we reported the expression of C5aR2 on murine natural killer cells, surprisingly without the usual co-expression of C5aR1. We observed that the systemic absence of C5aR2 leads to a strongly increased NKp46 expression in NK cells. This newly found NKp46 overexpression raises the proposition that systemic C5aR2 expression negatively regulates NKp46 surface levels and might therefore be involved in modulating NK cell cytotoxic activity, possibly relevant in the response towards metastatic tumors. Thus, we investigated the effects of a lack of C5aR2 in a NK cell-dependent murine pulmonary metastases model.

B16-F10 murine melanoma cells were injected intravenously into wild type, systemically and NK cell-specific C5aR2-deficient C57BL/6 mice through the tail vein. The disease progress was monitored daily. On day 7, 14 or 21 post injection, macroscopically observable surface metastatic foci were enumerated.

Our investigations reveal that systemically C5aR2-deficient mice developed fewer metastatic foci on the lung surface compared to their wild type counterparts after tumor cell injection. Consistently, C5aR2-deficient mice featured a lower disease score and a better overall survival. Surprisingly, NK cell-specific C5aR2-deficient mice did not show a reduced tumor burden, or a milder disease progress compared to wild type controls. In accordance with these findings, our experiments show for NK cell-specific C5aR2-deficient mice that NKp46 levels are similar to those in wild type mice.

Our findings demonstrate for the first time the relevance of systemic C5aR2 expression in context of NKp46 regulation and thus, metastatic cancer. We hypothesize that systemic C5aR2 expression has a negative effect on the regulation of NKp46 surface levels during NK cell development. Therefore, C5aR2 could prove a useful target to augment NKp46-dependent NK cell cytotoxicity, ultimately preventing metastases formation.

ID: 107

Complement activation and thromboinflammation in patients subjected to left ventricular assist device implantation

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Background: Left ventricular assist device (LVAD) implantation is a life-saving therapeutic intervention for patients with terminal heart failure that do not qualify for heart transplantation (1). Implantation can lead to thromboinflammatory complications, varying from bleeding to thromboembolic events, because of the mechanical shear stress and surface-induced activation of the blood cascade systems (1). The etiology of thrombosis is well characterized (2), but the inflammatory response is scarcely studied. Here, we aimed to investigate complement activation and thromboinflammatory response in patients supported by LVAD implantation.

Methods: Blood samples were collected from patients (n=8) before LVAD implantation (baseline), directly after implantation, for the next five following days, and at a three-month follow-up. Ten age- and gender-matched healthy individuals served as controls. Complement activation was

evaluated in EDTA-plasma by measuring C3bc, C3bBbP, and the terminal C5b-9 complement complex (TCC) by ELISA. Additionally, the samples were analyzed for cytokines and other thromboinflammatory markers by multiplex technology and ELISA.

Results: Elevated concentrations of complement activation products were observed directly after LVAD implantation; C3bc, C3bBbP, and TCC were increased by 1.8, 6.2, and 5.6 times respectively, compared to baseline. These levels decreased during the following days but remained significantly higher over the whole observation period compared to the control group ($p<0.05$ for C3bc and C3BbP, $p<0.01$ for TCC). All complement activation markers were significantly lower ($p<0.05$) at the three-month follow-up than at the baseline sampleobtained before implantation. Several thromboinflammatory markers, including tumor necrosis factor (TNF), interleukin (IL)-2, and IL-8, and matrix metalloproteinase (MMP)-8 and -9, were elevated after implantation. The difference was statistically significant for IL-8 ($p<0.05$).

Conclusions: This study shows that LVAD implantation mediated a substantial inflammatory response at implantation, including substantial complement activation, which potentially could contribute to thromboinflammatory complications in the acute phase.

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ID: 140

Complement activation in acute multisystem inflammatory syndrome in children

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Multisystem inflammatory syndrome in children (MIS-C) is a rare, life-threatening complication of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. MIS-C develops with high fever, marked inflammation and shock-like picture several weeks after exposure to, or mild infection with, SARS-CoV-2. Complement has been implicated to play a role in the pathogenesis of MIS-C, but detailed analysis of the complement profile, including all three pathways, as well as the presence and potential triggers of complement activation, such as anti SARS-CoV-2 antibodies caused by preceding or persistent COVID-19, was not performed until now.
We hence aimed to investigate complement activation in MIS-C during the acute stage of the disease,

and to analyze potential causes behind complement activation, especially possible relationships between classical pathway activation and the serological response following SARS-CoV-2 infection in MIS-C.

Concentrations of several complement parameters and anti SARS-CoV-2 antibody levels were determined in acute stage and remission samples of 34 MIS-C cases, as well as in 18 healthy control children.

Activation markers of the classical (C1s/C1-INH complex and C4d), alternative (Bb) and terminal pathways (sC5b-9) are highly elevated during acute stage of MIS-C, while the lectin pathway (measured by MASP-1/C1-INH complex levels) was not found to be activated. Besides that, activation of the classical pathway is largely independent of the anti-SARS-CoV-2 humoral immune response. Alternative pathway activation is stronger associated with terminal pathway activation than the classical pathway, indicating a more profound role of the alternative pathway in the pathomechanism of MIS-C.

In summary, multiple complement activation products are markedly elevated in acute MIS-C, and complement measurements are an appropriate tool to support diagnosis and to monitor response to therapy in MIS-C. Complement parameters showing closest association with acute MIS-C include classical pathway marker C1s/C1-INH complex, alternative pathway split product Bb, global marker C3a, and terminal pathway marker sC5b-9.

ID: 156

Complement activation is increased upon air contact in open- versus closed-circuit normothermic machine perfusion.

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Background and aim: The shortage of donor kidneys is a widespread problem. Normothermic machine perfusion (NMP) may increase the donor pool by enabling the use of extended criteria for donor kidneys, which today are discarded. The complement system gets activated upon blood-air contact [1] and activation of the complement system can lead to increased renal ischemia-reperfusion injury (IRI) [2]. The aim of this study was to investigate if a closed-circuit NMP, with minimal blood air interface, would reduce complement activation complement compared to standard open-circuit NMP.
Methods: Kidneys from Norwegian landrace pigs of either sex, bodyweight 60 kg +/- 10% (n=12) were static cold preserved at 4^0C for 18 hours, and then re-perfused with heparinized autologous whole blood for 4 hours at 39^0C. The kidneys from each pig were randomized to either an open- or a closed-circuit NMP system. Blood and urine samples were collected at set time points, and tissue samples were collected at the end of NMP.
Results: All kidneys showed rapidly stabilized renal resistance after the start of perfusion. There was no significant difference in urine production between the open- and the closed-circuit NMP. Both systems showed a similar trend of renal flow throughout the perfusion period. Terminal complement complex

(TCC) was significantly increased ($p<0.0001$) in open- compared to closed-circuit NMP in plasma; open system increased median 8.5 – 1275 (IQR 6.1–11.9 – 399-2536) and closed increased median 10 – 98.6 (IQR 5.7-12.5 – 52.9-159.8) from beginning to end of the experiment. No significant differences were seen in urine TCC between both groups.
Conclusion: Open-circuit NMP activates complement significantly compared to a closed-circuit NMP system. This may indicate that kidneys run on a closed-circuit NMP experience less IRI, and thus may have a prolonged long-term graft survival.

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ID: 84

Complement Alternative and Mannose-Binding Lectin Pathway Activation Is Associated With COVID-19 Mortality

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Background:
The SARS-CoV-2 infection triggers excessive immune response resulting in increased levels of pro-inflammatory cytokines, endothelial injury, and intravascular coagulopathy. Complement activation participates to this hyperinflammatory response. However, it is still unclear which activation pathways (classical, alternative, or mannose-binding lectin (MBL) pathway) pilot the effector mechanisms that contribute to critical illness. To better understand the immune correlates of disease severity, we performed an analysis of complement activation pathways and components in samples collected from COVID-19 patients and of their relationship with the clinical outcomes.

Methods:
We conducted a retrospective, single-center study cohort in 74 hospitalized patients with RT-PCR-proven COVID-19. The functional activities of pathways and the antigenic levels of the components C1q, C4, C3, C5, Factor B, and MBL were measured in patients' samples during hospital admission. Hierarchical clustering with the Ward method was performed to identify clusters of patients with similar characteristics of complement markers. Then, the clusters were compared with the patient clinical features: rate of intensive care unit (ICU) admission, corticoid treatment, oxygen requirement, and mortality.

Results:
Four clusters were identified according to complement parameters. Among them, two clusters revealed remarkable profiles: in one cluster (n=15), patients exhibited alternative and lectin pathways activation and low antigenic levels of MBL, C4, C3, Factor B, and C5 compared to all other clusters; this cluster had the higher proportion of patients who died (27%) and required oxygen support (80%) or ICU care (53%). In contrast, the second cluster (n=19) presented inflammatory profile with high classical pathway activity and antigenic levels of complement components; a low proportion of patients required ICU care (26%) and no patient died in this group.

Conclusion:
These findings argue in favor of prominent activation of the alternative and MBL pathways in severe COVID-19, but the spectrum of complement involvement seems to be heterogeneous requiring larger studies.

ID: 76

Complement and Haemostasis: Effects of complement inhibitors in a microfluidic bleeding model

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Background: Even though the coagulation cascade is responsible for the formation of clots, other physiological systems, like the complement system, can influence it. There have been several studies on the interactions between the complement system and clot formation, mainly in the context of atherosclerosis, inflammation, and thrombosis. To investigate a potential role of the complement system in the haemostatic response upon vessel injury, we have established a microvascular endothelialised whole blood flow model. This model allows the imaging of clot formation after vessel injury in real-time by confocal microscopy.

Aims: To investigate the role of the complement system during clot formation upon vascular injury in a microfluidic bleeding model.

Methods: Endothelial cells were used to create a synthetic vessel in a polydimethylsiloxane mould. Whole blood was then flown through the vessel and an injury was created by rupturing a collagen pouch

adjacent to the vessel. The formation of the clot was monitored in real-time with a confocal microscope, fluorescently labelled fibrinogen and antibodies against P-Selectin (CD62P). The fluorescence signal intensity over time was then compared with and without the following inhibitors targeting complement activation on different levels: inhibitory antibodies anti-C1s, anti-FD and anti-C5, the C3 inhibitor Cp20, and the MASP-1 inhibitor SGMI-1.

Results: We could show so far that inhibition of MASP-1 and FD prolonged the bleeding time after vessel injury, also platelet activation (CD62P) and fibrinogen/fibrin signal was lower compared with the controls. C1s inhibition however showed quite a variation. Preliminary experiments with C5 inhibitory antibodies showed a lower fibrinogen/fibrin signal at the injury site compared with the control. Experiments with Cp20 are underway.

Conclusions: Both the bleeding time and markers of clot formation at the injury site suggest an anticoagulant effect of complement inhibition on the clotting process. Further experiments are underway to confirm and extend our results.

ID: 8

Complement biomarker profiles across the spectrum of disease in COVID-19 reveal alternative pathway dysregulation as an early predictor of outcome and marker of severe disease.

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Background: Multiple lines of evidence implicate complement dysregulation as a driver of pathology in COVID-19, including the demonstration of complement activation products in plasma and in diseased tissues. However, several questions remain unanswered, notably, which parts of the complement system are dysregulated, when during the disease course complement dysregulation occurs and whether there is an optimal time to intervene with anti-complement drugs.

Methods: ELISA and MSD were used to measure 16 complement components, regulators and activation products, in 1084 plasma samples from 701 hospitalised COVID-19 patients (UK ISARIC4C cohort). Samples were collected at various intervals post-admission up to day 14 and segregated based on peak disease severity, severity at time of sampling, ICU admission and death as outcome. Data were subjected to unsupervised clustering, PCA, PLS-DA and ROC analysis to assess disease severity and outcome in admission samples and across the disease course.

Results: Cluster analysis of admission samples revealed four complement biomarker clusters that correlated with disease severity and outcome. Compared to heathy controls, most of the complement biomarkers were significantly increased in COVID-19 admission samples in all severity groups. Elevated levels of the alternative pathway activation markers Ba and iC3b and decreased levels of the regulator

properdin in admission samples were strongly predictive of COVID-19 disease severity and death as outcome. Properdin levels were better predictors of disease severity than either of the widely used markers CRP or neutrophil count. Decreased levels of the terminal pathway regulator clusterin also predicted more severe peak disease and death. When analysed across the timecourse, Ba levels were highly predictive with progressive elevation correlated with severe disease and death.

Conclusions: The data demonstrate complement dysregulation as an early event in COVID-19 and show that the degree of dysregulation predicts outcome. Early interventions with anti-complement drugs targeting alternative pathway might modulate the outcomes.

ID: 170

Complement C1s deficiency in a male Caucasian patient with systemic lupus erythematosus

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Deficiencies of the early complement components of the classical pathway are well described to be associated with systemic lupus erythematosus (SLE) or SLE-like syndromes and severe pyogenic infections. Among these deficiencies, complete C1s deficiency has been reported in 9 cases so far. Here we describe a 34-year old, male patient who presented with severe, recurrent infections (meningitis, erysipelas, subcutaneous abscess, infections of the upper airways) since childhood as well as adult-onset SLE (6/11 ACR criteria) with proliferative lupus nephritis (LN).
A screening of the complement cascade showed not measurably low CH50, while the function of the alternative pathway was normal. Subsequent determination of complement components revealed undetectable C1s with low levels of C1r and C1q and normal (C3) or slightly elevated (C4, C2) concentrations of other complement proteins. The patient had no anti-C1q antibodies. A renal biopsy showed class IVA LN with low positivity for complement C1q along the glomerular basement membranes and deposition of IgG, IgM and complement C3 in the mesangium and glomerular basement membranes. In an ELISA-based functional complement assay determining C4d deposition, the patients missing complement activity could be completely restored by the addition of active C1s. The genome of the patient was analyzed by whole genome sequencing showing two truncating variants in the C1s cDNA gene. One mutation was located at nucleotide 514 in exon 5, caused by a nucleotide substitution from G to T, resulting in a nonsense mutation from Gly172 (G172X). The other mutation was located at nucleotide 750 in exon 7, where C was replaced by a G, resulting in a nonsense mutation from Tyr250 (Y250X). Both mutations create a premature stop codon and have not previously been reported in the literature. The genetic findings in combination with absent C1s in circulation strongly argue for a total C1s deficiency in our patient.

ID: 13

Complement factor H promotes survival of regulatory T-cells via interaction with the inducible T-cells co-stimulator (ICOS)

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Background: Glioma is a common primary intracranial tumor with significant mortality and morbidity. The tumor microenvironment has a decisive role in tumor progression, and its components, including complement proteins and immune cells, often dictate the malignancy outcome. Like other checkpoint molecules, ICOS fine-tunes the immune response and regulates T-cell survival, activation, and suppressive abilities.

Materials and Methods: Regulatory T-cells (Tregs) were isolated from peripheral blood. H4 cells were stably transfected with factor H (FH). FH-ICOS binding was assessed using proximity ligation assay, co-immunoprecipitation, ELISA, and protein microarray. Viability was determined by flow cytometry. Immunosuppression was measured by thymidine incorporation. ICOS pathway was blocked with CAL-101. Cytokine profile was analyzed with ELISA. FH was detected in a glioma mice model using immunofluorescence.

Results: Strikingly, we discovered that plasma-purified and glioma cell line-derived FH binds to ICOS and promotes the survival of primary human Tregs. Only ICOS+ T-cells survived better in the presence of FH and this effect was diminished by blocking ICOS. Stimulation with FH also enhanced the primary function of Tregs – immunosuppression. Inhibition of the ICOS pathway decreased this immunosuppression, suggesting that FH-induced changes in cytokine expression follow from ICOS signaling. In a glioma mice model, we found that FH is expressed in brain tumors and colocalizes with tumor cells. We confirmed this finding using the Cancer Genome Atlas database. Additionally, we found that higher expression of FH positively correlated with the occurrence of Tregs, and also correlated with worse overall, and disease-free, survival amongst other clinical parameters.

Conclusion: Binding of FH to ICOS in the glioma microenvironment is associated with increased viability of Tregs and decreased patient survival. Since the accumulation of Tregs represents an auspicious prognostic and therapeutic target, FH expression should be scrutinized when considering the effectiveness of immunotherapies against glioma.

ID: 163

Complement feedback mechanism detaches MBL and C1q from their targets

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Regulation of the complement system is an essential feature of the cascade, preventing excessive activation and uncontrolled inflammatory conditions. Hence, there are soluble and membrane-bound inhibitory proteins within the complement system that maintain the balance by inhibiting enzymatic activity. This study shows a novel regulatory mechanism happening directly on the pattern recognition molecules (PRMs).
The binding of C1q and MBL to a clinical strain of *Aspergillus fumigatus* (strain 6871) was measured by ELISA and flow cytometry in parallel with C4b, C3b and TCC deposition over a time course of 64 minutes. MBL binding to *A. fumigatus* conidia in serum peaked after two minutes and afterwards started to decline, while C4b, C3b and TCC deposition increased. MBL binding was about 80% decreased after 64 min. By inhibiting the deposition of C4b, binding of MBL was preserved to a great extent, whereas inhibition of C3b only slightly affected the MBL release. This suggests that a feedback mechanism via C4b hinders continued activation via MBL. The same was observed using rMBL and MBL defect serum. Moreover, a fraction of released rMBL could re-bind to mannan. There is also a feedback mechanism working on C1q, as the binding decreased about 50% after 64 min of complement activation.
The underlying mechanism of these observations is elusive but suggests a novel intrinsic regulation of the activity of the lectin and classical pathway via C4 that dissolve complement PRMs interactions with ligands.

ID: 125

Complement FHR2 in patients with complement-mediated renal diseases

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Background: Two ultra-rare complement-mediated renal diseases (CMRD), C3 glomerulopathy (C3G) and atypical hemolytic uremic syndrome (aHUS) are caused by dysregulated complement activity of the alternative pathway (AP). Plasma factor H (FH) is the major negative regulator in the fluid phase and/or on cell surfaces. The *CFH* gene family includes five complement FH-related (*CFHR*) genes, which encode plasma proteins with a similar structural organization to FH [short consensus repeats (SCRs)]. Based on homologies of SCRs, FHRs are classified into two groups- type 1, which includes FHR1, 2 and 5; and type 2, which includes FHR3 and 4. Type 1 FHRs form homodimers and FHR1/2 heterodimers while type 2 FHRs circulate as monomers. FHR1 homodimers increase FHR1 avidity for C3b, deregulating FH and enhancing complement activation. However, the role of FHR2 remains unclear. We hypothesis that FHR2 plays a role in the pathogenesis of CMRD by modulating complement activity through its heterodimerization with FHR1, and that low FHR2 are a risk factor in developing CMRD.

Methods: Plasma FHR2 was quantified by Western blot and compared to serial dilutions of a standard.

Results: While six of 52 normal healthy individuals have low FHR2, in 56 patients with CMRD (C3G, n=35; aHUS, n=21), 15 or 27% of patients are FHR2 deficient ($P < 0.05$, patients vs controls). The difference in frequency of FHR2 deficiency between C3G and aHUS (8/35 *vs* 7/21 or 23% *vs* 33%) is not significant.

Conclusion: FHR2 deficiency is significantly high in patients with CMRD. Increased levels of FHR2 may reduce complement activity by heterodimerization with FHR1 and therefore may be protective. This study highlights the importance of the relative concentrations of FHR1, FHR2 and FH. When FH levels are low (below 250 mg/L), the relative concentrations of FHR1 and FHR2 are especially relevant if complement dysregulation is to be prevented.

ID: 159

Complement is activated during ex vivo porcine liver machine perfusion independent of existing liver injury

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Background and goal of study: Ischemia-reperfusion injury (IRI) is a key challenge in liver transplantation leading to both short- and long-term failure of the transplanted liver. Machine perfusion (MP) resembles extracorporeal membrane oxygenation to the liver *ex vivo* and is considered an important advance in organ preservation and evaluation prior transplantation. 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 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 vivo* liver MP was performed with 1h hypothermic, 1h rewarming and 4h normothermic perfusion. Heparinized white cell and platelet depleted blood was used as perfusate during rewarming and normothermic phases, while standard clinical preservation solution was used during hypothermic phase. Perfusate samples were collected at set timepoints and analysed for terminal complement complex (TCC) using ELISA.

Results: Prior MP, TCC was significantly higher in white cell and platelet depleted blood compared to whole blood ($p<0.01$). During MP, TCC increased significantly from start (median 24.75 interquartile range [12.21-36.83]) to end (100.48 [59.62-143.15], $p<0.001$), but there was no statistical difference between the biliary injury, the global injury, and the no liver injury groups ($p>0.5$).

Conclusion: These results indicates that standard clinical filtration of whole blood activates the complement system as measured by TCC. *Ex vivo* machine perfusion leads to high TCC formation and further analyses will be conducted to assess down-stream effects of complement activation. Inhibition of complement activation might be a therapeutic option during MP.

ID: 21

Complement mediated killing of Klebsiella pneumoniae is affected by colistin resistance mutations

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The rising numbers of multi-drug resistant bacteria is of great concern to healthcare worldwide. Special attention goes out to ESKAPE pathogens, including the Gram-negative bacterium *Klebsiella pneumoniae* which becomes increasingly resistant to β-lactam antibiotics. The complement system is a central part of the defence against Gram-negative bacteria as it can directly kill them via the membrane attack complex (MAC). MAC permeates the outer membrane, ultimately leading to destabilization of the inner membrane and lysis of the bacterium. Lipopolysaccharides (LPS) are a major component of the Gram-negative outer membrane and are crucial for protection against complement. But LPS is also the target of several antibiotics including colistin, which binds to LPS and destabilizes the Gram-negative cell envelope.

Because colistin and MAC both interact with the Gram-negative outer membrane, we wondered how development of colistin resistance in *K. pneumoniae* would affect bacterial sensitivity to MAC. Surprisingly, we found that colistin resistant (ColR) mutations, generated during an *in vitro* colistin evolution experiment, can sensitize *K. pneumoniae* to complement-mediated killing in human serum. Specifically, mutations in *phoQ* enabled the classical complement pathway to be more active on the ColR strain, thereby sensitizing it for killing by MAC. Mechanistic studies revealed that *phoQ* mutations specifically allowed IgM, but not IgG, to bind to the ColR strain and drive formation of bactericidal MAC pores.

Together this indicates that, due to the *phoQ* mutations, an epitope for IgM becomes reachable on the cell surface of the ColR strain that is important for the formation of bactericidal MAC pores. These findings demonstrate that mutations inducing antibiotic resistance can have an opposite effect on complement sensitivity and bactericidal clearance via the immune system.

ID: 5

Complement receptor 1 is expressed on brain cells and impacts roles of microglial relevant to Alzheimer's disease

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Background: Genome wide association studies in Alzheimer's disease (AD) have implicated complement in pathogenesis. Complement receptor 1 (CR1; CD35) is a top AD-associated GWAS hit; the CR1 long variant associates with increased AD risk. Roles of CR1 in brain health and disease are poorly understood; indeed, CR1 expression in brain is controversial. Our aim was to resolve this controversy by investigating

CR1 expression in brain cells and testing whether the AD-associated length polymorphism impacted CR1 expression, fate and function.
Methods: We tested CR1 expression in microglial cell lines and iPSC-derived microglia from long and short CR1 variant expressing donors. Cells were stained with CR1-specific monoclonal and polyclonal antibodies and analysed by immunofluorescence. RNA and protein were extracted and subjected to qRT-PCR and Western blotting. In iPSCs, CR1 was deleted using CRISPR. Human brain tissue was stained with CR1-specific antibodies and qRT-PCR was performed on RNA extracted from primary glial cells. Functional differences of microglia expressing long and short CR1 variants or knocked out for CR1 were tested in phagocytosis assays using diverse targets (E.coli bioparticles, synaptoneurosomes, amyloid β) either unopsonised or serum-opsonised.
Results: CR1 mRNA was detected in microglial lines and iPSC-derived microglia from different sources using qRT-PCR. CR1 protein was demonstrated using Western blotting and immunofluorescence in cell lines and iPS-derived microglia expressing long or short variant CR1. CR1 transcript and protein were detected in primary glia extracted from human brain and on microglia and astrocytes in situ. Phagocytosis of serum-opsonised targets was impaired in CR1 knockout microglia and significantly different in iPSC-microglia expressing long or short CR1 variants.
Conclusion: CR1 is expressed in the human brain. CR1 expression is an important component of microglial phagocytic activity; expression of the long (risk) variant of CR1 impacts phagocytosis, perhaps explaining its association with AD risk.

ID: 175

Complement- and antibody-mediated phagocytosis of Staphylococcus aureus: macrophages and neutrophils in comparison

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Staphylococcus aureus evolved multiple strategies to evade immune system recognition and killing mechanisms in addition to developing resistance to antibiotic treatments. Altogether, these features make *S. aureus* infections one of the most challenging infections to treat by traditional antibacterial therapies. Monoclonal antibody therapy could represent a valid therapeutic alternative, providing host immune cells more effective tools to deal with invading pathogens.
Bacterial opsonization by antibodies in combination with efficient complement activation drives pathogen phagocytosis and killing. This bacterial clearance is mainly controlled by a close collaboration between neutrophils and macrophages, the first immune cells recruited at the infection site.
In this work, we want to recreate *in vitro* a system where neutrophils and macrophages act in concert to fight *S. aureus*. Their phagocytic capacity is usually determined under adherent conditions (macrophages) versus suspension (neutrophils). We need to adapt the conditions for the two cell types to enable a fair comparison of their phagocytosis and killing capacity.
When infected in suspension, we observed a higher serum-dependent uptake of *S. aureus* by neutrophils compared to macrophages. However, when bacteria were opsonized with antibody-depleted serum, phagocytosis did not occur for both cell types. On the other hand, when cultured in adhesion, the two immune cells were able to uptake bacteria opsonized by both types of sera. Regardless of the culture conditions, complement and Fc-γ receptors expression was not affected in both cell types.
Next, we want to assess how the phagocytosis ability of the two immune cells can be affected by

opsonizing *S. aureus* with a combination of serum and monoclonal antibodies targeting staphylococcal surface structures. Eventually a proper variant of co-incubation of macrophages and neutrophils will teach us what is the best strategy to kill *S. aureus* and avoid persistence in long living cells.

ID: 77

Complementary Amplicon-Based and Capture-Based NGS Approaches for Accurate and Efficient Complement (CFH and CFHR Locus) Genetic Testing

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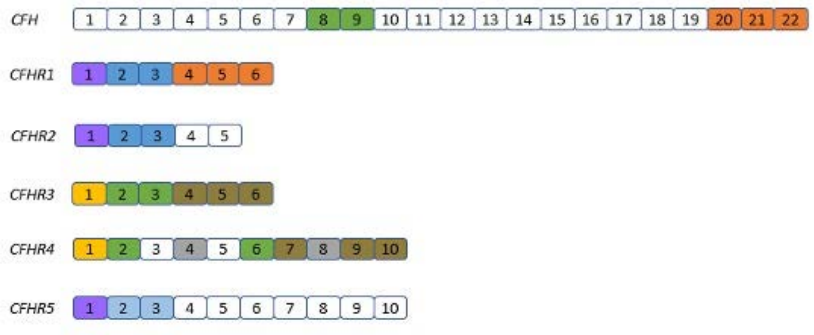
Factor H, which is encoded by the *CFH* gene, is a complement regulatory protein. The Complement Factor H Related proteins, encoded by *CFHR1* through *CFHR5*, share regions of homology with Factor H, both at the protein and gene level. This high degree of homology results in challenges for short-read next generation sequencing (NGS) chemistry due to inability to uniquely map reads. Therefore, NGS is often supplemented by long-range PCR (LR-PCR) and Sanger sequencing, along with multiplex ligation-dependent probe amplification (MLPA) for detection of variants in these genes.

We developed a complementary capture-based and amplicon-based NGS approach to interrogate the CFH/CFHR locus. The amplicon-based portion includes LR-PCR to specifically amplify select regions, which are then pooled together and sequenced by NGS, followed by bioinformatic analysis against a custom genome reference file that has all homologous regions removed that were not amplified by LR-PCR. The capture-based NGS portion includes interrogation of all regions in the genes of interest, alignment against GRCh37 reference file, and a bioinformatic pipeline that includes CNV calling. The results from the two NGS methods are combined, and any variants detected can be assigned to the appropriate gene by taking into consideration the presence or absence of the variant in both methods. 18 samples run with this combined method against our previous NGS method with supplemental Sanger sequencing and MLPA demonstrated 100% concordance for SNVs and 97.5% for CNV (*CFH* exon 20 yielded 7 false positives in the setting of *CFHR1* deletion). A single duplication (*CFHR5* c.254-2_266dup) at a low variant fraction (~20%) was not detected, likely representing a limitation of the bioinformatic pipeline.

This accurate and efficient method will decrease the time required for data review, as well as the cost of diagnostic testing, while yielding both CNV and sequence variant data for the complex CFH locus.



CFH and *CFHR* Family Homology at a Genetic Level. Each numbered box represents an exon, which are colored to indicate sequence homology.

ID: 91

Complementary effects of bacteriophages and complement on the killing of Acinetobacter baumannii

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Infections caused by multidrug-resistant (MDR) bacteria are becoming an emerging global health threat. Due to the lack of effective and novel antibiotics, there is an urgency to develop alternative therapeutic methods to treat infections caused by MDR bacteria. Phage therapy, which utilizes bacteriophages (phages) to infect and kill bacteria, has shown promising results to treat bacterial infections. However, the interactions between bacteria, phages and the human immune system are not well understood. Especially, we were wondering whether the phages would be neutralized by complement. Thus, we analyzed the interaction between phages and the complement system and its effect on bacterial killing. We followed the growth of serum-resistant clinical isolates of *Acinetobacter baumannii* by monitoring the optical density with Bioscreen analyzer up to 23 h. Isolate-specific phages effectively attenuated bacterial growth up to 5 h. Strikingly, when the bacteria and phages were cultured together, there was no growth at all in the presence of complement. Following exposure of the bacteria to the phages they became phage-resistant within 23 h. However, the phage-resistant bacteria became serum sensitive and showed an apparent loss of their capsules. Interestingly, the phage-resistant isolates were susceptible to serum-mediated killing but not completely, because within another 23 h of cultivation in the presence of serum, the bacteria were able to revert to serum resistance. The bacteria without capsule showed increased C5b-9 deposition compared to the wild-type, suggesting that the capsule shields *Acinetobacter* from opsonization and C5b-9-mediated bactericidal activity. Together, our results show that phages in the context of innate immune system could be a powerful approach to counteract bacterial adaptation to phage resistance. Furthermore, *Acinetobacter* capsule is essential for phage infection, but shields against complement-mediated bactericidal activity.

ID: 131

Contrasting effects of the factor H-related protein 1 & 3 gene deletion on renal allograft rejection in living versus deceased-donor kidney transplant.

Gaya da Costa, Mariana

Introduction Acute rejection after renal transplantation negatively impacts allograft function and survival. Local complement activation in the donor kidney plays a key role in the pathogenesis of renal injury inherent to transplantation. Recently, a combined gene deletion of factor H–related protein 1 and 3 (CFHR3,1Δ) was implicated in the pathogenesis of renal diseases. This single-nucleotide polymorphism (SNP) results in the deletion of CFHR1 and CHFR3, higher serum levels of factor H, and an increased risk

for autoantibodies against factor H. The effect of the SNP can, therefore, be harmful or protective. We explored the clinical effects of the CFHR3,1Δ SNP in the kidney donor on renal outcome after transplantation. Method The rs6677604 polymorphism was determined in 871 kidney donors, consisting of 159 living, 718 deceased donors. The association of the rs6677604-A allele with acute rejection was evaluated using Cox regression models. Furthermore, we performed a subgroup analysis among living and deceased donors. Results Overall, 31.8% of the donors were heterozygous and 3.0% were homozygous for CFHR3,1Δ. The frequency of the deletion was similar among living and deceased donors. During follow-up, 320 recipients developed acute rejection. Considering donor types, there was no association of the donor CFHR3,1Δ allotype with acute rejection. However, subgroup analysis revealed a hazardous association of the polymorphism in living donors and a protective association among deceased donors with the occurrence of acute rejection. In adjusted models, the deletion in living donors was independently associated with an increased risk for acute rejection.(hazard ratio 3.10; 95% CI 1.05–9.14; P=0.04). In deceased donors, the deletion was independently associated with a decreased risk for acute rejection (hazard ratio 0.73; 95% CI 0.51–0.96; P=0.02). Conclusion CFHR3-1Δ deletion in the kidney donor might impact the development of acute rejection, providing new insights over the role of complement regulators in acute rejection.

ID: 115

Copy number and sequence variations in the FHR1 gene influence complement regulation and cooperation with complement regulators

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FHR1 is a member of Factor H (FH) protein family which is involves in regulating the innate immune complement system and inflammation. However, the exact interplay between FHR1 and other complement regulators is still not completly understood. Several studies demonstrated that FHR1 competes with FH by binding same cell surfaces ligands, including complement C3 fragments and glycosaminoglycans, thereby impairing FH regulation and leading to complement activation. Other results showed that FHR1 inhibits complement C5 convertase activity and terminal complex formation (Skerka et al, 2021). Two common haplotypes of FHR1, respectively having in their SCR3 domain either "YVQ" or "HLE" residue, were found equally distributed in the population but the relevance of the two isoforms is much less documented.
By generating recombinant FHR1 variants, which either have "YVQ" or "HLE", we demonstrate that YVQ/HLE exchange influences FHR1 interaction with glycosaminoglycans and cell surfaces as $FHR1_{YVQ}$ binds more efficiently to heparin and modified endothelial cells surfaces compared to $FHR1_{HLE}$. Furthermore, by changing in SCR5 the FHR1 characteristic "LA" residue with the "SV" residue of FH in SCR20, we observe that, in contrast with modified $FHR1_{SV}$ constructs, $FHR1_{LA}$ significantly reduced erythrocytes lysis showing that the "LA" residue in SCR5 conferred to FHR1 a protective role against complement-mediated lysis. Our data also revealed that compared to FH, both FHR1 natural haplotypes binds more efficiently to heparin-C3d suggesting that FHR1 and FH may interact differently to C3-opsinized fragments exposed. In addition, using human FHR1-deficient serum, we observe that FHR1

levels directly affect the degree of FH cell-surface attachment. Overall, we demonstrate that genetic variations on the FHR1 gene have an impact on its role in complement. Our data also support that FHR1 is not solely a FH competitor but acts as a counterpart with FH in a dynamic control on the innate immunity.

Skerka et al, 2021; DOI: 10.1111/bph.15290

ID: 99

Copy Number Variations: A Missing Genetic Driver in C3G

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C3 glomerulopathy (C3G) defines a group of rare renal diseases, including C3 glomerulonephritis (C3GN) and Dense Deposit Disease (DDD), characterized by C3 deposition in the glomerulus associated with dysregulation of the alternative pathway of complement. A large portion of patients have an identifiable driver of disease, either genetic (~25%) and/or acquired (~50-80%). Genetic drivers are, in large part, identified by targeted genomic enrichment and massively parallel sequencing (TGE+MPS) of *C3, CFH, CFI, CFHR5, CFB*, *CD46* (*MCP*), and *DGKE*, together with multiplex ligation-dependent probe amplification (MLPA) of the *CFH-CFHR5* genomic region to detect copy number variations (CNV) and rearrangements. Screening of all C3G candidate genes for CNVs is not routinely performed. To address this knowledge gap, we compared MPS read-depth ratios across a large pool of samples (n=88) using a sliding window approach to identify potentially pathogenic CNVs. With this method, we identified a potential heterozygous deletion of four exons (exons 10-13) in the MG3-MG4 domains of *C3* in a patient diagnosed with C3GN who has a chronically low C3 levels. The deletion was predicted to result in a frameshift with a premature stop codon, consequently resulting in a null allele. The presence of the truncated allele (Δ ~2500 base pairs) was confirmed using long-range PCR and gel electrophoresis. Complement functional testing confirmed low C3 levels at 0.7 g/L (normal: 0.9-1.8 g/L); Ba was elevated (3.4 mg/L, normal: <1.2 mg/L) consistent with chronic kidney disease. To our knowledge, this is the first heterozygous multi-exon deletion in *C3* detected via TGE-MPS based CNV analysis. This case supports the importance of CNV detection in C3G and other complement-mediated renal diseases, and proposes an efficient, accessible method for identifying CNVs as drivers of disease that have been elusive to traditional detection methods previously.

ID: 38

CORVOS – facilitating more PhD student exchanges and working towards a European Complement PhD

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Ten European universities (Innsbruck, Lübeck, Mailand, Lund, Utrecht, Helsinki, Paris, Copenhagen, Oslo, Budapest), three biomedical companies (SVAR, Hycult, MSD), two research institutes (Statens Serum Institut, Research Centre Borstel) and two major hospitals (Helsinki, Innsbruck) have been granted with an ambitious European Horizon 2020 Marie Skłodowska Curie European Joint Doctoral programme, which started December 2019.

Opportunistic infections are defined as infections caused by pathogens primarily affecting immunocompromised subjects. Due to the immunodeficiency, they are difficult to treat and together the top-2 killers worldwide. Opportunistic pathogens comprise bacteria (e.g. *Mycobacterium tuberculosis)*, viruses (e.g., HIV), fungi (e.g., *Aspergillus fumigatus*) and parasites (e.g., *Plasmodium falciparum).*

Complement as a chief conductor of innate immunity plays a crucial role in immune defence against opportunistic pathogens. CORVOS - for COmplement Regulation and Variations in Opportunistic infectionS presently trains 15 widely recruited and highly motivated early stage researchers (ESRs). They will fill the current scientific gap in the understanding of the role of complement in opportunistic infections, and translate this knowledge into clinical practice.

The four ambitious research work packages include **Regulation & Variations, Pathogen evasion**, **Diagnostic tools,** and **Prevention & Treatment**.

Research and training are conveyed by the scientifically well recognized CORVOS faculty in a collaborative effort. CORVOS involves research secondments at partner universities and multiple training stays at the different intersectoral consortium members. All ESRs will receive highly attractive stipends and will be enrolled in existing interdisciplinary doctoral programmes always at two European universities which will jointly award Double PhDs. The ESRs will become creative alumni, equipped with entrepreneurial and clinical skills, making them pleasant human resources to academia and industry alike.

In conclusion, CORVOS is an attractive European PhD programme, focusing on complement research, and it is envisaged that the European Complement Network will carry on eventually, especially with facilitating student exchanges between complement labs.

ID: 66

Cross-species performance of iLite(R) C5a-specific reporter cell line and other functional and biomarker assays

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In the past decades we have witnessed an increasing focus on complement targeting therapeutics. This focus naturally follows the growing knowledge and understanding of this system in homeostasis and its involvement in various indications and in onset of condition. Consisting of more than 30 proteins, the complement system is a key component in both innate and adaptive immune response. It has been

shown that hyper- as well as hypo-activation are associated with a variety of severe diseases. Its involvement in a broad range of indications also shows why it must be tightly regulated by nature. With distinct triggering pathways, the complement cascade in human merge at the level of C3, where C3 and the very next step in the cascade, C5, are important targets for controlling the entire complement cascade.

Many novel drugs, especially when it comes to quenching of the activity, will target a complement component at proteome level. Unlike up and downstream products of the drug target, one cannot measure the effectiveness of the drug by relying solely on the concentration of the target component, rather, functional assessment is a necessity to show how the novel treatment interferes with a target molecule. C5a/C5aR1 binding interaction is an example for such an important target for functional assessment. C5a, the cleavage product of C5, is a strong chemoattractant and involved in recruitment of inflammatory cells of the immune system.

As the complement cascade is a preserved defence system across different species, studies are often performed on animal models to initially assess the efficacy and side effects of a novel drug. In this poster we show in which common animal models C5a reporter cell line (iLite ®, Svar) can be used for, along with other commercially available functional and biomarker assays

ID: 116

Crystal structure of the Factor H domains 8-14 uncovers the significance of the central domains for the discrimination between self and nonself by Factor H

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The plasma glycoprotein Factor H (FH) protects from alternative pathway activation in the fluid phase and on surfaces. Binding to sialic acid moieties on opsonised surfaces induces increased FH regulatory activity whereas absence of sialic acids results in a regulatory penalty of FH activity, increasing the selectivity factor between self and foreign surfaces[1]. Previous structural efforts employing nuclear magnetic resonance of CCP module pairs and small-angle-x-ray-scattering (SAXS) of larger domain constructs, *i.e.* FH10-15 and FH8-15, revealed a compact domain arrangement in the centre of FH, but failed to provide evidence that these domains indeed direct the FH termini to modulate its regulatory activity.
Several recombinant FH constructs spanning the complement control protein (CCP) domains 8-15 were recombinantly expressed, purified and subjected to crystallisation trials. The crystal structure of FH CCPs 8-14 was solved by molecular replacement at a resolution of 2.2 Å. While CCPs 8-9 are elongated and relatively free of inter-domain contacts (as is typical for CCP domain proteins), CCPs 10-14 form a complete loop introducing a 'U-turn' into the centre of FH. This brings CCP14 into close proximity of CCPs 10 and 11 to uniquely form intimate contacts with CCP domains that are positioned at least three CCPs domains further N-terminal. The close interaction between CCP14 and the CCPs 10-11, buries a total surface area of 1160 $Å^2$ and stabilises the U-turn structure of FH that directs both termini into the same direction. Concatenating the FH8-14 structure with other, already available structural data of FH domain stretches (*i.e.* high-resolution crystal structures and SAXS shape envelops) further illustrates how the FH

termini can be positioned against each other to modulate the regulatory function of FH in a context dependent manner.

Dopler A, Guntau L, Harder MJ, et al. Self versus Nonself Discrimination by the Soluble Complement Regulators Factor H and FHL-1. J. Immunol. Baltim. Md 1950. 2019;202(7):2082–2094.

ID: 132

Design and characterization of KP104, a bi-functional anti-C5 mAb and FH1-5 fusion protein that synergistically inhibits alternative and terminal pathways of complement activation

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Anti-complement drugs hold great promise for treating many human autoimmune and inflammatory diseases. Achieving desirable pharmacokinetics and pharmacodynamics of anti-complement drugs is challenging due to target abundance and target-mediated drug disposition (TMDD), exponential amplification of activation cascade, and multiple effectors implicated in a disease. For example, Eculizumab and Ravulizumab, two approved anti-C5 mAb drugs for paroxysmal nocturnal hemoglobinuria (PNH), require frequent and/or large dosing but many treated patients are still anemic and transfusion-dependent. Thus, there remains a need to develop more efficacious and convenient complement drugs. We have designed and tested a bi-functional antibody fusion protein, KP104, by attaching the N-terminal 5 short consensus repeats of factor H (FH1-5) to the Fc domain of a humanized murine anti-C5 mAb, 2G1-3. 2G1-3 recognizes C5 beta-chain and blocks C5 cleavage. It also binds to C5b in soluble and membrane-bound C5b-9 complex. Humanized 2G1-3 was further engineered in Fab and IgG4 Fc domains to stabilize dimerization and improve plasma recycling and half-life before fusing with human FH1-5 to produce KP104. In sheep RBC lysis assays, KP104 showed comparable activity to Ravulizumab, whereas in rabbit RBC or human PNH RBC lysis assays, KP104 but not Eculizumab/Ravulizumab achieved complete inhibition. As expected, KP104 but not Eculizumab/Ravulizumab dose-dependently inhibited alternative pathway complement activation in a LPS-based ELISA assay, C3b opsonization of rabbit and PNH RBCs, and complement-mediated hemolysis in a mouse model of extravascular hemolysis (1). Notably, KP104 more potently inhibited rabbit RBC lysis than the corresponding anti-C5 mAb and FH1-5 added together as two independent molecules, suggesting a synergistic effect between the two moieties in KP104. Finally, KP104 but not Fc-FH1-5 avidly bound to cells with C5b-9 deposition. Collectively, these data showed KP104 to be a potent bi-functional complement inhibitor of alternative and terminal pathways and possess tissue-targeting property for cells with C5b-9 deposition.

Miwa, T., Zhou, L., Hilliard, B., Molina, H. and Song, W.-C. Crry but not CD59 and DAF is indispensable for murine erythrocyte protection in vivo from spontaneous complement attack Blood 99: 3707-3716. (2002).

ID: 147

Design and evaluation of C3 inhibitors of the compstatin family with prolonged target residence time

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Despite the growing recognition of aberrant complement activation as contributor to various clinical conditions[1], the arsenal of complement therapeutics has long been limited both in numbers and target selectivity[2]. A second class of complement-specific drugs has only received FDA approval in 2021 with pegcetacoplan (Empaveli, Apellis), a PEGylated peptide based on an early analogue of the C3 inhibitor compstatin. Meanwhile, non-PEGylated compstatin derivatives such as Cp40 (AMY-101, Amyndas) have reached clinical development for the treatment of periodontal disease, transplantation, and COVID-19-induced acute respiratory distress syndrome, among others. The omission of the PEG moiety in Cp40 was largely enabled by the long target residence on C3.
In this study, we therefore investigated whether the binding affinity for C3 and, thereby the target residence can be further approved and whether this leads to a pharmacokinetic benefit. By a thorough review of published compstatin structure-activity relationship data, we identified unexplored optimization opportunities in the peptidic compstatin scaffold. A panel of novel compstatin derivatives was produced to provide a detailed SAR profile of hitherto underexplored amino acid positions, which led to the identification of candidates with enhanced binding affinities and target residence when compared to Cp40. In addition to the kinetic and thermodynamic binding profiles, we present initial pharmacokinetic data in non-human primates.
The insight provided by this study adds to our understanding of the PK/PD properties of compstatin-based C3 inhibitors and is expected to facilitate the further development of this promising family of broadband complement inhibitors for various indications.

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ID: 178

Detection of renal C3d deposition as a marker of disease activity in lupus nephritis.

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Lupus nephritis (LN) is a renal disease affecting most patients with systemic lupus erythematosus (SLE). Early diagnosis of LN is essential, since starting treatment quickly leads to better outcomes. A kidney

biopsy remains indispensable in the diagnosis of LN. Although generally safe, biopsies are invasive procedures, and they are also subject to sampling error. Consequently, there is a need for improved tests to accurately recognize the onset of LN before irreversible damage occurs. We, therefore, wanted to determine whether non-invasive detection of renal C3d deposits can serve as an indicator of disease onset and severity.

First, we retrospectively analyzed renal biopsy reports from patients with LN that were previously immunostained for C3. The C3 intensity in renal biopsies correlated with the histologic disease activity scores. Next, MRL/lpr mice (a model of LN) were studied for renal C3d deposition at different ages. Glomerular C3d deposition was detected at 8 weeks (very mild disease) and stepwise increased at 12, 16, and 20 weeks of age, but showed a small decrease at 24 weeks. Finally, we developed a method for non-invasive detection of C3d using a monoclonal antibody (mAb) labeled with a bioluminescence resonance energy transfer (BRET) system. As a proof-of-concept, we first tested the BRET labeled anti-C3d mAb in CFH knockout mice, a model of C3 glomerulopathy. BRET imaging revealed high intensities in the kidneys and liver, whereas low signal or only background was detected in wild-type mice and C3 knockout mice, respectively. Preliminary experiments in MRL/lpr mice demonstrated that renal C3d deposition could also be detected by BRET imaging at 12 weeks and significantly increased at 16 weeks as disease activity worsened. In conclusion, renal C3 deposition is an important marker of disease activity in LN. C3d-imaging can be used to non-invasively monitor inflammation in the kidneys of patients with LN.

ID: 37

Development and evaluation of a parasite-derived modulator to attenuate adverse host defense responses

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BD001, a leech derived protein, inhibits two serine proteases C1s (classical, CP) and MASP2 (lectin, LP) needed for complement activation. However, the structural determinants for activity and target selectivity have only partially been described, due to its complex structure, also hampering the assessment of the protein as therapeutic option. We aimed at improving the production of BD001 in *E.coli*. We used computational tools to perform a mutational scan of BD001 and predict changes in C1s affinity. We selected several mutants for experimental testing. The activity of recombinant BD001 and derivatives was tested in functional complement inhibition assays. Direct binding studies with C1s and other host defense proteases were performed with SPR. Finally, we developed complement activation ELISAs in animal sera of translational interest to determine the species specificity of BD001.
Despite its disulfide-rich nature, we managed to express BD001 and its mutants in *E. coli*. The panel of BD001 mutants for our SAR study showed distinct activity profiles in the functional assays, the experimental activity changes did not always correlate with *in silico* predictions. Intriguingly, some mutants differentially affected the overall efficacy or selectivity profiles, thereby suggesting a path for rational optimization of BD001. In addition to its reported activity for human complement, BD001 showed potent CP and LP inhibition in monkey and mouse serum.
Our initial SAR study provided insight into the molecular determinant of target activity and selectivity of BD001, thereby paving the way for more precise protein engineering. As the use of off-the-shelf tools for mutant screening is of limited predictive value, we are establishing enhanced computational methods for

future optimization steps. Overall, our findings establish BD001 as an interesting lead for further development towards treatment options for complement-related diseases, with the species specificity studies indicating the feasibility for preclinical evaluation of the inhibitor in disease models.

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ID: 31

Development of a functional complement assay based on liposomes for total complement activity determination

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The importance of the complement system in various diseases such as age-related macular degeneration, Alzheimer's or autoimmune diseases [1] and its potential as target for new drug development highlights the importance of research gaining fundamental understanding of the functional roles of its participating proteins. Such research remains difficult, as most relevant assays are based on fresh erythrocyte lysis or on mere protein quantification via ELISAs. In contrast, liposome-based assays have long been proposed and offer alternate strategies to detect pore formation and activity [2]. Here, a new class of liposomes is developed to function as biomimic replacing the animal-derived erythrocytes. These liposomes enable a highly targeted, specific and reliable functional assay, and provide quantitative, standardized data analyses. Initial studies focused on designing liposomes that resist complement-induced lysis or other lysis events when incubated with human serum for a prolonged period of time at 37 °C. By entrapping a self-quenching fluorescent dye within the liposomes, their lysis can easily be determined through an appearing, quantifyable fluorescent signal upon release of the dye as self-quenching is non-existent in diluted solutions. The lipid composition, surface tags and encapsulant concentrations were varied and resulted in stealth liposome compositions with anionic, cationic, or polyethylene glycol surfaces, respectively. Furthermore, through careful surface ligand design, the lysis of these liposomes by the membrane attack complex upon triggered activation of the complement system was accomplished. These liposomes can now be applied to study the total complement activity (TCA) by providing simplified handling and avoiding the use of sheep erythrocytes. We were able to compare and correlate this liposome-based assay to a conventional TCA CH50 assay. Further development of this bioassays will focus on point-of-care diagnostics applicable for probing a wide range of diseases as part of the SciFiMed consortium.

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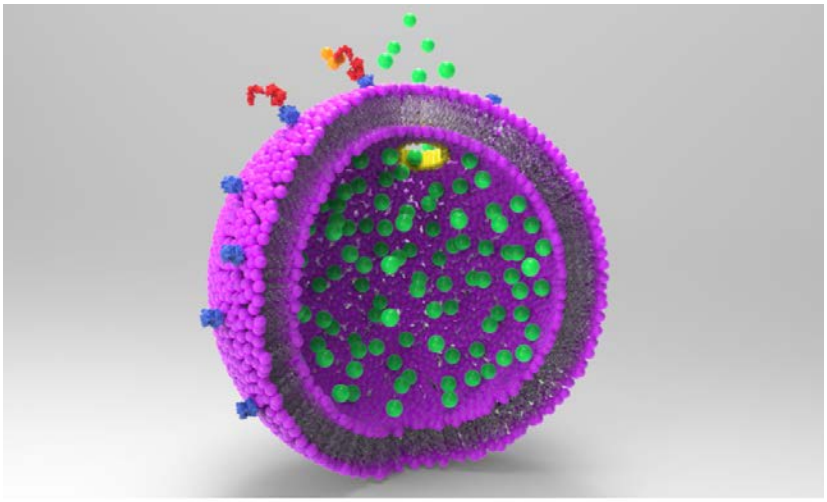

Schematic illustration of a liposome encapsulating sulforhodamine B (SRB, green) in its cavity surrounded by the lipid bilayer (purple). Trigger modifications (e.g. LPS) on the liposome surface (blue, red, orange) enable pore formation (yellow) via the membrane-attack-complex. Thus, SRB is released and an increase in fluorescence is detectable.

ID: 172

Development of bioassays to evaluate the interaction profiles of carbohydrate-based ligands and modulators of lectin targets within the complement system.

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The interaction of lectins with carbohydrate signatures on cells and other surfaces is a critical component of many biological processes, including complement activation and regulation (1). And while the diversity of carbohydrate structures and their biological targets render carbohydrate-derived drugs an enticing subject for drug design, their implementation as therapeutic agents remains limited due to inherently poor pharmacokinetic properties. Moreover, the specificity and affinity of carbohydrate ligands is often mediated by complex linking of saccharide units and/or multivalent binding. To overcome some of these limitations, our group is developing glycomimetic structures that functionally and structurally emulate bioactive carbohydrates yet display enhanced target binding affinities, target selectivity, and *in vivo* stability (2).
Yet, the unusual molecular properties and binding modes for their lectin targets not only impose challenges for drug development but also for the design of bioassays that are capable of monitoring weak monovalent and/or strong polyvalent interactions. In the context of an in-house project aiming at the design of collectin-11 (CL-11) antagonists, we are establishing a platform for the assessment of complement-relevant carbohydrate-lectin interactions based on recombinantly expressed carbohydrate-binding domains, natural and synthetic ligands, and various *in vitro* screening methods. These include thermal denaturation assays based on nano-differential scanning fluorimetry and a glycopolymer-based competitive binding assay, among others. The established bioassays enable protein-ligand affinity ranking

and have identified synthetic CL-11 antagonists with more than 100-fold improvements in binding affinity. First concluding results encourage further developments in favor of glycomimetic libraries and provide valuable information to further develop therapies against reperfusion-induced ischemic injury.

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ID: 190

Devils Dance: Complement, NETs in Immuno-Thrombosis of COVID-19

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Background
COVID-19 is characterized by multiple organ dysfunction syndrome (MOS). Microvascular complications in the form of arterial and venous thrombosis are involved in the pathogenesis of MODS and fatality in COVID-19 (1). There is evidence that complement system and neutrophil activation in the form of neutrophil extracellular traps (NETs) seem to be main drivers for the development microvascular complications in COVID-19 (2). Here we measured complement activation (C4bc, C3bc), markers for neutrophil activation in the form of NETs with nucleosomes, Elastase-α1 antitrypsin complexex (EA), and DNase 1 activity in patients suffering of increasing severity of COVID-19.
Materials and Methods
87 patients infected by SARS-CoV-2 with different severity of disease have been included. Plasma and serum were collected at enrollment (baseline), day 11 (D11) and day 28 (D28). EA, nucleosome and complement activation products (C4bc, C3bc) have been measured by ELISA, DNase 1 activity by a Picogreen assay and mtDNA by ddPCR, respectively.
Results
Nucleosomes and EA were similar in mild disease compared to controls; both markers were significantly increased in moderate disease at baseline then decreased overtime reaching controls level at D28; in severe disease both levels were significantly increased and remained high at D28. DNase 1 activity was significantly higher in severe disease compared to controls (***p=0.0004). C3bc levels decreased over time, whereas in severe disease it remained high. C4bc levels significantly increased over time (*p=0.032 baseline vs D11, *p=0.039 baseline vs D28). mtDNA was significantly increased in severe disease as compared to controls.
Conclusion

Neutrophil activation, complement activation, cell-free DNA and D-dimer levels increased with disease severity. Our data suggest neutrophil and complement activation are an important river of microvascular complications and that they reflect the occurence of immunothrombosis in these patients.

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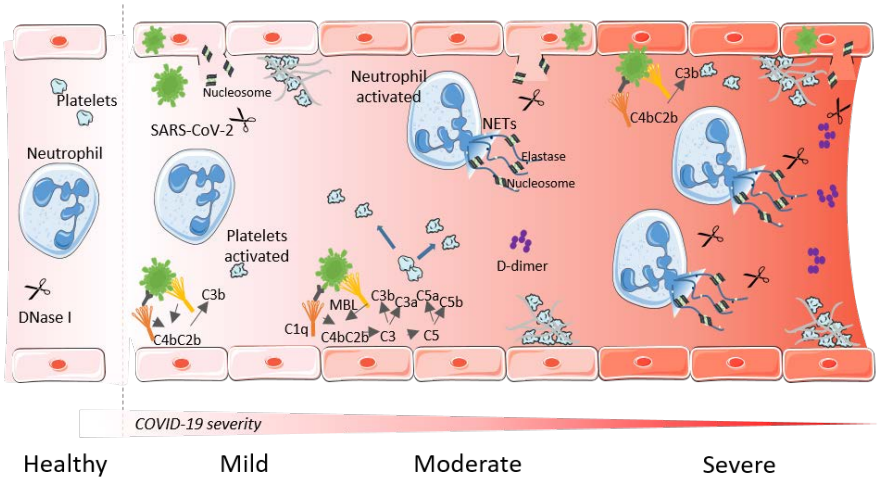

ID: 19

Dilutional linearity issues in complement activation marker assays

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To yield an accurate result of a marker of interest using ELISA, samples may be analyzed in different dilutions. This allows to calculate a concentration compared to a standard line over the range of the assay. However, when samples do not dilute linearly, it is difficult to judge if an observed result is accurate or affected by non-linearity, resulting in an over- or underestimation of the real content. When validating several complement activation markers, we observed non-linearity upon dilution. For C3a and Bb, commercial kits were used, whereas for C3d and sC5b-9 in house developed assays were used. Samples for C3d were precipitated and then analyzed for C3d content with in-house and commercial antibodies. sC5b-9 was setup according to publication by *Bergseth et al (2013),* detection was with an anti-C6 monoclonal Ab. For C3a and Bb, the optimal dilution was dependent on the concentration of these markers in the plasma sample. In samples with low levels of Bb, the recovery in lower dilutions than the standard dilution suggested by the kit insert was poor: 30-70% only. For samples with a high concentration, recovery was better in those cases where the results was out of range in the lower

dilutions. For C3d, at the higher concentrations an underestimation of the C3d concentration was observed. Whereas sC5b-9 low samples showed aspecific signals in lower dilutions that did not dilute out linearly. This latter aspecificity could not be pinpointed to complement activation in the plate. In conclusion: it is essential to choose the correct dilution when analyzing complement activation markers. Testing one dilution may lead to an over- or underestimation, depending on the concentration in the sample. In context of complement targeted therapies, these assays may be used to detect a decrease in complement activation markers compared to normal, indicating requirements of other dilution schemes.

DOI: 10.1016/j.molimm.2013.05.221

ID: 148

Ecotin homologs from ESKAPE microbes are potent complement inhibitors that have characteristically different specificities

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Ecotin, a serine protease inhibitor isolated from *Escherichia coli,* potently blocks lectin pathway activation and protects the ecotin-producing bacteria from complement-mediated lysis (1). Ecotin orthologs are expressed by many opportunistic pathogens, including four members of the infamous ESKAPE group. ESKAPE is an acronym for the six bacterial pathogens, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter spp.* that quickly develop or acquire multidrug resistance against antibiotics and cause the majority of lethal nosocomial infections. In 2019, five members of the ESKAPE group were responsible for the majority of diagnosed lethal infections (2). To efficiently combat such pathogens, we need to better understand how they establish an infection and avoid the attack of the human immune system. As ecotin had been shown to be a virulence factor for various opportunistic pathogenic microbes, we recombinantly produced several ecotin orthologs from pathogenic microbes and characterized their functional properties. Our data demonstrate that these orthologs have characteristically different specificity profiles. We suggest that the observed functional differences might indicate different ecotin-based immune system evading mechanisms shaped by adaptive evolution, which can reflect the different *niche* occupied by the corresponding pathogens in their host.

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ID: 135

Effects of complement C3, C5 and CD14 inhibitors on bacterial survival and thromboinflammation induced by two different live E. coli-strains in human whole blood

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Background
The complement system and Toll-like receptor (TLR) co-receptor CD14 are crucial in the innate immune response during sepsis. This study examined the relative importance of complement and CD14 on bacterial survival and the thromboinflammatory response in human whole blood incubated with two different live *E. coli* strains.
Methods
Fresh human whole blood anticoagulated with lepirudin was incubated at 37˚C for 120 min in the presence of live *E. coli* strains ATCC 33572 or ATCC 25922 (1×10^7/mL). The C3 inhibitor compstatin, anti-C5 monoclonal antibody (mAb) eculizumab, C5aR1 antagonist PMX53 and anti-CD14 mAb r18D11 were used as inhibitors and isotype-matched mAb as control. Bacterial survival was determined by cultivation techniques and results expressed as colony forming units. Tissue factor (TF) on monocytes was measured using flow cytometry. The soluble terminal C5b-9 complement complex (TCC) and prothrombin 1+2 (PTF1.2) levels were analyzed using ELISA. A total of 27 inflammatory mediators including cytokines were measured using multiplex technology.
Results
The two strains differed substantially with respect to inhibition. The ATCC 33572 strain: eculizumab increased bacterial survival ($P<0{\cdot}05$), while compstatin had no effect. Selective complement C3 and C5 inhibition significantly reduced the TF-levels on monocytes ($P<0{\cdot}05$) with no additional effect of anti-CD14. Combined inhibition of C3 and CD14 decreased PTF1.2 levels ($P<0{\cdot}05$). The ATCC 25922 strain: eculizumab and compstatin significantly increased bacterial growth up to 7·8-fold, while PMX53 alone had no effect. Only combined C3 and CD14 inhibition lowered TF-levels ($P<0{\cdot}05$), while the PTF1.2 level was non-significantly reduced. Both strains induced 26 of 27 measured inflammatory mediators ($P<0{\cdot}05$). Combined inhibition of C3 and CD14 significantly reduced TNF, IL-1β and IL-6 release ($P<0{\cdot}05$). Both strains: combined inhibition of complement C5 and CD14 reduced thromboinflammation.
Conclusion
Dual inhibition of complement and CD14 significantly inhibited the thromboinflammatory response, but increased bacterial growth may occur.

ID: 4

Elevated Factor H-related 1 and Factor H-related 5 Associate with End State Kidney Disease in C3 Glomerulopathy

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C3 Glomerulopathy (C3G) is an ultra-rare complement-mediated renal disease characterized by dysregulation of the alternative pathway (AP) of complement. Control of the AP is presumed to be mediated by a network of complement proteins including complement Factor H (FH), Factor H-realted 1 (FHR1), and Factor H-related 5 (FHR5). FH is known to regulate the C3 convertase through three mechanisms: 1) decay accelerating activity, 2) cofactor activity, and 3) inhibition of Factor B binding. FHR1 and FHR5 on the other hand, are suggested to promote formation of C3 convertase and therefore complement activation. In the glomerular microenvironment, FHR1 and FHR5 foster C3 convertase formation by recruiting native C3 and increasing avidity for C3b, respectively. The disparate roles of FH and FHR1/FHR5 suggest that an alteration in the balance of these proteins may lead to AP dysregulation and thus potentiate C3G development. To test this hypothesis, we determined the ratios of FH to FHR1 and FHR5 in a general C3G patient cohort (n=100; controls, n=85) and also one stratified by chronic kidney disease (CKD) stage. Our data showed a significant increase in the ratios of FHR1:FH and FHR5:FH in the C3G cohort compared to healthy controls ($p = 0.0001$ and $p = 0.0481$, respectively). Subgroup analysis by CKD stage also showed that the ratios of FHR1:FH and FHR5:Fh increased significantly from CKD stage 1 to 5 ($p = 0.0166$ and $p = 0.0053$, respectively). These results support a primary role for FH and FHR1/FHR5 in the pathogenesis and possibly prognosis of C3G. (Supported in part by NIH Grant R01 DK110023 (RJS))

ID: 81

Environmental stimuli and strain-characteristics regulate proteolytic cleavage of complement factors C3b and C5 by Pseudomonas aeruginosa

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The last decade has seen a sharp rise in high-risk clones of multidrug-resistant *Pseudomonas aeruginosa*, causing an estimated 20% of all nosocomial infections in Europe, leading to life-threatening clinical conditions, increased hospital stays and morbidity in those with a generally compromised immune defence. The virulence of *P. aeruginosa* largely relies on its diverse and variable collection of virulence factors and immune evasion proteins, with the major virulence contributor being its large arsenal of extracellular proteases. The proteases are known to degrade a wide array of host proteins, impair host defences and destroy physical barriers that prevent attachment and penetration of the bacteria. Target processes include complement-mediated opsonophagocytosis and complement lysis. Complement component-degrading proteolytic enzymes of opportunistic pathogens have been described previously. We analyzed proteolytic cleavage of human complement proteins C3b and C5 by *P. aeruginosa* clinical isolates and selected serotype strains (per IATS). In some isolates, expression of proteases was directly

influenced by the environmental factors and changes in the physicochemical properties of the culture medium (e.g., nutrients, inorganic salts, and the presence of amino acids). Results suggest that there are several different inducible proteases because the cleavage patterns across the studied *P. aeruginosa* strains varied. Also, changes in the bacterial lipopolysaccharides in response to environmental stimuli may contribute to the differences seen in the cleavage patterns. Examining mechanisms that regulate proteolytic degradation of complement factors by opportunistic pathogens, such as *Pseudomonas,* can yield clues about the surprisingly varied types of evasion mechanisms within the same species and pave the way toward designing personalized antimicrobial therapies for those in need.

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ID: 136

Escherichia coli-induced platelet activation is not reduced by complement inhibition

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Background
Platelets are important players both in hemostasis and in the immune response. The complement system is involved in thromboinflammation, but the role of complement in platelet aggregation and activation is only partly known. Since the inflammatory response to bacterial challenge in whole blood is largely complement dependent, we studied the role of complement in bacteria-induced platelet activation.

Methods
Fresh human whole blood anticoagulated with lepirudin was incubated at 37°C for 30 min together with heat-inactivated *Escherichia coli* (*E. coli*) 3x10e9/mL or phosphate buffered saline (PBS). The following inhibitors were included; the glycoprotein IIb/IIIa inhibitor tirofiban, compstatin, eculizumab, the C5a receptor antagonist PMX53, anti-CD14, an isotype matched control antibody and a control peptide. Platelet activation markers P-selectin (Invitrogen) and β-thromboglobulin (BTG) were measured in plasma by ELISA (Elabscience). The *E. coli*-induced (5x10e9/mL) expression of P-selectin on platelets was detected by flow cytometry using P-selectin (BV650) and CD61 (FITC) specific antibodies.

Results
E. coli significantly increased plasma levels of P-selectin and BTG from 37 ng/mL to 63 ng/mL (p=0.0003) and from 6.1 ng/mL to 12 ng/mL (p=0.0313), respectively. The here tested complement inhibitors, alone or in combination, had no effect on *E. coli*-induced plasma P-selectin or BTG. Further, *E. coli* significantly increased P-selectin expression on platelets by 2.3-fold (p=0.0015). Here, the combination of compstatin and tirofiban had a significant effect and reduced the *E. coli*-induced P-selectin expression with 42% (p=0.0148).

Conclusion
Complement C3 and C5 inhibitors did not reduce the *E. coli*-induced platelet activation in whole blood when measured as plasma P-selectin and BTG. However, combined inhibition of C3 and the glycoprotein IIb/IIIa significantly reduced *E. coli*-induced P-selectin expression on platelets.

ID: 39

Evaluation of a novel cell depletion model and the characterization of cytokine response by individual cell populations in the lepirudin human whole blood model

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Background: Anticoagulants are important for conducting research in whole blood. The lepirudin whole blood model has been shown to be effective for the study of complement in inflammation, as it shows no adverse effects on the complement system (1). However, the role of individual cell populations in the expression of inflammatory markers in the lepirudin whole blood model is unknown. The aim of this study was to develop a model to elucidate the cellular source of inflammatory markers in the lepirudin whole blood model.
Method: Using Miltenyi Straight from Whole Blood Microbeads we have developed a negative selection model, enabling selective cell depletion of whole blood. Analysis of complement was performed using previously proven in-house ELISAs and cytokine analysis using commercial multiplex kits. Flow cytometry of cell- and platelet activation markers were used to study quantity and activation of individual cell populations.
Results: Analysis of specific cell markers showed >90% depletion of CD14+ and CD15+ cells, respectively. The background activation in the model showed negligible complement activation (C3bc and TCC) and cell activation (CD11b and CD62p), supporting a high degree of biocompatibility of the model. When activating the depleted blood using *Escherichia coli*, a distinctive pattern of cytokine release occurred. Most cytokines, such as IL2, TNF, and IFNg were released solely by the CD14+ cells. A few, such as IL8 and IL10, were released by both CD14+ and CD15+ cells. Only two cytokines, PDGF-bb and RANTES, were not released by the leukocytes, but were platelet derived.
Conclusion: We can document an effective model for the depletion of individual cell populations from

whole blood without causing inflammatory background activation. The model is a useful tool to elucidate the role of individual cell populations in their release of inflammatory mediators, including cytokines, by studying the whole blood selectively depleted from each population.

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ID: 95

Evasion mechanisms used by Acinetobacter spp. to escape the human complement system.

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BACKGROUND: *Acinetobacter* spp. are gram-negative bacteria that cause life-threatening hospital-related infections such as pneumonia, septicemia, and meningitis, especially in immunocompromised patients. The World Health Organization has listed *Acinetobacter baumannii* among the priority pathogens due to their multi- and extensive-drug resistance. Therefore, the development of new antibiotics and antibacterial treatment options are urgently required. The complement system is the crucial cascade in the innate immunity, which plays a significant role in protection from bacterial invasion, however, a knowledge gap exists on complement activation during Acinetobacter-dependent infection. A better understanding of escape mechanisms in *Acinetobacter* spp. would lend insight into the development of novel treatment options not based exclusively on antibiotics.
AIM OF THE STUDY: Previously, we showed clinical isolates of *Acinetobacter* spp. (n=50) are highly serum-resistant despite being efficiently recognized by the complement system (1). Opsonization with C3 fragments and deposition of MAC are crucial complement mechanisms against gram-negative bacteria. Here, we studied whether clinical isolates of *Acinetobacter* spp. express virulence factors targeting C3 and MAC.
RESULTS: Flow cytometry analyses showed binding of C3 protein mostly by *A. calcoaceticus* isolates, further confirmed in western blot. Pathogens bound C3 in a concentration-dependent manner. Even though MAC was deposited on the surface, bacteria survived treatment with the antibiotic nisin, which cannot pass the outer membrane of gram-negatives, suggesting non-functional MAC deposits. Western blot analyses showed the binding of C9 to bacteria.
CONCLUSION: Our data showed potential mechanisms of *Acinetobacter* spp. to evade the complement system attack. Hijacking C3 by surface proteins could prevent the opsonization of bacteria, thus protecting them from phagocytosis. Being gram-negative bacteria *Acinetobacter* should be sensitive to complement-mediated lysis. By preventing MAC deposition and/or insertion into the membrane, bacteria could escape from killing and spread during the infection.

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ID: 58

Evasion strategies of clinical Klebsiella pneumoniae isolates to prevent recognition and killing by human complement system

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Background: The gram-negative bacterium *Klebsiella pneumoniae* has been associated with pneumonia and sepsis. Due to the rising emergence of multi-drug resistance in these bacteria, new treatment options such as boosting the human immune response are under consideration. As part of the human innate immune response, the complement system can kill *Klebsiella* via insertion of the membrane attack complex (MAC) into the bacterial cell envelope. However, it has previously been shown that some *K. pneumoniae* strains can evade this insertion and withstand killing in human serum. How complement is activated on different clinical *K. pneumoniae* isolates, and whether it modulates bacteria survival, will be subject of this study.

Methods: Several distinct clinical isolate strains of *K. pneumoniae* were collected. Complement-mediated killing was assessed by SytoxBlue, a non-membrane permeating DNA-stain that becomes fluorescent upon damage of the inner membrane. The antimicrobial peptide nisin was used to assess for the occurrence of outer membrane damage. Bacteria were treated with normal human serum or fluorescence-labelled complement components to determine complement deposition and MAC-formation using flow cytometry.

Results: Serum-resistant isolates did not undergo membrane damage caused by the MAC. This is in contrast to serum-susceptible *K. pneumoniae* isolates, where membrane damage correlates with decreased bacterial viability. Of the serum-resistant strains, some showed C9 deposition on the bacterial surface, while circumventing killing via the MAC by an unknown mechanism. We further found that distinct isolate strains prevent deposition of several complement components at different earlier stages the complement cascade.

Conclusion: Our data show that *K. pneumoniae* has several distinct mechanisms to prevent complement deposition at different stages of the complement cascade, greatly enhancing their survival in a serum environment. Understanding the mechanisms how *K. pneumoniae* resists complement activation is essential to explore novel antimicrobial strategies, such as boosting the immune system to effectively prevent infection.

ID: 72

Fabry disease patients exert strong complement activation independent of enzyme replacement therapy

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Background
Lysosomal storage disorders (LSDs) are characterized by accumulation of specific substrates in lysosomes resulting from mutations encoding lysosomal enzymes/receptors. Previously, we found strong complement activation in Gaucher disease, driving the inflammation in this LSD (Pandey et al. Nature 2017). Here, we determined complement activation and autoantibody formation in Fabry disease (FD), an X-linked LSD associated with cardiac/cerebrovascular disease and renal failure.

Methods
We analyzed blood samples from "classic" male FD patients and healthy controls (18-70 years). Patients were either treatment-naïve or received enzyme replacement therapy (ERT). Samples were collected by five European hospitals. The concentrations of C3a and C5a from healthy controls (n=10), treatment-naïve FD patients (n=20) and ERT-treated FD patients (n=18) were determined as well as autoantibodies against extractable nuclear antigens (ENAs) and G-protein-coupled receptors by ELISA. The study was approved by the Ethics Committee of the University of Lübeck (Ref No: 20-151).

Results
C3a and C5a serum levels in treatment-naïve and ERT-treated FD patients were significantly higher than in healthy controls but similar in treatment-naïve and ERT-treated FD patients. Of note, 45% of treatment-naïve and 11% of ERT-treated FD patients had C5a levels in the range of healthy controls. In contrast, C3a serum levels from treatment-naïve and ERT-treated FD patients were consistently higher than those in healthy controls. No autoantibodies against ENAs in FD patients were detected. Angiotensin II type 1 receptor but not C5a-receptor 1 autoantibodies were significantly reduced in FD patients as compared to healthy controls.

Conclusion
We identified a hitherto unrecognized strong complement activation in treatment-naïve and ERT-treated classical FD patients with a dominant C3 activation. The high C3a and C5a serum levels after ERT treatment suggest sustained complement activation despite enzyme substitution. This ongoing complement activation may explain endothelial dysfunction and the high risk of thrombotic events observed in FD patients.

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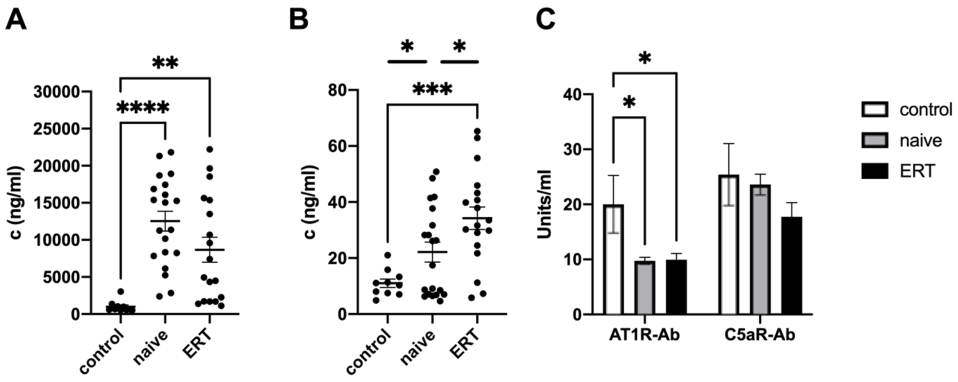

Figure 1: Serum concentrations of C3a and C5a and autoantibody formation in FD patients. C3a (A) or C5a (B) concentrations in serum samples and level of AT1R and C5aR autoantibodies (C) from healthy controls (n=10) as well as from treatment-naïve (n=20) and ERT-treated (n=18) patients were assessed by ELISA. Data shown are the mean ± SEM. Differences between groups were determined by unpaired t-test (A, B) or two-way ANOVA with Tukey posthoc test (C). *p<0.05; **p<0,01; ***p<0,0002; ****p<0,0001.

ID: 168

Factor H and Factor H-Related Proteins show different expression profiles in healthy controls and IgA Nephropathy patients

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Factor H–related proteins (FHRs) are structural homologues of FH, but they lack its complement regulatory domains. It has been proposed that FHRs act as FH deregulators and/or complement activators, as recently demonstrated for FHR-1. Therefore, an imbalance in the relative levels of FH and FHRs proteins can perturb the complement alternative pathway, and predispose to renal disease. We previously identified different expression profiles of FH and FHRs in plasma associated with atypical Haemolytic Uraemic Syndrome (aHUS) and C3 glomerulopathy (C3G). In this work, we aimed to determine FH and FHRs levels and expression profiles in an IgA nephropathy (IgAN) cohort.
A cohort of 69 biopsy-proven IgAN patients and 79 healthy controls were studied. The common genetic variant *ΔCFHR3-CFHR1*, and the aHUS-risks variants *CFH/H3*, *CFHR3*B* and *CFHR1*B* were analysed. Plasma levels of FH, FHR-1, FHR-2, FHR-4A and FHR-5 in controls and patients were determined by in-house ELISAs, and statistically compared by using GraphPad Prism. For the identification of the FH/FHRs

expression profiles, a hierarchical clustering analysis was performed in Heatmapper (http://www.heatmapper.ca/).
Plasma levels of FH, FHR-1, FHR-2 and FHR-4A were significantly higher in IgAN patients than in controls, while FHR-5 levels were similar. These results agree with published data on elevated FHR-1 levels in IgAN, but we did not observe increased FHR-5 levels. Clustering analysis identified multiple FH/FHRs expression profiles, which can be classified into 7 major subgroups with a different distribution in patients and controls ($p<0.0001$, χ^2 test). The subgroup that includes the expression profiles with elevated FH, FHR-1, FHR-2, and FHR-4A levels was only observed in IgAN patients, who also presented a higher frequency of expression profiles characterized by elevated FHR-2 and FHR-4A. These findings provide new information for understanding the relevance of FH and FHRs levels in the development of IgAN that should be confirmed in larger patient cohorts.

ID: 110

Flick the switch – subclass switching generates a complement-fixing monoclonal antibody that inhibits malaria transmission

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Plasmodium falciparum causes millions of malaria cases yearly and effective strategies to target this parasite are urgently needed. *Plasmodium* parasites are solely and efficiently transmitted via mosquitoes. Transmission commences with the uptake of gametocyte-infected erythrocytes from an infected person through a mosquito blood meal. In the mosquito midgut, the gametocyte egresses from the erythrocyte and activates into a gamete that is exposed to human complement also present in the bloodmeal. Transmission blocking vaccines (TBVs) aim to induce human antibodies that target gametes, inhibit parasite development in the mosquito midgut and hence block transmission. A leading TBV candidate is the gamete surface protein Pfs230. Interestingly, all functional Pfs230 (monoclonal) antibodies are complement-dependent [1]. We previously generated a panel of mouse anti-Pfs230 monoclonal antibodies (mAbs), but despite high affinity for gametes, these were non-functional [1]. Here we tested whether switching one of these mAbs, 18F25, to a complement fixing subclass would result in functional activity.

Employing a recently published CRISPR-Cas9-based technique [2], we engineered the 18F25 mouse hybridoma to produce complement fixing IgG2a instead of non-fixing IgG1. We confirmed that the affinity and specificity of the subclass-switched mAb 18F25 was identical to the parental. 18F25-IgG2a induced full gamete lysis *in vitro* in the presence of active complement, whereas 18F25-IgG1 did not induce substantial lysis. We next tested the transmission-reducing activity (TRA) of the 18F25 mAbs in a standard membrane feeding assay in which *in vitro* cultured gametocytes are mixed with mAbs and fed to mosquitoes. We found that 18F25 IgG2a had high TRA at 10 µg/ml, whilst 18F25 IgG1 had no TRA at any of the concentrations tested (Fig. 1). In summary, we used an efficient method to switch the subclass of a mouse hybridoma and concurrently generated a highly potent mAb that blocks *Plasmodium* transmission in a complement-dependent manner.

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ID: 97

Functional characterization of aHUS-associated genetic variants in the serine protease domain of factor B suggests a novel mechanism to enhance complement activation

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Atypical hemolytic uremic syndrome (aHUS) is an ultra-rare renal disease associated with dysregulation of the alternative pathway of complement at the endothelial surfaces. Amongst the genetic factors associated with the development of aHUS are gain-of-function mutations in the vWA domain of factor B (FB) that increase formation of the AP C3 proconvertase or prolong the half-life of the C3 convertase by reducing spontaneous decay and increasing resistance to accelerated decay by complemnt regulators. To gain further insights into the role of FB in aHUS, we have selected and functionally characterized 44 FB variants that have been identified in the genetic screening of aHUS patients. Normal human serum completely depleted of FB was reconstituted with equal concentrations of either a recombinant mutant FB protein or WT FB protein. Functional analysis using a sheep hemolytic assay showed that 12 FB variants had increased lysis as compared to WT FB protein. Six of these FB variants are located in the vWA domain and behave like prototypical aHUS.associated FB variants in the sense that they confer to the AP C3 convertase reduced spontaneous decay and increased resistance to FH accelerated decay. Interestingly, the other 6 FB variants are located in the serin protease (SP) domain and additional hemolytic assays demonstrated that they do not affect the formation, stability or decay of the AP C3 convertase. The location of these FB variants suggests that they mediate binding of the FB SP domain to the C3 substrate, accelerating activarion; a possibility that we are currently testing computationally and experimentally. Although it remains to be determined whether enhanced activation of C3 has pathological consequences, these novel FB variants will be useful to derive additional structural information of how the AP C3 convertase interacts with the C3 substrate.

ID: 3

Functional characterization of PASylated forms of moss produced human complement factor H in vitro.

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Background:
Atypical haemolytic uremic syndrome (aHUS) is a rare complement mediated kidney disease mostly caused by mutations in complement factor H (FH). Currently available drugs cause a complete blockade of the complement system impairing its natural protective role against pathogens. A replacement therapy with recombinant factor H would offer a much better chance to prevent complement overactivation while retaining its physiological function.
Recently, a moss based expression system leading to high yields of recombinant, moss produced human FH has been developed. However, *in vivo* experiments in FH deficient mice showed that mossFH suffered from a short half-life compared to plasma-isolated FH.
In order to increase the serum half-life of moss FH we therefore examined the effects of single and double PASylation of recombinant mossFH on its activity *in vitro*.

Methods/Results:
PASylation of Moss FH had little effect on the protein's cofactor activity in vitro. In contrast, the PASylated mossFH variants have a significantly reduced complement regulatory activity compared to normal mossFH. The ability to suppress TCC was 10 times lower with 1xPAS MoosFH and 30 times lower with 2xPAS than MoosFH. In the hemolysis assay we could observe similar effects with a reduced activity of 1xPAS by a factor of 4 and 2xPAS by a factor of 9 compared to mossFH. Furthermore, there was a significantly lower binding of the PASylated proteins to C3b and on endothelial cell surfaces.

Conclusion:
While PASylations are able to increase the half-life of recombinant proteins, the modification in Moos FH leads to a reduction in complement regulatory activity. The modifications add long peptide chains to the protein, which likely lead to steric hindrance of the FH molecule. However, the reduced potency *in vitro* could be compensated for by a prolonged serum half-life *in vivo*.

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ID: 54

Functional consequences of missense mutations at position 263 in complement C2 protein

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The complement system is one of the first defense lines protecting from invading pathogens. However, it may turn offensive to the body's own cells and tissues when deregulated by the presence of rare genetic variants that impair physiological regulation and/or provoke abnormal activity of key enzymatic components. Factor B and complement C2 are examples of paralogs engaged in the alternative and classical/lectin complement pathway, respectively. Pathogenic mutations in the von Willebrand factor A domain (vWA) of FB have been known for years. Despite substantial homology between two proteins and the demonstration that certain substitutions in FB translated to C2 result in analogous phenotype, there was a limited number of reports on pathogenic C2 variants in patients. Recently, we studied a cohort of

patients suffering from rare kidney diseases and confirmed the existence of two gain-of-function and three loss-of-function mutations within the C2 gene sequences coding for the vWA domain (amino acids 254-452) or nearly located unstructured region (243-253). Herein, we report functional consequences of amino acid substitution of glutamine at position 263. p.Q263P variant found in a patient with C3 glomerulopathy resulted in the loss of C2 function whereas the p.Q263G variant resulted in the gain-of-function phenotype, similarly to a homologous mutation p.D279G in FB. Our results confirm that the N-terminal part of the vWA domain is a mutational hot spot crucial for complement C2 function.

ID: 113

Functional tests of the complement system in serum samples before and after hemodialysis

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The complement system consists of about 50 proteins that can be circulating or membrane bound. System plays an important part of innate immunity. Some diseases are related to excessive / uncontrolled activation of complements, so the balance of activation / inactivation is extremely important. More than 2 million people suffer from end-stage kidney disease (ESKD) worldwide, and most of them dialysis-dependent. Hemodialysis (HD) is a life-sustaining therapy but despite considerable technical and scientific improvements, results are still not fully satisfactory in terms of morbidity and mortality. Blood contact with foreign surface (such hemodialising membrane) may induce activation of contact systems including complement. Serum samples of 125 patients before and after hemodialysis been collected. Complement activity was determinate by laser kinetic method (classic-CHU and alternative-CAHU pathway). Concentration of beta 2 microglobuline measured by laser nephelometric method. Reduction concentrate of beta 2 microgloblulin (40,19 ± 20,00 g/L vs 26,94 ± 19,48g/L) was obtain and was statistical significant p <0,001, indication of successful removing small molecules. CHU and CAHU results analyzed done in two groups. The first group includes patients with decrease test values after HD CHU (13,31 ±2,54 HU/ml vs12,03 ± 32,27 HU/ml) and CAHU (1,32 ± 0,67 HU/ml vs 1,19 ±0,73 HU/ml), the second group consists of patients with increase test values CHU (10,22 ± 3,84 vs 12,85 ±3,82HU/ml,) and CAHU (1,02 ± 0,67 HU/ml vs 1,37 ± 0,6 HU/ml). Determinate values was statistical significant p<0,01.HD membranes can cause complement activation and lower CHU and CAH values. The results of the second group were interesting; a possible explanation is the presence of complement inhibitors (primary disease) removed during HD.

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ID: 137

Functional validation and reference range definition of early complement activation markers C1s/C1-INH and MASP-1/C1-INH complexes

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The most commonly used markers to assess complement activation are split products downstream of C3, that are produced through activation of all three pathways. In contrast, C4d derives from the cleavage of C4 and hence indicates either classical (CP) or lectin pathway (LP) activation. Although C4d is perfectly able to distinguish between CP/LP and AP activation, no well-established markers to differ between early classical and early lectin pathway activation are available. Active enzymes of both pathways (C1s/C1r for the CP, MASP-1/MASP-2 for the LP) are regulated by C1 esterase inhibitor (C1-INH) through the formation of covalent complexes.
Aim of this study was to investigate whether C1s/C1-INH and MASP-1/C1-INH complexes could serve as markers for either early CP or LP activation. In addition, we aimed to define a reference range for these complexes in healthy adults and examine whether levels differ in pathological samples.

Complement-preserved normal human serum was incubated *in-vitro* with zymosan and complex formation was monitored over time using two newly developed immunoassays for the quantification of either C1s/C1-INH or MASP-1/C1-INH complexes in human samples. Next, these assays were utilized to measure C1-INH complex levels in EDTA-plasma samples obtained from healthy adults and diseased individuals.

Both complex levels showed a time-dependent increase in serum when activated *in-vitro*.
Measurements of C1-INH complexes in healthy adults showed normally distributed C1s/C1-INH complex levels with a physiological concentration of 1846 ±1060 ng/ml (mean ±2SD) and skewed distribution of MASP-1/C1-INH complex levels with a median concentration of 36.9 (13.18-87.89) ng/ml (2.5-97.5 percentile range). Levels did not differ between subgroups (gender/age), but were increased in pathological samples.

To conclude, C1s/C1-INH and MASP-1/C1-INH complexes are suitable markers to assess early classical and early lectin pathway activation. A reference range was set and first studies showed that these markers have added value for investigating and unraveling complement activation in human disease.

ID: 33

Generation of monoclonal antibodies targeting murine C5aR2

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Background
C5aR2 is an enigmatic receptor binding C5a and C5adesArg. It serves as a decoy receptor for C5aR1, acts synergistically with C5aR1, or exerts functions independent of C5aR1. Previously, we have generated tdTomato-C5aR2$^{fl/fl}$ reporter mice to track and cell-specifically delete C5aR2 (Karsten et al. J. Immunol 2017). However, mAbs that specifically bind and/or block binding of C5a/C5adesArg to murine C5aR2 are still lacking. Here, we aim to close this gap and designed an immunization strategy to generate mAbs targeting murine C5aR2.
Methods
Three C57BL/6 *C5ar2*$^{-/-}$ mice were immunized with HEK293-transfected cells expressing murine (m)C5aR2. The antibody response was tested with BSA-coupled peptides corresponding to the extracellular domains of mC5aR2 by ELISA. Further, immune sera were tested for mC5aR2 reactivity with Ly6G^{+} primary bone marrow cells (pBMCs) from C57BL/6 wildtype mice by intracellular staining using flow cytometry.
Results
We observed a strong IgG serum response towards the mC5aR2-derived N-terminal peptide (Np) and Ly6G^{+} pBMCs. After fusion, we identified two priority groups of clones that: (i) reacted strongly with Ly6G+ pPBMCs and Np; (ii) reacted strongly with Ly6G+ pPBMCs but not with Np (both Priority group I); or (iii) reacted strongly with pB but only moderately with Ly6G+ pBMCs (Priority group II). Work is in progress to purify these clones, test their subclasses, cross-reactivity with C5aR1/C3aR and their ability to inhibit binding of C5a/C5adesArg.
Conclusions
We have implemented an immunization scheme to generate several mAbs against mC5aR2. Our results identify the N-Terminus as one immunodominant region of mC5aR2 which is also accessible in the naïve mC5aR2 as evidenced by the strong reactivity of some antibodies with Ly6G+ pBMCs. In addition epitopes outside the N-terminus contributed to the immune response resulting in mAbs that also reacted with naïve mC5aR2. Future experiments will determine their viability for tracking and functional inhibition of mC5aR2.

ID: 181

Granzyme K Elicits a New Pathway for Complement Activation

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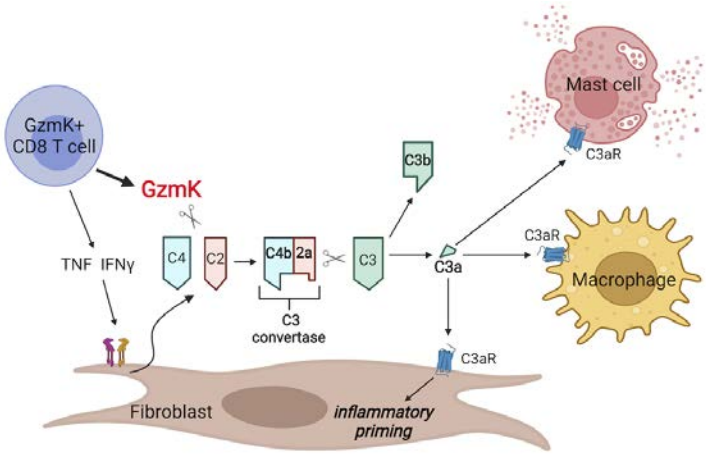
The role of T cells as major drivers of rheumatoid arthritis (RA) pathogenesis is well established, but nearly all research has focused on CD4^{+} T cells. We have found that CD8^{+} T cells are strikingly abundant in inflamed RA synovium and make more IFNg and nearly as much TNF as their CD4^{+} T cell counterparts.

Unlike cytotoxic T lymphocytes, these $CD8^+$ T cells have low cytotoxic potential, expressing low levels of granzyme B and perforin. Instead, these $CD8^+$ T cells express high levels of granzyme K (GzmK), a granzyme that does not drive apoptosis of target cells. Similar $GzmK^+$ $CD8^+$ T cells are also highly enriched in many other inflamed tissues, including gut, kidney, and lung affected by inflammatory bowel disease, lupus nephritis, and COVID-19, respectively. Here, we describe that GzmK activates a new complement pathway. GzmK cleaves C4 to C4b and C2 to C2a to form an active C3 convertase (C4b + C2a) that cleaves C3 to C3a and C3b. Further, we show that synovial fibroblasts, important drivers of chronic inflammation in RA, are the dominant cellular source of complement components in the synovium. IFNg and TNF, cytokines abundantly produced by $CD8^+$ T cells in RA synovium, drive fibroblasts to synthesize and release C2, C3, and C4. Strikingly, we show that GzmK cleaves C4 and C2 secreted by fibroblasts, eliciting formation of an extracellular C3 convertase that generates C3a and C3b. Our findings support a two-cell model for complement activation, where $CD8^+$ T cell-derived GzmK acts extracellularly on fibroblast-derived complement components. This novel pathway is independent of the well-known classical, alternative, and lectin pathways of complement activation, and the C3a and C3b it generates may act on fibroblasts and other cells to perpetuate tissue inflammation in RA and other diseases.



ID: 185

Group B streptococcus virulence factors and their interactions with the complement system

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Streptococcus agalactiae or Group B Streptococcus (GBS) is a leading cause of adverse pregnancy outcomes, neonatal sepsis, pneumonia and meningitis. This is a growing concern, as the number of antibiotic resistant pathogens is rising worldwide. High prevalence of GBS infections could be due to the interactions and evasion of the immune system. Of related bacteria, it has been well-known that they use many ways and proteins to evade the immune system. Earlier research showed that some GBS proteins

interact with the complement system. Most GBS proteins found have homology to proteins found in other species. However, compared to other streptococci, there is a lack of knowledge about the pathophysiology and virulence factors of GBS.
The aim of this study is to find host factors and novel GBS virulence factors associated with severe infections in newborn babies and GBS colonization in pregnancy.
Using the bulk mRNA sequencing, host transcriptional responses from cells were analyzed. Samples included 100 GBS positive and 100 negative vaginal swab samples obtained from pregnant women. Eighteen upregulated genes specific for GBS infection were found. Based on these genes, strains that showed most significant changes in maternal mRNA levels were selected and cultured. Secreted proteins were isolated from the culture supernatant, surface-associated proteins were isolated using lysozyme and mutanolysin. Proteins were fractionated using size-exclusion chromatography. Activity of isolated protein fractions to lyse and activate primary blood cells and cultured C5aR expressing HEK cells was analyzed by flow cytometry. Results showed that several fractionated GBS proteins were able to lyse white blood cells, interact with C5a-receptor and amplify the complement-mediated inflammatory response in serum. Our goal in the future is to identify these virulence factors and characterize their pathogenic role in host-microbe interactions.

ID: 10

High-resolution imaging of complement molecules on Gram-negative bacteria

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The complement system is a part of the immune system that potently kills invading Gram-negative bacteria via the formation of lytic pores, called Membrane Attack Complexes (MACs). Whereas MAC pores can efficiently kill a broad range of Gram-negative bacteria by damaging their outer and inner membrane, the latter of which is essential for bacterial killing, bacteria can also resist killing by MAC pores. In order to better understand how our immune system clears infections with Gram-negative bacteria, it is key to study what determines whether a bacterium can be killed by MAC pores or not. Here, we used fluorescence microscopy to study MAC-dependent killing of *E. coli*. By combining widefield fluorescence microscopy and microfluidics, MAC formation and subsequent inner membrane damage was visualized in time. Specifically, we incubated *E. coli* with fluorescent MAC components in the presence of a naturally impermeable DNA dye and observed that a limited number of MAC pores correlated with bacterial inner membrane damage. In addition, we used single-molecule super-resolution microscopy to localize MAC pores on the surface of *E. coli* with high resolution. For this, we fluorescently labeled the core of the lipopolysaccharides (LPS) of *E. coli*, which is the major component of the bacterial outer membrane. When we incubated these bacteria with fluorescent MAC components, we observed that functional MAC pores form high-density fluorescent spots that colocalize with the LPS core, suggesting that they deeply anchor into the bacterial outer membrane. In contrast, MAC pores are deposited further away from the LPS core in MAC-resistant bacteria, suggesting that bacteria can evade MAC-dependent killing by preventing proper MAC insertion into the outer membrane. Altogether, we show how high-resolution imaging techniques can give crucial insights into complement-mediated killing of Gram-negative bacteria.

ID: 109

HIV-1 Trans Infection via TNTs Is Impeded by Targeting C5aR

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Non-adjacent immune cells communicate through a complex network of tunneling nanotubes (TNTs). HIV-1 is able to hijack TNTs, spreading between connected cells protected from the extracellular environment threats. Dendritic cells (DCs) are among the first cells to encounter HIV-1 at mucosal sites, but they are usually efficiently infected only at low levels. However, complement opsonized HIV-1 (HIV-C) was demonstrated to productively infect DCs. Such HIV-C exposed DCs mediated an improved antiviral and T-cell stimulatory capacity. The role of TNTs in combination with local complement production in enhancing DC infection with HIV-C remains to be addressed. To this aim, we first compared TNT formation on the surface of DCs or DC/CD4+ T-cell co-coltures incubated with non- or complement-opsonized HIV-1 (HIV, HIV-1). In a second step, we evaluated the influence of TNT or locally producing complement on HIV-1 infection incubating DCs or DC/CD4+ T-cell co-coltures with either two different TNT inhibitors or C3aR and C5aR receptors antagonists. We found that HIV-C significantly increased the formation of TNTs between DCs or DC/CD4+ T cell co-coltures compared to HIV-exposed DCs or co-coltures. While augementd TNT formation in DCs promoted productive infection, as previously observed, a significant reduction in productive infection was observed in DC/CD4+ T-cell co-coltures, indicating antiviral activity in this setting. As expected, TNT inhibitors significantly decreased infection of HIV-C-loaded-DCs as well as HIV- and HIV-C infected-DC/CD4+ T-cell co-coltures. Thus, local complement mobilization via DC stimulation of complement receptors plays a pivotal role in TNT formation and our findings might offer an exciting opportunity for novel therapeutic approaches to inhibit Trans infection via C5aR targeting.

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ID: 74

Human Complement C3 gain-of-function variant is resistant to cofactor activity leading to excessive complement activation and renal disease

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As one of the most abundant plasma proteins, C3 is the convergent point for the three complement activation pathways. C3 links innate and adaptive immunity and plays a critical role via its fragments generated during complement activation. We have systematically analyzed a family with a C3 variant. The proband is a 2-year-old female with episodic gross and microscopic hematuria in association with febrile illness and with a positive family history of renal disease. We completed trio-based whole-exome sequencing and identified a paternally inherited C3 variant which was a three-nucleotide, single isoleucine deletion at the junction of C3a and C3b. This variant, missing 734 isoleucine, was absent from public databases including gnomAD and ExAC. Western blot analysis of the father's serum demonstrated reduced serum C3 with normal-sized α and β chains and no other fragments. The patient's FB, FI and FH were in the normal range. Rabbit RBC hemolysis assay demonstrated that patient has a significant defect that could be corrected by adding purified human C3. No enhancing agents such as nephritic factors were identified in the patient's serum through our mixing experiments with normal human and variant sera. To further analyze the behavior of the C3 variant, we produced recombinant proteins. WT and variant C3 were employed as substrates in cofactor assays with FI and FH. The C3 variant protein was significantly less efficiently cleaved. Structural analysis pointed out that the missing isoleucine is in a region that is important for binding to FH. Our results indicate that the deletion of isoleucine in the C3 variant impacts its regulation by FH. In conclusion, we suggest that less efficient cleavage of the C3 variant via cofactor activity leads to excessive complement activation because of an inadequately controlled C3 in the kidney.

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ID: 62

Human complement system inhibits therapeutic bacteriophages targeting Pseudomonas aeruginosa

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Bacteriophages (phages) are the natural predators of bacteria. Their ability to specifically recognize and kill bacteria without infecting eukaryotic cells has made phage therapy gain interest as an alternative to antibiotics. However, its application is met by several challenges. One of them is the limited knowledge

about the interactions of phages and the complement system.

To learn about this, we have developed and validated an *in vitro* method that enables rapid screening of phages in the presence of human serum. It is based on a fluorescent dye that stains damaged bacteria, in such a way that killing can be monitored in real time. Using this method, we have detected that certain phages targeting *Pseudomonas aeruginosa*, including some used in therapy, are inhibited by human serum. Based on our observations, this effect seems restricted to phages of the *Myoviridae* family. The activity of these phages in presence of serum can be rescued by the C3 cleavage inhibitor compstatin. In addition, depletion of C1q from serum also prevents the inhibitory effect. It therefore seems like the early stages of the complement cascade are involved in this phenomenon. To investigate why this occurs, we labeled the phages with a fluorescent tag and analyzed their binding to *P. aeruginosa* by flow cytometry. This revealed that human serum hampers the adsorption of *Myoviridae* phages to the bacterial surface.

These observations indicate that the complement system may interact in different ways with phages of different families. Thus, developing tools to assess phage activity in combination with complement system components is necessary to find the best candidates for therapy. Our results bring us a step closer to understanding what happens at the site of infection when a patient receives phage therapy, and may contribute to designing better therapeutic strategies against multi-drug resistant bacteria.

ID: 112

In human microvascular endothelial cells (HMEC-1) C5a/C5aR1 induces von Willebrand factor (vWF) release from Weibel-Palade bodies (WPBs) via adenylyl-cyclase activation.

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Introduction: We previously documented that C5a/C5aR1 axis is a key player of endothelial dysfunction and loss of antithrombogenic properties in conditions associated with complement activation, spanning from genetic rare diseases to viral infections. C5a caused RalA-mediated vWF exocytosis from WPBs which favoured thrombus formation (doi:10.1182/bloodadvances.2021005246).
C5aR1 is a poorly characterized G protein-coupled receptor (GPCR). Regulated secretion of WPB is induced upon engagement of different GPCRs coupled with either Ga-s or Ga-q subunits, however neither subunit has been previously involved in C5aR1 signaling in endothelial cells.

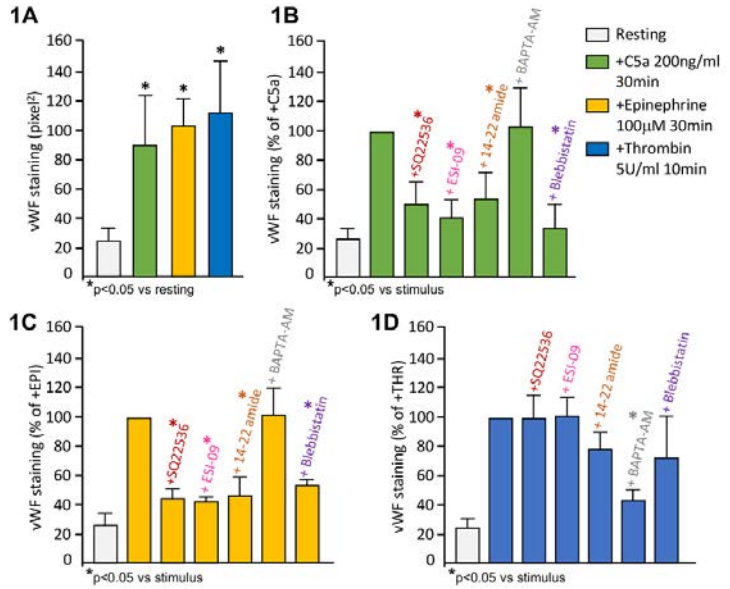
Objectives: To identify the C5a/C5aR1-induced intracellular pathway/s leading to WPBs exocytosis.

Methods: HMEC-1 were unstimulated or stimulated with C5a, epinephrine or thrombin (positive controls for Ga-s/cAMP-mediated or Ga-q/Ca^{2+}-mediated secretagogues, respectively), with or without the following cell permeable specific inhibitors: SQ22536 (adenylyl-cyclase inhibitor), or BAPTA-AM (Ca^{2+} chelator), or ESI09 (EPAC inhibitor), or 14-22 amide (PKA inhibitor), or blebbistatin (Myosin IIa inhibitor) (Fig.1A-D). Cell surface vWF staining was evaluated by immunofluorescence.

Results: C5a, epinephrine and thrombin induced vWF exocytosis (Fig.1A). vWF release from C5a-stimulated HMEC-1 was inhibited by blocking the adenylyl-cyclase or the downstream cAMP-dependent activation of EPAC or PKA (Fig.1B). The same results were obtained with the Ga-s/cAMP-mediated

secretagogue epinephrine (Fig.1C). No inhibition of C5a- or epinephrine-induced vWF release was observed with a Ca^{2+} chelator, excluding the involvement of Ca^{2+}-mediated pathways (Fig.1B-C). Instead, thrombin-induced vWF exocytosis was blocked by Ca^{2+} chelator as expected, whereas the specific inhibitors of the adenylyl-cyclase pathway had no effect (Fig.1D).
vWF release induced by C5a and epinephrine was inhibited by a myosin IIa inhibitor (Fig.1B-D), which is in line with published data that expulsion of WPBs by cAMP-raising secretagogues requires myosin IIa activation and reorganization.

Conclusion: WPB exocytosis induced by C5aR1 engagement on endothelial cells occurs via activation of adenylyl-cyclase suggesting that C5aR1 is a GPCR signalling through a Ga-s subunit.



Inhibitors: SQ22536 (Adenylyl Cyclase inhibitor, 200µM), ESI-09 (EPAC inhibitor, 10µM), 14-22 amide (PKA inhibitor, 10µM), BAPTA-AM (Ca2+ chelator, 10µM), Blebbistatin (Myolla inhibitor, 50µM). Data are mean±sd. N=3-5 experiment each inhibitor.

ID: 45

In Vivo Inhibition of Alternative Pathway Activity via MASP-3 Inhibitor OMS906

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Background: Dysregulation of the alternative pathway of complement is implicated in a variety of human diseases. Complement Factor D (CFD) catalyzes complement Factor B cleavage, allowing formation of the alternative pathway C3 convertase, which drives the amplification cycle of C3b generation. Mature CFD is generated upon cleavage of its zymogen form (pro-CFD) by mannan-binding lectin associated serine protease 3 (MASP-3). Herein we demonstrate *in vivo* inhibition of the alternative pathway by OMS906, a humanized monoclonal antibody that binds the serine protease domain of MASP-

3 and prevents its cleavage of pro-CFD.

Methods: Potency of OMS906 across species was assessed *in vitro* by inhibition of pro-CFD cleavage in plasma. Pharmacokinetics (PK) and pharmacodynamics (PD) were evaluated in cynomolgus monkeys. PD assessments included evaluation of changes in mature CFD and pro-CFD concentrations and *ex vivo* alternative pathway activity measurements. In addition, pharmacologic activity of OMS906 in blocking *in vivo* alternative pathway activity was assessed in mice exposed to bacterial lipopolysaccharide (LPS) and in a mouse collagen antibody-induced arthritis (CAIA) model.

Results: OMS906 demonstrated single-digit nM functional potency in mouse, rabbit, monkey, and human plasma. In monkeys, OMS906 decreased mature CFD concentrations and inhibited Ba induction in a dose-dependent manner, demonstrating inhibition of alternative pathway activity. In an experimental mouse model, OMS906 inhibited LPS-induced alternative pathway activity *in vivo* in a dose-dependent fashion. In a CAIA mouse model, OMS906 significantly reduced joint inflammation.

Conclusions: Preclinical studies demonstrate inhibition of alternative pathway activity through MASP-3 inhibition, suggesting therapeutic potential of OMS906 in the treatment of human diseases associated with alternative pathway dysregulation. A Phase 1 clinical trial of OMS906 is underway.

ID: 68

Inducing complement activation and ischaemic injury in a whole blood model of ex vivo normothermic perfusion of porcine kidneys

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Background: Complement activation is a key mechanism in the process of ischaemia reperfusion injury (IRI). Ex vivo normothermic perfusion (EVNP) is being used to recondition marginal organs and also offers a means to deliver drugs to organs prior to transplant. We hypothesised that homodimeric mini factor H (HDM-FH; PMID:29588430) may protect the transplanted kidney from complement mediated damage. To reach that goal, we have developed a model of EVNP in which complement activation and ischaemic damage is up-regulated and quantifiable in porcine kidneys.

Methods: 6 pairs of porcine kidneys were perfused for 6 hours with anti-coagulated autologous whole blood at 37 °C on a circuit using extracorporeal membrane oxygenation at a mean arterial pressure of 75 mmHg. The cold ischaemic time and the initial and secondary warm ischaemic times were altered to induce IRI. To quantify the IRI and complement activation, levels of complement and damage markers in the circulating perfusate, urine and tissue were measured, alongsidephysiological parameters. Complement activation from the EVNP circuit alone was measured by running a circuit without a kidney – using only the perfusate components.

Results: Controlled increases in warm and cold ischaemic times are tolerated by porcine kidneys. Longer cold ischaemic time leads to increased deposition of C3, C5b-9, and markers of fibrosis and endothelial activation in porcine kidneys, which exacerbates inflammation and damage. Circulating levels of pro-inflammatory cytokines and physiological measures of kidney function including urine output and

creatinine clearance confirmed increased damage. Complement is activated in the EVNP system in the absence of kidneys. Administration of HDM-FM to kidneys is ongoing.

Conclusion: Whole blood EVNP of porcine kidneys with extended cold ischaemic time leads to significant complement activation and IRI. This platform will be ideal to assess the ability complement inhibitors and prevent IRI damage in kidneys

Yang Y, Denton H, Davies OR, Smith-Jackson K, Kerr H, Herbert AP, Barlow PN, Pickering MC, Marchbank KJ. An Engineered Complement Factor H Construct for Treatment of C3 Glomerulopathy. Journal of the American Society of Nephrology. 2018;29:1649.

ID: 41

Inflammation and Fatigue in Patients with Cold Agglutinin Disease (CAD): Analysis From the Phase 3 Cardinal Study

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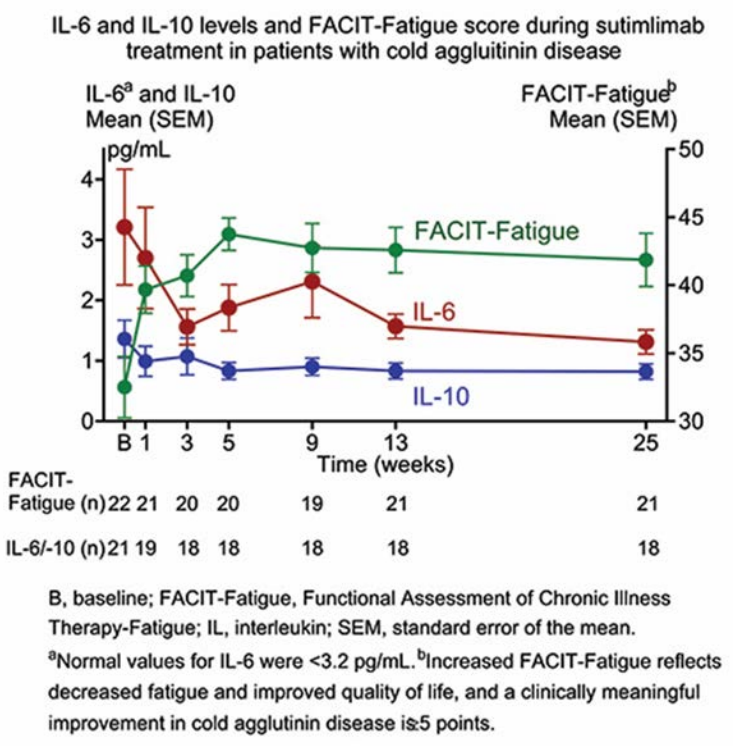
Background: CAD is a rare autoimmune hemolytic anemia driven by activation of the classical complement pathway. Complement activation increases vascular inflammatory markers via generation of C3a/C5a-mediated response of increased cytokine production (eg, interleukins [IL]-6 and IL-10). In CAD, classical complement pathway activation and chronic inflammation may contribute to fatigue beyond anemia. Sutimlimab is a humanized monoclonal anti-C1s antibody that selectively inhibits the classical complement pathway. We assessed the inflammatory cytokine expression and fatigue over the Part A 26-week treatment period of CARDINAL.
Methods: Twenty-four CAD patients with recent history of transfusion received intravenous sutimlimab on Days 0 and 7, then biweekly infusions (6.5g dose for <75kg [n=17]; 7.5g dose for ≥75kg [n=7]). Cytokine profiles and Functional Assessment of Chronic Illness Therapy (FACIT)-Fatigue scores were assessed from baseline to Weeks 1, 3, 5, 9, 13, and 25.
Results: In CARDINAL Part A, baseline mean FACIT-Fatigue score (pg/mL [standard error of the mean]) was 32.5. Sutimlimab treatment inhibited the classical complement pathway with improvement in mean FACIT-Fatigue score by Week 1 that was maintained through Week 25 (Figure). Mean IL-6 level was 3.21pg/mL at baseline, decreased as early as Week 1 (2.70pg/mL) after starting sutimlimab treatment and was 1.31pg/mL at Week 25 (Figure). A similar pattern of change was observed for IL-10, with the mean values of 1.36pg/mL at baseline and 0.82pg/mL at Week 25 (Figure). Decreased inflammation, demonstrated by IL-6 and IL-10 activity, inversely correlated with FACIT-Fatigue score improvements over time.
Conclusion/Summary: Sutimlimab treatment was associated with rapid and steady decrease in IL-6 and IL-10 levels that coincided with clinically meaningful improvements in fatigue. These results suggest that, in addition to anemia, complement-mediated inflammation may contribute to fatigue in CAD patients and

improves with complement inhibition. Furthermore, they highlight the influence of classical complement pathway inhibition on inflammatory activity in CAD.



B, baseline; FACIT-Fatigue, Functional Assessment of Chronic Illness Therapy-Fatigue; IL, interleukin; SEM, standard error of the mean.
[a]Normal values for IL-6 were <3.2 pg/mL. [b]Increased FACIT-Fatigue reflects decreased fatigue and improved quality of life, and a clinically meaningful improvement in cold agglutinin disease is≥5 points.

ID: 166

Inflammatory stimuli reveal a differential glycocalyx response between venous and arterial endothelial cells leading to differences in complement deposition

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Introduction:
The glycocalyx is a layer of sugars on endothelial cells (EC) enriched in heparan sulfate (HS). During inflammation, altered sulfation and shedding of HS occurs, activating pro-inflammatory and pro-coagulant pathways. Arterial and venous EC respond differently to inflammatory cues, whether this is due to distinct glycocalyx/HS dynamics is not known. Here we investigate differences in shedding of HS between arterial and venous EC in response to inflammatory stimuli and analyze its effect on complement deposition.

Methods:
Porcine arterial and venous EC were cultured under 12 dyne/cm^2 shear stress using a 3D microfluidic system. HS coverage and -shedding was quantified after EC activation with human serum (as a model of xenotransplantation), TNFa, or LPS. EC activation and complement deposition were analyzed by

immunofluorescence.

Results:
HS was detected on arterial EC exclusively under flow but was observed on venous EC in static and flow conditions, suggesting different glycocalyx dynamics. Next, glycocalyx was detected upon EC activation. HS remained intact on venous EC when activated with human serum, TNFa or LPS, whereas HS was shed from arterial EC (75% with human serum, 37% with TNFa and 28% with LPS) (Fig. 1A, B). Next, expression of E-selectin and deposition of C3b/c was analyzed. While C3b/c deposition was comparable between arterial and venous EC after stimulation with human serum, it was reduced by 60% ($p < 0.05$) on venous EC stimulated with LPS or TNFa (Fig. 1C).

Discussion:
We show for the first time that the glycocalyx on venous but not arterial EC is resistant to shedding upon inflammatory cues. Interestingly, complement deposition still occurs on EC with an intact glycocalyx, but is reduced on venous EC after stimulation with TNFa or LPS. This suggests that arterial and venous EC respond differently to pro-inflammatory signals.

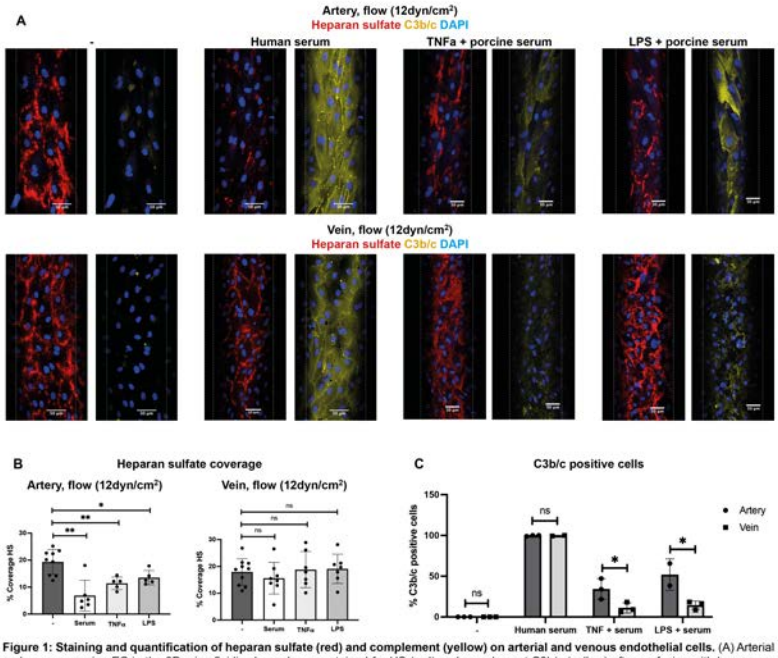

Figure 1: Staining and quantification of heparan sulfate (red) and complement (yellow) on arterial and venous endothelial cells. (A) Arterial and venous porcine EC in the 3D microfluidic channels were stained for HS (red) and complement C3b/c (yellow) after perfusion with human serum, TNFa or LPS and porcine serum to evaluate HS coverage and complement deposition Nuceli were stained with DAPI (blue). (B) HS coverage was calculated based on immunofluorescence staining and is reported in % coverage of the channel area for n=6-8 experiments. (C) The number of C3b/c positive cells was evaluated by immunofluorescence staining and is reported in % positive cells comparing arterial to venous EC for n=3 experiments. * p<0.05, ** p< 0.01, student's t-test. Both arterial and venous EC were cultured under 12 dyne/cm² shear shear stress as culturing venous EC under low shear stress (2 dyne/cm²) had no additional effect on HS or complement deposition.

ID: 102

Influence of the C5a/C5aR1 Axis on B-1 Lymphocyte Trafficking and Function

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B-1 lymphocytes are a subtype of B cells that belong to the innate immune system and contribute as well as the complement system to a rapid first-line of defense against invading pathogens.

Interestingly, our previous results show a role for the complement component 5a in regulation of B-1 cells in steady state. Mice that lack the first receptor for the complement component 5a (C5aR1) show decreased numbers of B-1 lymphocytes in the peritoneal cavity and increased numbers of these cells in the spleen compared to wild-type controls. Additionally, we found a decreased concentration of the chemokine CXCL13 in peritoneal lavage fluids of C5aR1-deficient mice which is heavily involved in B-1 cell trafficking. Our data also propose an influence of C5aR1 on the function of B-1 cells as we saw increased serum titers of natural antibodies against pneumococcal polysaccharides and phosphorylcholine in C5aR1-deficient mice[1].

Since our previous results showed that C5aR1 is playing a significant role in the homeostasis of B-1 lymphocytes, a thorough examination of the impact on activated B-1 cells is required. Therefore, B-1 cells from wildtype and C5aR1-deficient mice were stimulated intraperitoneally with the TLR2 ligand Pam3CSK4. After several timepoints organs were sampled, and cell surface antigen expression was analyzed with flow cytometry. Additionally, serum antibody levels and cytokine levels in the peritoneal lavage fluid were analyzed with immunoassays.

Our results indicate a clear influence of the C5a/C5aR1 axis on B-1 cells in an activated state, as B-1 cells in C5aR1-deficient mice are unable to leave the peritoneal cavity after 3h of stimulation as seen in the wild-type controls. Concomitantly, there is no increase of B-1 cells in secondary lymphoid organs like spleen and lymph nodes. The analysis of migration markers like CD9 and CXCR5 additionally verified the impact of C5aR1 on B-1 cell trafficking.

Reference

Bröker, K., Figge, J., Magnusen, A.F., Manz, R.A., Köhl, J., and Karsten, C.M. (2018). A novel role for C5a in B-1 cell homeostasis. Front. Immunol. 9, 1–14.

ID: 40

Inhibition of Complement C1s by Sutimlimab in Patients with Cold Agglutinin Disease (CAD): Efficacy and Safety Results from the Randomized, Placebo-Controlled Phase 3 CADENZA Study

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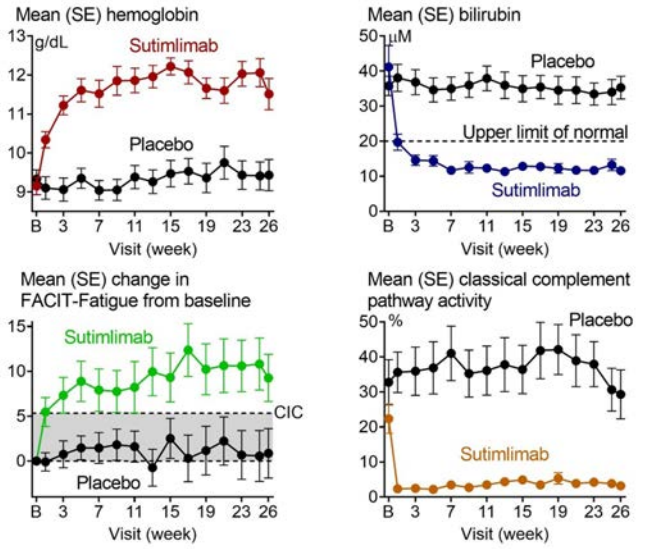
Background: CAD is a rare autoimmune hemolytic anemia mediated by classical complement pathway activation. Sutimlimab, an anti-C1s antibody, halted hemolysis, improved hemoglobin, and quality of life (QOL) in CAD patients with recent transfusion (CARDINAL). CADENZA is a randomized, double-blind, placebo-controlled Phase 3 study assessing sutimlimab in CAD patients without recent transfusion.
Methods: CAD patients (baseline hemoglobin ≤10g/dL, elevated bilirubin, transfusion independence, ≥1 CAD symptom) were randomized to receive sutimlimab (N=22) or placebo (N=20) on Days 0, 7, then biweekly. Primary endpoint was the proportion of patients with hemoglobin increase ≥1.5g/dL at the treatment assessment timepoint (TAT; mean of Wks23, 25, 26) and avoidance of transfusion and study-prohibited CAD therapy (Wks5–26). Secondary endpoints included hemolysis markers, FACIT-Fatigue, and pharmacodynamic outcomes. Safety was evaluated.
Results: Sixteen (73%) sutimlimab patients met the primary endpoint criteria, versus 3 (15%) placebo patients (P<0.001). Sutimlimab, but not placebo, increased mean hemoglobin and FACIT-Fatigue, and normalized bilirubin by Wk1, which were sustained to TAT (Figure). At TAT, the mean (standard error) difference in hemoglobin and FACIT-Fatigue between sutimlimab and placebo was 2.6 (0.4)g/dL (P<0.001) and 8.9 (2.5) points (P<0.001). With sutimlimab, improvements in anemia, hemolysis, and fatigue coincided with normalized C4 levels and reductions in classical pathway activity and CH50. C1q remained unchanged from baseline to Wk26. Twenty-one sutimlimab (96%) and 20 (100%) placebo patients experienced ≥1 treatment-emergent adverse event (TEAE). Serious infections were reported (none meningococcal). There were no TEAE of serious hypersensitivity, anaphylaxis, systemic lupus erythematosus, or death. Three sutimlimab patients discontinued from study due to acrocyanosis and Raynaud's phenomenon (n=1), increased blood IgM (n=1), and infusion-related reaction (n=1). TEAEs more common in sutimlimab than placebo (difference of ≥3 patients) were hypertension, headache, Raynaud's, rhinitis, and acrocyanosis.
Conclusion: Sutimlimab rapidly halted hemolysis, increased hemoglobin, and improved QOL over placebo, and was generally well tolerated.

Figure. Effects of sutimlimab and placebo on hemoglobin, bilirubin, FACIT-Fatigue, classical complement pathway activity, and incidence of solicited symptomatic anemia.



ID: 36

Insight into the interaction profiles and functional modulation of complement-related integrin receptors using an integrated screening platform

Plüss, Carla[1]; Lamers, Christina[2,1]; Leiggener, Céline[1]; Vogt, Stephanie Anita[1]; Ciullo, Lorella[1]; Tschanz, Marco[1]; Mancuso, Riccardo[1]; Rabbani, Said[1]; Ricklin, Daniel[1]
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While the complement system serves as a first line of defense against pathogens and endogenous threats, inappropriate complement activation is involved in several clinical conditions. Cell adhesion and inflammatory signalling via complement receptors (CR) of the β_2 integrin family can serve as critical disease contributors, but the poor druggability of integrin targets and the shortage of validated assay systems render CR a scarcely explored therapeutic option. In this project, we therefore compiled a library of the major ligand-binding domains (aI) of the β_2 integrins to enable direct binding and functional assay, with an emphasis on the complement receptors CR3 and CR4. Furthermore, we employ this platform to develop and assess tool compounds able to disentangle the promiscuous ligand binding profile of CR3 and its implication in pathologies. (1)
By optimizing the expression parameters and selecting appropriate purification tags, we expressed the αI domains of all four β_2 integrins with high yield and purity. SPR studies confirmed activity and selectivity of the recombinant CR3 and CR4 aI domains for complement opsonins C3b, iC3b and C3d with distinct kinetic profiles. Bead- and cell-based adhesion assays were employed to confirm and expand upon the molecular characterization. Moreover, the effect of cofactors and peptidic and small molecule modulators are currently investigated with this platform.
It is anticipated to use the recombinant αI domains of β_2 integrins in functional studies and for CR3/CR4-targeted drug development, with a potential expansion to the other β_2 family members at a later stage. Tool compounds developed within this project are expected to provide valuable insight into the (patho-

)physiology of CR3 and may help to identify potential therapeutic approaches for autoimmune, inflammatory, and age-related diseases.

Reference

Lamers C, Plüss CJ, Ricklin D. The Promiscuous Profile of Complement Receptor 3 in Ligand Binding, Immune Modulation, and Pathophysiology. Front Immunol. 2021 Apr 29;12:662164. doi: 10.3389/fimmu.2021.662164.

ID: 51

Interaction of a distinct Fcα interdomain oligopeptide of camel IgA with the Human Complement System

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The complement system is a well-known part of the innate immune system and can be activated by different triggers. One way to activate the cascade of the complement system is by the binding of an antibody to an antigen. Old-world and New-world camelids own unique, homodimeric heavy chain-only antibodies, which exist beside conventional heavy-light chain heterotetrameric antibodies. These single-domain antibodies, with their special characteristics, are of pharmaceutical interest. Sequence analysis of camel IgA revealed a species-specific pentapeptide situated between the α2 and α3 domain of Fcα of cryptic function. We hypothesized that the oligopeptide containing 2 anionic amino acids (glutamic and aspartic acid) has the potential to either activate or inhibit the complement system.

A specific 20-mer fragment of a camelid IgA Fcα region was aligned and synthesized. As a control, a 19-mer sequence from human IgA Fcα was used. Both oligopeptides are derived from the same region between α2 and α3 domains in the Fc region of IgA. Complement activation was investigated by incubation of peptides with normal human serum (NHS) and subsequent measurement of the terminal complement component (TCC) by a customized TCC-ELISA.

Preliminary experiments showed inconclusive results (n = 3). However, the experimental setup is finalized and major experiments will be conducted within the next weeks. Upon availability of results, we will be able to conclude whether these camelid-derived oligopeptides might play a role in modulating complement-related diseases.

ID: 24

Investigating the role of C3 in type 2 diabetes onset using mouse models

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[1]Lund University

Type 2 diabetes (T2D), and its risk factor metabolic syndrome are associated with amplified inflammatory reactions. C3, as the central player of the complement system, contributes to the inflammation seen in T2D and obesity. Serum C3 levels correlate with body fat mass, BMI, and insulin resistance. The cleavage product C3a has also been linked to inflammation and subsequent insulin resistance in obese adipose tissue.
Our lab recently discovered that C3 is highly expressed in human pancreatic islets, and upregulated in diabetic donors and 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, but a proinflammatory role in the extracellular environment.
To understand the role of C3 in pancreatic islets, we are using an animal model of high-fat diet induced obesity and type 2 diabetes. We first compared full body C3KO to WT mice, as well as a beta cell-specific C3KO mice created using a Cre-lox system. Mice were weighed weekly and fasting blood glucose and insulin were measured. After six months on high fat diet, tissues were collected for gene expression analysis and immunohistochemistry to assess inflammation in the tissues. We did not see any difference between total C3KO and WT mice so far, probably due to the contrasting effects of serum C3 and beta cell-derived C3. Consistent with this, beta-cell specific C3KO mice displayed an increased weight gain and an impaired glucose homeostasis. Ultimately, we believe that better understanding of mechanisms controlling and alleviating beta cell stress could lead to the development of new strategies for extending beta cell survival and function.

ID: 98

Involvement of classical complement pathway activation in anti-neutrophil cytoplasmic antibody-associated vasculitis

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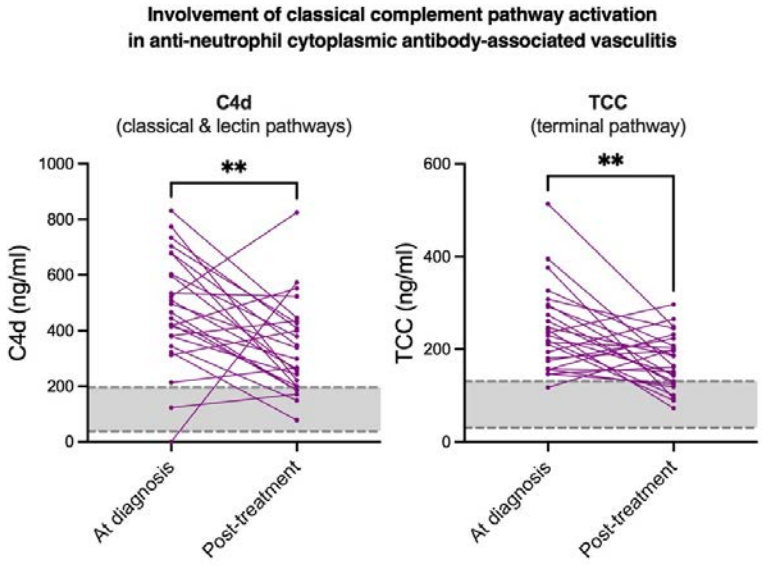
OBJECTIVES: There is strong evidence that complement activation plays an important role in anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV). Most studies suggest that the alternative complement pathway is crucial for the pathogenesis of AAV. Using a novel C4d assay detecting a neoepitope in C4d that exclusively arises upon complement-mediated cleavage, we investigated the involvement of classical pathway activation.
METHODS: The study included 42 patients with established AAV. Plasma samples at diagnosis were available for all patients of which 35 had renal involvement. For 26 patients an additional sample after induction therapy (6-12 months) was available. Sixteen patients were myeloperoxidase (MPO) ANCA positive and 25 were proteinase 3 (PR3) ANCA positive. ï»¿Levels of plasma C4d and terminal complement complex (TCC) were measured by ELISA. Forty population-based control samples were included for

comparison.
RESULTS: Compared to population-based controls, both plasma C4d and TCC levels were significantly increased in AAV patients at diagnosis (p=0.0362 and p<0.0001). Importantly, paired analysis revealed that both C4d and TCC plasma levels were significantly decreased after induction therapy (p=0.0067 and p=0.0039). C4d levels at diagnosis were significantly higher in PR3-positive patients than in MPO-positive patients (p=0.0042). Considering only patients with renal involvement, TCC levels correlated significantly with BVAS at diagnosis (r_s=0.3403, p=0.0455). TCC levels were significantly higher in untreated than in treated patients at diagnosis (p=0.0394). Post-treatment, C4d and TCC levels were lower, although not significantly, in remission than in active patients.
CONCLUSIONS: We have shown that the classical complement pathway might play a more significant role in AAV pathogenesis than widely established. We suggest that the C4d assay could be added to the repertoire of complement biomarkers used to identify patients amenable to treatment with complement inhibitors and for monitoring treatment outcomes in clinical trials of AAV. However, these results need to be confirmed in larger cohorts.



ID: 141

Irradiated apoptotic peripheral blood mononuclear cell secretome (APOSEC) attenuates complement activation in an in vitro xenotransplantation model.

Salimi Afjani, Neda; Sorvillo, Nicoletta; Ankersmit, Hendrik Jan; Rieben, Robert

Background:
Uncontrolled complement activation causes an exacerbated inflammatory response and tissue damage and is observed in several pathological conditions. Hence, fine regulation of the complement system is the key to achieving a balanced immune response. We investigated the effect of registered anti-inflammatory biological drug APOSEC (Lyophilized gamma-irradiated and viral cleared apoptotic

periphery blood mononuclear cells' secretome (APOSEC)), on complement activation and deposition on endothelial cells.

Method:

Complement activation was achieved by incubating wild type porcine aortic endothelial cells (PAEC) with 10% pooled normal human serum (NHS). Either pure medium (as control) or three concentrations of APOSEC (1, 2.5, 10 U/ml) were added to the NHS before incubation with PAEC. Complement deposition was then assessed by immunofluorescence staining for C3b/c, C4b/c and C5b-9. Complement activation markers C3a and C5a were measured by ELISA, and the viability of the cells was analysed by flow cytometry.

Results:

Incubation of PAEC with 10U/ml APOSEC let to a significant reduction in complement deposition on PAEC (Figure 1). Binding of C3b/c, C4b/c and C5b-9 to PAEC was reduced by 64%, 82% and 80%, respectively. Moreover, lower levels of C3a (36%) and C5a (76%) anaphylatoxins in the cell culture supernatant were observed. The addition of 10U/ml of APOSEC to NHS also led to an increased cell viability by more than 50%.

Conclusion:

Our data indicate that APOSEC can reduce complement activation leading to a reduction in cellular deposition and increase in endothelial cell viability in a xenotransplantation model. Our data also suggest that modulation of complement activation might contribute to the known anti-inflammatory effects of APOSEC.

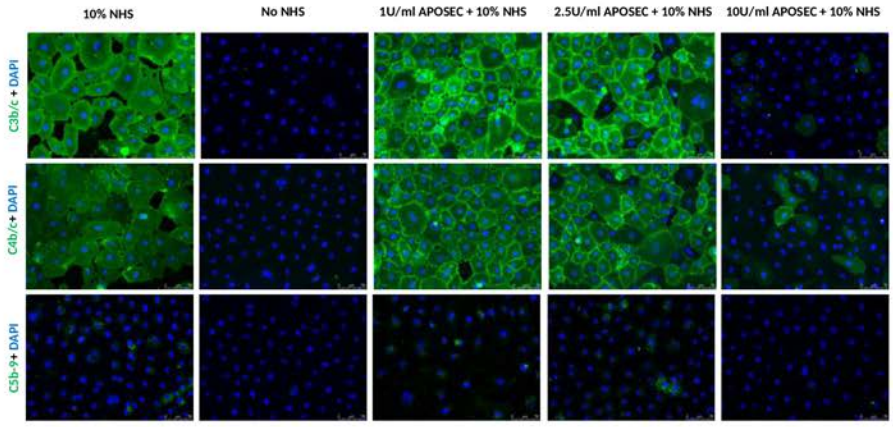


Figure 1: Deposition of complement proteins in PAEC was inhibited by applying APOSEC. All three complement deposition markers C3b/c, C4b/c and C5b-9 have the highest signal in positive control (1st column from left). In negative control there isn't any signal of complement deposition markers (2nd column from left) . 1U/ml and 2.5U/ml APOSEC do not decrease the complement deposition on PAEC (3rd and 4th column from left) , where as 10U/ml APOSEC inhibits complement activation and deposition significantly (5th column from left).

ID: 143

Is the combined heterozygous genetic defect in C2 and C8B a reason for multifaceted immune imbalance?

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Several genetic abnormalities in central complement genes have been linked to diseases and can help to explain their clinical phenotype on a mechanistic level. In the present case study, a patient presented at our institute in a reduced general condition, depicting a wide variety of autoimmune diseases (e.g., Morbus Basedow, SJörgen syndrome). While no disease-specific autoantibodies could be determined, whole exome sequencing revealed mutations in the complement genes C2 and C8B. Investigation of the cellular and humoral innate immune system identified slight impairment of leukocyte (PMN, monocyte) functionality and a more prominent dysfunction in complement functionality. In vitro addition of purified C2 and C8 into the patients serum did not improve hemolytic activity while the addition of purified C3 partially normalized complement function. Besides, we identified low levels of properdin and Factor B in comparison to sex- and age-matched controls, indicating a putative combination of several factors resulting in this phenotype. In a clinical transfer experiment, we backfilled complement components by the addition of blood group matched fresh frozen plasma and could improve complement functionality in terms of surface opsonization and hemolytic activity. Without elucidating the direct link between the genetic complement defects and the clinical phenotype, this present sudies shows that the patient would benefit from a complement subsitution therapy, thus paving the way for further complement deficiency research with its therapeutical potential.

ID: 49

Isoform-specific binding of apolipoprotein E to complement factor H alters amyloid-β-mediated neurotoxicity in vitro and in vivo

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Idiopathic Normal Pressure Hydrocephalus (iNPH) is a neurodegenerative disease seen in aging population. The frontal cortex biopsy samples taken from these patients at shunt placement show a high frequency of amyloid β (Aβ) pathology and expression of markers that correlate with early Alzheimer's disease (AD). AD is characterized by accumulation of Aβ, formation of neuronal plaques and neuroinflammation in the brain. The *APOE4* variant of apolipoprotein E (apoE) is the strongest risk factor for late-onset AD. ApoE is believed to be involved in Aβ clearance, but the mechanisms in this process are not fully understood. Mouse models have demonstrated that the classical pathway component C1q contribute to the microglia-mediated loss of brain synapses in early AD. Interestingly, apoE interacts with complement regulator factor H (FH), but the role of this interaction in AD pathogenesis is unknown.

Here, we studied unique frontal cortex biopsy samples and sera obtained from living subjects with iNPH to analyze the apoE isoform-specificity of the apoE-FH interaction and whether this interaction plays a role in early stages of AD. We show here that FH binds both lipid-free and high-density lipoprotein-associated native apoE in an apoE isoform-dependent manner (apoE2 > apoE3 > apoE4) both *in vitro* and *in vivo*. The immunohistochemistry images from the biopsy samples reveal colocalization of apoE and FH near Aβ plaques and complement activator C1q, indicating role of apoE in FH-mediated complement regulation on C1q-containing Aβ plaques. Increased expression of AD associated proteins *in vitro* by microglia and complement factors *in vivo* in the brains of apoE4 allele carriers provides further evidence for the role of complement-apoE interaction in regulation of Aβ-induced neuroinflammation. In conclusion, this study suggests an important mechanism of how the strongest genetic risk factor for AD predisposes to neuroinflammation in the early stages of the disease pathology.

ID: 69

Labelling bacterial surfaces with artificial antigens to unravel how antibodies and complement drive bacterial killing

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Infections with Gram-negative bacteria are increasingly difficult to treat because of the emergence of antibiotic resistant strains. Antibody therapy is a promising alternative treatment strategy since antibodies can activate the complement system, and thereby trigger the direct killing of Gram-negative bacteria via formation of the Membrane Attack Complex. However, it remains unknown what antigen-antibody combinations can potently trigger complement-mediated lysis of Gram-negative bacteria. Because monoclonal antibodies targeting Gram-negative surface structures are lacking, we developed a model to artificially couple well-defined antigens to live *Escherichia coli* cells. Specifically, we the fused the 8-amino acid StrepTagII to outer membrane protein X (OmpX) via genetic engineering. Alternatively, we used click chemistry to couple StrepTagII to 3-Deoxy-D-manno-oct-2-ulosonic acid (KDO), an important component of the lipopolysacharide inner core. Next, we used monoclonal antibodies against StrepTagII to evaluate binding and complement activation on these cells. We observed equal binding of anti-StrepTagII IgM and IgG antibodies to the StrepTagII on both targets. We subsequently compared the activity of IgM and IgG in a purified classical pathway system, to exclude interference of the alternative and lectin pathways, which revealed that IgM has superior capacity to induce complement lysis of *E. coli*, compared to IgG. No significant differences were observed between pentameric and hexameric IgM in binding, nor complement activation. Finally, we show that the presence of the O-antigen completely blocks the binding of anti-StrepTagII monoclonals to the StrepTagII on both OmpX and KDO. Altogether, we developed a well-defined antigen-antibody system to study antibody-dependent complement activation on bacterial cells.

ID: 27

Lectin pathway enzyme MASP-2 and downstream complement activation in COVID-19

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Mannose-binding lectin-associated serine protease 2 (MASP-2) is the main activator of the lectin complement pathway and has been suggested to be involved in the pathophysiology of coronavirus disease 2019 (COVID-19). Circulating in heterocomplexes with the pattern recognition molecules (collectin-10, collectin-11, mannose-binding lectin, ficolin-1, -2 and -3) it is activated upon binding to carbohydrates and acetylated compounds, found on the surface of various pathogens and endogenous ligands. MASP-2, when activated, can cleave C4 and C2 into C4a, C4b and C2a and C2b, respectively. To study a possible association between MASP-2 and COVID-19, we aimed at developing a sensitive and reliable MASP-2 ELISA. From an array of novel mouse-monoclonal antibodies using recombinant MASP-2 as antigen two clones were selected to create a sandwich ELISA. Plasma samples were obtained from 216 healthy controls, 347 convalescent COVID-19 patients, and 147 prospectively-followed COVID-19 patients. The assay was specific towards MASP-2 and did not recognize the truncated *MASP2* splice variant MAP-2 (MAp19). The limit of quantification was shown to be 0.1 ng/ml. MASP-2 concentration was found to be stable after multiple freeze-thaw cycles. In healthy controls, the mean MASP-2 concentration was 524 ng/ml (95% CI: 496.5–551.6). No significant difference was found in the MASP-2 concentrations between COVID-19 convalescent samples and controls. However, a significant increase was observed in prospectively-followed COVID-19 patients (mean: 834 ng/ml (95% CI: 765.3–902.7, $p<0.0001$). In these patients MASP-2 concentration correlated significantly with the concentrations of the terminal complement complex ($\rho = 0.3596$, $p<0.0001$), with the lectin pathway pattern recognition molecules ficolin-2 ($\rho=0.2906$, $p=0.0004$) and ficolin-3 ($\rho=0.3952$, $p< 0.0001$), and with C-reactive protein ($\rho=0.3292$, $p=0.0002$). Overall, we developed a specific quantitative MASP-2 sandwich ELISA. MASP-2 correlated with complement activation and inflammatory markers in COVID-19 patients and underscore a possible role of MASP-2 in COVID-19 pathophysiology.

ID: 103

Levels of plasma serine protease/C1-inhibitor complexes in SARS-CoV-2 infection

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C1-inhibitor (C1-INH) is a main inhibitor of the serine proteases of the complement, kinin-contact and coagulation systems. Decreased level of functionally active C1-INH is behind the symptoms and may lead to life-threatening angioedematous episodes in a rare disease: hereditary angioedema due to C1-INH deficiency (C1-INH-HAE). Some of the serine proteases controlled by C1-INH are responsible for bradykinin (BK) genesis (e.g.: kallikrein, FXII, MASP-1) which is thought to be the main cause of the edema formation in C1-INH-HAE. In our laboratory we developed a set of ELISA tests to detect the amount of the serine protease/C1-INH complexes in parallel from plasma samples to reveal which serine proteases and cascade systems are involved most in the pathomechanism of C1-INH-HAE. BK was also thought to contribute to the pathomechanism of coronavirus disease (COVID-19) caused by SARS-CoV-2. Furthermore, the generation of BK correlates with C1-INH levels and the activation of the serine proteases of the above mentioned systems.

Thus, we measured the levels of total C1-INH, active C1-INH and the C1r-, C1s-, MASP-1-, MASP-2-, FXI-, FXII-, trombin-, and kallikrein/C1-INH complexes with our ELISA system in parallel, in 126 COVID-19 patients divided into five severity categories.

We found that the total C1-INH level was the highest in most severe patients group, but the active/total C1-INH ratio was the lowest in this group. Nonetheless, the highest total complex level was found in a moderate severity category. Significantly higher complement system activation (indicated by higher C1r-+C1s-+MASP-1-+MASP-2/C1-INH complex levels) was observed in hospitalized versus non-hospitalized, or mild severity groups.

Our results provide further insight into the role of C1-INH in the pathogenesis of COVID-19. However, our future aim is to find characteristic patterns and roles of the serine protease/C1-INH complexes in the pathomechanism of COVID-19 disease.

ID: 134

Low FH-to-FD ratio increases complement activity and exacerbates complement-mediated diseases

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Introduction: Atypical hemolytic uremic syndrome (aHUS) and C3 Glomerulopathy (C3G) are ultra-rare renal diseases defined by complement dysregulation of the alternative pathway (AP). The AP is initiated through the hydrolysis of C3 to C3(H2O). Factor D (FD) cleaves factor B (FB) bound to C3(H2O) or C3b to form C3 convertase [C3(H2O)Bb, C3bBb], which cleaves, in turn, more C3 to C3b. Factor H (FH) regulates C3 convertase activity by inhibiting the formation of C3bB, accelerating the decay of C3bBb, and acting as a cofactor for factor I-mediated C3b degradation. FD levels increase as renal function declines, while FH levels remain relatively stable at different stages of chronic kidney disease.
Methods: The C3b deposition (C3bD) assay recapitulates complement activity on cell surfaces. We sought to use this assay to determine how FD levels modulate complement activity using spiking tests. We also measured FH and FD levels in 20 patients with C3G or aHUS, and correlated FH/FD ratios to C3bD assay

results.
Results: With increasing amounts of FH, C3bD declines or normalizes. With the addition of FD, C3bD increases in a dose-dependent manner. C3bD occurs when the FH/FD ratio drops below 110 in patients with aHUS or C3G. Most importantly, transplant patients whose samples are positive for C3bD pre-transplant show negative (normalized) results on post-transplantation samples, if there is no ongoing complement dysregulation.
Discussion: These data indicate that a low FH-to-FD ratio (< 110) drives complement activity. As renal function declines, FD increases and AP dysregulation increases. Anti-FD therapy can be challenging in patients with chronic kidney disease, especially if the FH levels are also relatively low FH (< 250 mg/L). When targeting complete AP blockade in CKD, the excess accumulation of FD may be a variable that will require consideration.

ID: 179

Mannose binding lectin specific Nano-inhibitors and their potential use as neuroprotective agents

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Background: Experimental evidence indicates that circulating mannose binding lectin (MBL) binds the sugar moieties exposed on endothelial cell membrane after brain ischemia (damage associated molecular patterns, DAMPs) and eventually, MBL drives pathogenic thromboinflammation on damage tissue, as a initiators of lectin pathway of the complement system. Therefore, inhibitory sugar-coated nanoparticles with a high affinity to MBL have been proposed as a novel protective therapeutic strategy for the secondary brain injury of stroke patients.
Methods: We set up a novel Surface Plasmon Resonance (SPR) based assay to screen MBL inhibitors. The selected inhibitor was tested for toxicity and efficacy on a cell model of ischemia-induced endothelial damage.
Results: The new SPR-based-assay showed that recombinant MBL (rhMBL) bound the sugar-coated SPR chips with high affinity (KD 2.3±0.3 nM). This binding was reduced if rhMBL was pre-incubated with monovalent mannose (IC50=5 mM), with a nine mannose-residues carrying glycan (IC50=0.33 mg/mL) and more effectively with mannose-coated gold nanoparticles (man-GNP, IC50=1.1 µg/mL). The method could also measure the inhibition of serum endogenous MBL. Man-GNP ability to inhibit MBL's toxic functions were analyzed ihBMECs subjected to hypoxia and re-oxygenation in presence of serum MBL. In vitro ischemia induced structural damage and inflammation (increased IL-1α, MMP-2 and ICAM-1 gene expression). These effects were not ameliorated by administering 40 µg/mL of man-GNPs, possibly due to excessive dosing of GNP. Next smaller doses, namely 5 and 20 µg/mL, were tested with the latter showing reduction of MMP2 overexpression to a similar extent than when MBL-deprived serum was applied.
Conclusions: In conclusion, we successfully developed a SPR tool for screening MBL inhibitors and identified a promising dosing before in vivo studies.

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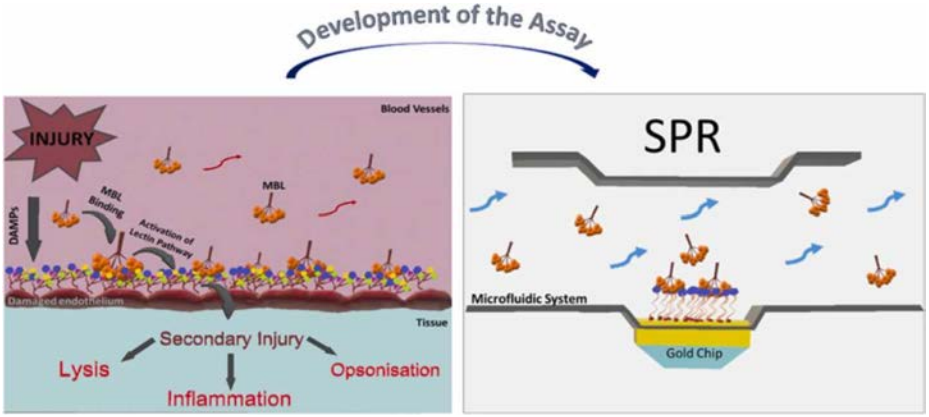

ID: 9

Mice with hyper functional complement develop age-related non-alcoholic steatohepatitis.

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Complement (C) is essential for liver regeneration and homeostasis, with both $C3^{-/-}$ and $C5^{-/-}$ mice displaying impaired response to acute injury. C dysregulation is associated with inflammation and disease; $CFH^{-/-}$ mice show increased incidence of non-alcoholic steatohepatitis (NASH) and hepatocellular carcinoma (HCC). Our mouse model of hyperfunctional complement (C3 D1115N) displays reduced binding to Factor H and impaired surface regulation (1) and so was investigated for alterations in liver homeostasis.
Wild type, $C3^{+/D1115N}$, $C3^{D1115N/D1115N}$, $C3^{D1115N/D1115N}$;$C5^{-/-}$ and $C3^{D1115N/D1115N}$;$C7^{-/-}$ mice were aged to 18 months (1,2). Liver morphology, inflammation, steatosis and fibrosis were assessed histologically by a liver pathologist. Immunohistochemistry was used to assess stellate cell activation, C deposition, immune infiltrate, proliferation, senescence and apoptosis.
$C3^{+/D1115N}$ and $C3^{D1115N/D1115N}$ mice >12 months of age showed liver pathology consistent with NASH, including cellular stress (hepatocellular ballooning, karyomegaly) inflammation (lipogranuloma) moderate-severe mixed pattern steatosis and perisinusoidal fibrosis. Two animals presented with malignant lesions, one a $CD31^{+}$, HNF4 $alpha^{-}$ vascular lesion (likely a primary hepatic angiosarcoma) and the other an HNF4 $alpha^{+}$ neoplasm with features consistent with HCC. Histological analysis showed further signs of liver injury such as stellate cell activation, apoptosis and cellular proliferation. Mice displayed C deposition and increased abundance of T cells, neutrophils, dendritic cells and macrophage, known to mediate inflammation. There were no significant differences in whole body and fat pad mass indicative of metabolic syndrome. Constitutive knock-out of C5 or C7 in $C3^{D1115N/D1115N}$ animals only partially ameliorated the hepatic phenotype.
C3 D1115N mice have a NASH phenotype similar to the $cFH^{-/-}$ mouse and further work is ongoing to assess HCC incidence in mice aged 18-24 months of age. These data suggest that that even partial loss of

C regulation through Factor H impairs liver homeostasis and that C dysregulation and chronic inflammation are sufficient to cause cellular stress and hepatic fat accumulation.

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ID: 128

Mitochondrial DNA activates the complement system in a dose- and time-dependent manner

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Background: Mitochondria evolved from α-proteobacteria; hence, mitochondrial DNA (mtDNA) is structurally similar to bacterial DNA and identified as a damage-associated molecular pattern (DAMP) in sterile and infectious inflammation. Recently, we found that synthetic analogues of mtDNA are potent activators of the complement system. This study aimed to examine the effect of native mtDNA on complement activation.
Materials and Methods: mtDNA and nuclear DNA (nDNA) were extracted from human placental tissue and purified by differential centrifugation, the DNA was isolated using phenol-chloroform-isoamyl lysis with proteinase-K digestion. The extraction yields of mtDNA and nDNA were determined by qPCR. Increasing concentrations of purified mtDNA and nDNA were incubated in lepirudin-anticoagulated human whole blood and plasma. The activation of the complement system was quantified by its activations products, using in-house developed ELISAs. mtDNA and nDNA plasma levels of 55 resuscitated out-of-hospital cardiac arrest patients were measured at the day of hospital admission using qPCR, and correlated to complement activation products C3bc and the soluble terminal complement complex (sC5b-9). Complement activation products were analyzed by in-house developed ELISA.
Results: C3bc, C3bBbP and sC5b-9 were elevated in a dose- and time-dependent manner upon the incubation with mtDNA, but not with nDNA, in whole blood and plasma. High concentration of mtDNA induced C3bc levels similar to the potent complement activator zymosan, indicating a substantial degree

of activation. C3bc and in particular sC5b-9 correlated significantly with cell-free circulating mtDNA (r=0.28, p=0.02; r=0.47, p<0.001, respectively) and even more strongly with nDNA (r=0.55, p<0.001; r=0.61, p<0.001, respectively) in these patients.
Conclusions: We conclude that mtDNA, but not nDNA, activated the complement system in a dose- and time-dependent manner. Plasma mtDNA and nDNA in resuscitated out-of-hospital cardiac arrest patients correlated with complement activation. Thus, a new activation mode of human complement is described, which might be of relevance for novel therapeutic strategies.

ID: 183

Modulation of the classical complement pathway by factor H-related proteins

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The main soluble regulatory proteins of the classical/lectin pathways, C4b-binding protein (C4BP), and that of the alternative pathway (AP), factor H (FH), do not only act in solution on C4b or C3b but also interact with ligands such as C-reactive protein (CRP) and extracellular matrix (ECM) proteins, and regulate complement on cellular and non-cellular surfaces. Next to FH, in humans there are five structurally related proteins, the FH-related (FHR) proteins, whose function is poorly understood. Accumulating evidence support a role for FHRs as competitors of FH for binding to certain ligands and enhancers of AP activation. Due to their homology to FH, FHRs were mainly studied for their effect on the AP. Since the FHRs have overlapping ligand binding profile with both FH and C4BP, we studied the potential effect of FHR-1, FHR-2 and FHR-5 on the binding and regulatory activity of C4BP and on classical pathway (CP) activation.

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

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

ID: 78

Modulation of the complement terminal pathway ameliorates peritoneal injuries in a rat peritoneal dialysis model.

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Background and objective:
Peritonitis and the resulting peritoneal injuries are common problems that prevent long-term peritoneal dialysis (PD) therapy in patients with end-stage kidney diseases (ESKD). Previously, we have analyzed the relationship between the complement system and progression of peritoneal injuries associated with PD, particularly focusing on the early activation pathways and effects of the anaphylatoxins. In the present study, we utilized a novel mAb (2H2) that blocks the C5b-7 complex and membrane attack complex (MAC) formation to investigate roles of the terminal pathway in PD-associated peritoneal injury.
Methods:
We intraperitoneally injected mAb 2H2 (2.5mg or 5mg/rat) once or twice over the course of the experiment to investigate the effects of neutralizing the C5b67 complex to prevent formation of MAC in a fungal rat peritonitis model caused by 5 sequential intraperitoneal administrations of zymosan in peritoneal dialysate after methylglyoxal pretreatment (Zy/MGO model). We evaluated macro- and microscopic changes pathological changes of both parietal and visceral peritoneum on day 5 and also assessed the degree of C3b and MAC deposition and accumulation of inflammatory cells.
Results:
mAb 2H2, but not control mAb, reduced the zymosan-triggered increase in peritoneal thickness and accumulation of inflammatory cells in a dose and frequency dependent manner. These effects were accompanied by decreased C3b, MAC and fibrinogen depositions in peritoneum.
Conclusion:
In the rat Zy/MGO peritonitis, complement terminal pathway activation contributed to development of peritoneal injuries, suggesting that it might be a target to prevent development of peritoneal injuries in human PD.

ID: 151

Molecular conditions for the assembly of initial C3 AP convertases formed by native C3 at biomaterial surfaces

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Background: Native C3 binding to abiotic surfaces is believed to be one of the initiators of the complement cascade, possibly induced by the conformational change of C3 to C3(H_2O). We have previously shown that fluid phase C3(H_2O) can form an active but not very effective alternative pathway (AP) convertase. However, the formation of C3(H_2O) leading to AP activation is likely to be greatly

accelerated by the interaction with biomaterial surfaces. Therefore, the purpose of this study was to investigate the link between surface properties and the conformation of adsorbed C3, leading to convertase formation, which is of great importance for the development of new, improved biomaterials.
Materials and methods: Native C3 was studied at the interface of systematically varied biomaterial surfaces, e.g. rigid/soft acrylate polymers, SiO_2, polystyrene (PS) and liposomes. C3 conformation and AP convertase assembly were assessed with Quartz Crystal Microbalance with Dissipation (QCM-D), ELISA and capillary immune electrophoresis.
Results: Native C3 adsorbed to hydrophobic PS and rigid acrylate surfaces had changed conformation to C3(H_2O), while C3 adsorbed to the soft acrylate and hydrophilic SiO_2 surfaces had retained most of its native conformation. Factor B bound exclusively to C3(H_2O) on the hydrophobic and rigid surfaces, and required properdin for binding. The addition of Factor D resulted in an active convertase. In contrast, no activation complexes were assembled on the hydrophilic and soft surfaces. Furthermore, C3 was binding to negatively charged liposomes, but only to those containing cholesterol. Some of these C3 molecules had formed C3(H_2O), which were able to form AP convertases. Again, properdin was essential for the initial Factor B binding.
Conclusion: The surface properties have a large impact on the conformation of bound C3, which in turn regulates the formation of AP convertases and complement activation. Surface softness and hydrophilicity are important properties for improved biomaterial biocompatibility.

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ID: 57

Molecular Insight into the Target Interaction Profile of the Factor H-Binding Peptide 5C6

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Upon contact with blood, non-self surfaces such as biomaterials or transplants can trigger the complement system and generate inflammatory mediators. These undesirable host defence reactions are enabled by the absence of complement regulators on such surfaces. One option to mitigate adverse complement activation is the recruitment of the abundant regulator factor H (FH) to the material surface using FH-binding coatings. Our groups previously described a cyclic peptide, termed 5C6, with potent FH-recruiting capacity and assigned the broad core section of FH, comprising complement control protein (CCP) domains 5-18, as general contact region (1,2).
To further characterize the binding mode and selectivity of 5C6 for proteins of the FH family, we combined recombinant protein expression with direct binding and functional studies. Using a truncation library of FH segments produced in HEK cells, we identified a five-domain segment of FH as minimum binding site for 5C6. A recombinant FH fragment containing CCP10-14 (rFH10-14) was expressed in *E. coli*

and assessed for its interaction with fluorescently-labelled 5C6 by microscale thermophoresis. rFH10-14 produced in bacteria showed potent binding to 5C6, with a comparable profile to FH10-14 and FH8-15 produced in eukaryotic cells or plasma-purified FH. Considering the homology of FH10-14 with FH-related protein 5 (FHR5), we explored potential cross-reactivities of 5C6 with FHR5 but did not detect any relevant interactions. The FH-recruiting and complement-modulating capacities of 5C6 coatings were validated by a bead-based assays. 5C6, but not a scrambled derivative, was able to recruit rFH10-14 and full-length FH. The active 5C6 coating also prevented C3b deposition on beads when exposed to serum. Despite the absence of structural data, the combination of segment libraries and recombinant FH segments enabled us to map the 5C6-capturing site to a defined area in the core section of FH. The availability of a functionally active rFH10-14 fragment that can be produced in bacteria will largely facilitate future structural studies.

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ID: 160

New Biomarkers for non-C1INH Associated Hereditary Angioedema.

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Background: Hereditary angioedema (HAE) is a genetic disorder of the C1-INH gene, primarily affecting the kallikrein-kinin system, although complement evaluation has traditionally been used for diagnosis. Mutations in Factor XII, plasminogen, kininogen 1 and angiopoietin-1 genes have also been found in some patients with HAE with normal C1-INH (nC1INH-HAE) concentration but with a similar clinical presentation, which indicates that there is a need for new diagnostic biomarkers as a supplement to the presently used genetic and immunochemical analyses. By using protease-C1-INH or antithrombin (AT) complex assays, we investigated if these assays could distinguish nC1INH-HAE patients from HAE type I/II patients.

Materials and methods: Circulating complexes between proteases (FXIa, FXIIa, Kallikrein, MASP1, MASP2 and Thrombin) and serpins C1INH and AT were measured in EDTA plasma samples from HAE type I/II patients (n = 48), HAE-FXII patients (n = 9) and controls (n=42) using multiplex sandwich immunoassay. The levels of prekallikrein and high molecular weight kininogen (HMWK) were assessed using an automated capillary immune electrophoresis device. Chromogenic functional tests and determination of C1INH, C4, C4a and C1q concentrations were also performed.

Results: The levels of both C1-INH- and AT-protease complexes were normal in the HAE type I/II patient group compared to the control group. In HAE-FXII patients no or low levels of complexes was formed.

The HAE type I/II patients generally had a lower level of C4 and a higher C4a/C4 ratio compared to the HAE-FXII patients and there was also a difference in other protein levels.

Conclusions: Assessment of the levels of complexes between proteases and C1-INH or AT can be used to differentiate between different mechanisms of HAE. Extended analysis of parameters such as prekallikrein, HMWK, C4a and C1q add further valuable information in HAE patients for the diagnosis and response to treatment.

ID: 53

Non-canonical source of elevated C5a levels in convalescents from severe COVID-19

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Numerous publications have underlined the link between complement C5a and the clinical course of COVID-19. We previously reported that levels of C5a remain high in the group of severely ill patients up to 90 days after hospital discharge [1]. We have now evaluated which complement pathway fuels the elevated levels of C5a during hospitalization and follow-up. The alternative pathway (AP) activation marker C3bBbP and the soluble fraction of C4d, a footprint of the classical/lectin (CP/LP) pathway, were assessed by immunoenzymatic assay in a total of 188 serial samples from 49 patients infected with SARS-CoV-2. Unlike C5a, neither C3bBbP nor C4d readouts rose proportionally to the severity of the disease. Detailed correlation analyses in hospitalization and follow-up samples collected from patients of different disease severity showed significant positive correlations of AP and CP/LP markers with C5a in certain groups, except for the follow-up samples of the patients who suffered from highly severe COVID-19 and presented the highest C5a readouts. In conclusion, there is not a clear link between persistently high levels of C5a after hospital discharge and markers of upstream complement activation, suggesting the existence of a non-canonical source of C5a in patients with a severe course of COVID-19.

The project was supported by the National Science Centre (NCN) grant no. 2019/35/B/NZ6/02450

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ID: 93

Ocular phenotype in the C3Del923_924 C3-Glomerulopathy mouse model

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C3 glomerulopathy (C3G) is a heterogeneous group of rare kidney diseases characterized by complement dysregulation and a predominant C3 deposition in the kidney biopsy. In 20% of patients with C3G the pathogenic mechanism involves rare pathogenic variants in complement components or regulators. To understand how pathogenic C3 mutations result in C3G, we have used Crispr/cas9 methodologies to generate a mouse carrying a murine version of the C3G-associated human $C3_{del923\_924DG}$ protein. Previously, we reported that this murine mutant C3 protein replicates functionally its human counterpart and the mice exhibit a renal phenotype that resembles the C3-glomerulonephritis (C3GN) subtype of C3G. They show normal levels of plasma C3 and C5 and strong C3 deposition in mesangial regions of the glomeruli, but not in the glomerular basement membrane (GBM). These deposits co-localize with other complement proteins. Histopathological examination of the kidneys illustrated glomerular hypercellularity and mesangial expansion and ultrastructural findings showed non-organized, electron-dense subendothelial and mesangial deposits. Notably, no dense deposits within the GBM or evidence of endothelial injury or thrombi were observed in the glomeruli of these mice. In humans, C3G occasionally associates with early forms of age-related macular degeneration (AMD). To check whether this was also the case of our murine C3GN model we search for an eye phenotype and found an intense C3 deposition in the Bruch membrane (BM). Functional studies using cryostat sections of mouse eyes demonstrate that the BM of our $C3_{del923\_924DG}$ mice promotes activation of the alternative pathway, whilst no complement activation was observed in the BM of normal C57BL/6 mice. We also observed that $C3_{del923\_924DG}$ mice present intense lipofuscin deposits in the retinal pigment epithelium much earlier than wildtype mice, suggesting an early retinal aging in our C3GN model. These data reveal that our $C3_{del923\_924DG}$ mice is also a potential a model to study AMD.

ID: 55

Off-label use of eculizumab for the treatment of patient with neurological symptoms and non-AMD progressive loss of vision carrying pathogenic FH p.H402Y mutation

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We present the case of a 30-year-old female patient with a several-year history of epilepsy and non-AMD progressive loss of vision caused by the involvement of various eye structures. In 2021, there was a progression of neurological symptoms resulting from the involvement of the central nervous system. Epileptic seizures and behavioral disorders intensified. Based on imaging examinations, pachymeningitis, the presence of inflammatory infiltration of the left frontal lobe, and the presence of a pericerebral hematoma were diagnosed. Infectious and autoimmune causes of the observed abnormalities were excluded. Despite steroid treatment, which was initially effective, and attempts of immunosuppressive therapy, gradual disease progression was observed. In laboratory tests, signs of excessive activation of the complement system were reported, with elevated levels of the alternative pathway activity (AP50), Bb, C5b-9, and concurrent low levels of FH. Anti-FH autoantibodies were present. NGS analysis revealed seven rare genetic variants of unknown character in complement genes C8B, C8G, CFHR4, CFI, CR1, CR2, and FCN1 and pathogenic CFH p.H402Y mutation. An attempt to treat the patient with eculizumab, a blocker of C5 breakdown to C5a and C5b, has been made. During the treatment, neurological symptoms subsided, vision loss was inhibited, and laboratory improvement was achieved. It was possible to discontinue the steroid therapy, which was already starting to show side effects.

ID: 15

Peritransfusional C1-inhibitor in patients with severe complement-mediated autoimmune hemolytic anemia: an open-label phase 2 trial

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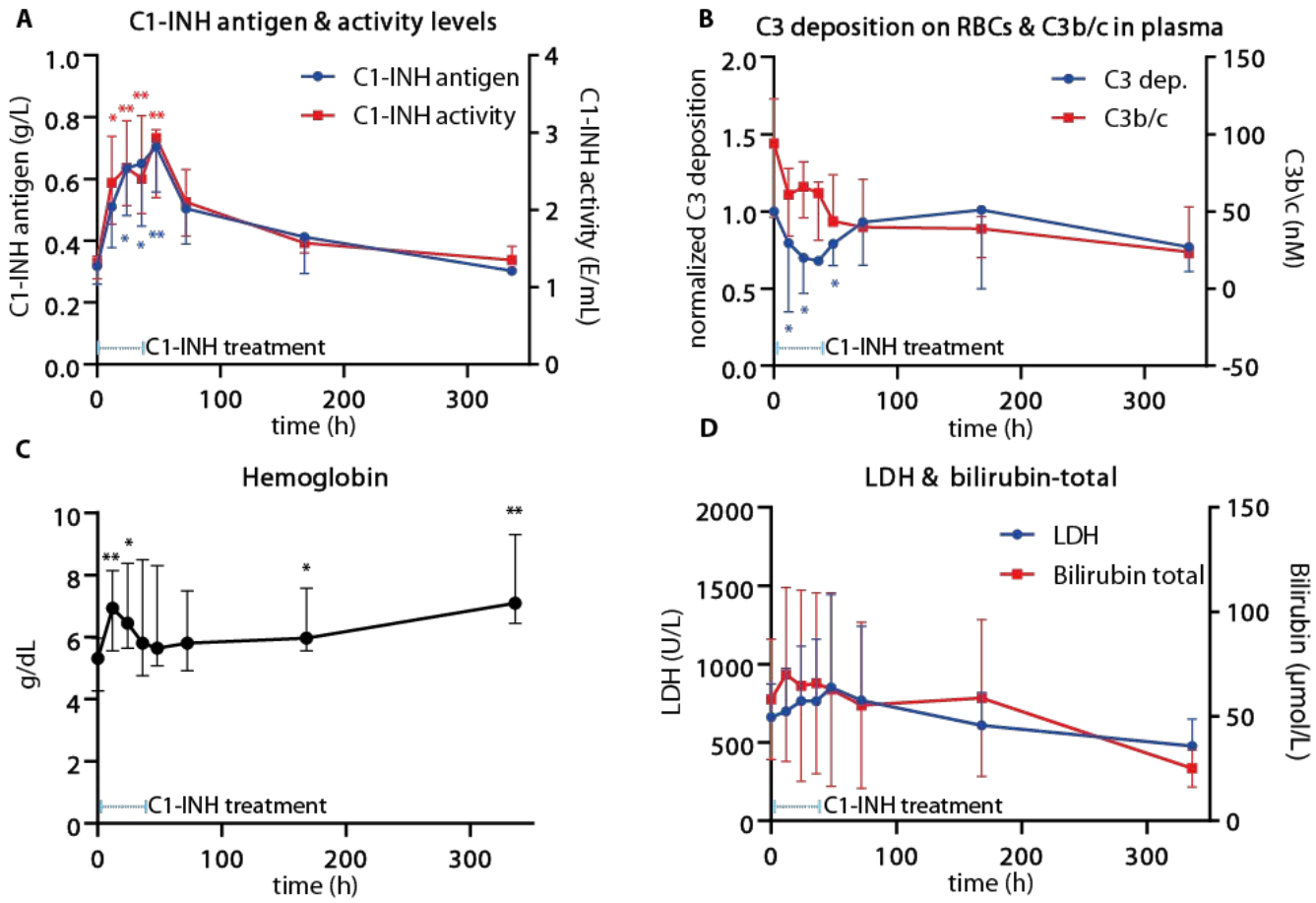
Background
Complement-mediated autoimmune hemolytic anemia (AIHA) is characterized by destruction of red blood cells (RBCs) by autoantibodies that initiate classical pathway activation. C1-inhibitor (C1-INH; Cinryze) is an endogenous plasma regulator of the classical and lectin pathway approved for patients with hereditary angioedema. In a case report and in vitro, we observed that C1-INH attenuated complement deposition on RBCs and subsequent hemolysis. Therefore, we hypothesized that peritransfusional C1-INH administration in complement-mediated AIHA would reduce complement activation, hemolysis and thus improve transfusion outcome.
Methods
We conducted a prospective, single center, phase 2 open-label trial. Patients with confirmed complement-mediated AIHA and indication for two RBC concentrates were included. Treatment consisted of four peritransfusional C1-INH doses intravenously (6000, 3000, 2000 and 1000 U) with 12 hr intervals. Blood samples at 0, 12, 24, 36, 48, 72 hrs, 7 and 14 days were analyzed for hemolytic parameters, RBC opsonization, complement activation and inflammation (nucleosomes and neutrophil elastase-α1-antitrypsin complexes) markers. Safety was monitored and scored according to the CTCAE version 4.0.
Results
Ten patients were included in the study. C1-INH administration resulted in an increase of C1-INH antigen and -activity, peaking at 48 hrs. At this timepoint, treatment significantly reduced C3d deposition on RBCs, but not plasma C3b/c levels. Transfusion briefly increased hemoglobin levels, but overall hemolytic and inflammation parameters did not improve. No serious adverse events were reported.
Conclusion

Peritransfusional C1-INH in AIHA temporarily reduced local complement activation as evidenced by C3d deposition, but did not affect hemolysis or systemic complement activation. Increased hemoglobin levels at later timepoints could be caused by additional treatments and transfusions. Altogether, we found no (clinically) significant effect of C1-INH on hemolysis and systemic complement activation. This may be explained by the dosing of C1-INH, which possibly was too low to counter the severe complement-mediated AIHA and additional transfusion effects.



ID: 176

Persistent complement activation following SARS-CoV-2 infection

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Following recovery from acute SARS-CoV-2-infections patients may experience respiratory, cardiovascular and neuropsychological post-infectious sequelae. If these symptoms occur within 3 months after acute

infection and last more than 2 months, patients suffer from post-acute sequelae of COVID-19 (PASC) according to a recent consensus definition. With an estimated prevalence of 5-20%, PASC represents a significant disease burden. Molecular mechanisms underlying the development of PASC are largely unknown. Yet, persistent immune cell activation and elevation of inflammatory cytokine serum concentration suggest immunity-related processes being involved in the pathogenesis of PASC.

Complement activation has been observed to be a feature of acute COVID-19 correlating with disease severity. However, the state of the complement system in patients with PASC has -to our knowledge- not been examined so far. In this exploratory single-center study, we determined plasma concentrations of complement activation products of the classical (C1rs/C1Inh complex, C4d), alternative (Bb) and the terminal complement pathway (C3d, sC5b-9) in a cohort of 143 patients with previously mild to severe acute COVID-19 5 months after acute infection. Furthermore, complement activation was correlated with (a) the severity of acute COVID-19 disease, (b) PASC symptoms and (c) the levels of the coagulation parameters (vWF, Factor VIII, fibrinogen and other laboratory biomarkers of severe acute COVID-19 as determined in this patient cohort 5 months post onset of acute COVID-19.
Our results demonstrate increased plasma levels of sC5b-9 (above reference range) in 46%, of C1Inh/C1rs complexes in 26% and C4d in 41% of the study participants 5 months post-infection. Bb levels were elevated in 8% of the patients while -except for one patient- C3d plasma concentrations were in the normal range. Correlation analyses with clinical and laboratory parameters are ongoing.
These findings indicate a persistent complement activation in a significant fraction of our patient cohort 5 months after SARS-COV-2 infection.

ID: 126

Pharmacokinetics/pharmacodynamics of KP104, a bi-functional antibody fusion protein inhibitor of complement, in C5/FcRn-humanized transgenic mice and Cynomolgus monkeys

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Dysregulated complement contributes to many human autoimmune and inflammatory diseases. Although several anti-complement drugs are now available, there remain significant unmet medical needs for next generation anti-complement drugs with better efficacy/convenience. KP104, a bi-functional inhibitor of complement alternative/terminal pathways, is an engineered anti-C5 IgG4 mAb fused with the N-terminal 5 short consensus repeats of human factor H, a potent alternative pathway inhibitor. *In vitro* studies have shown a differentiating and desirable profile of KP104 compared with currently approved anti-complement C5 or C3 drugs. In this study, we characterized the pharmacokinetics/pharmacodynamics of KP104 in C5/FcRn-humanized SCID mice (hC5/FcRn-Tg) and cynomolgus monkeys. hC5/FcRn-Tg mice were generated by hydrodynamic injection and expressed 80-120 mcg/ml human C5 in plasma. Following single IV administration to hC5/FcRn-Tg mice (5, 40 mg/kg), KP104 remained stable *in vivo*, showed target-mediated drug disposition behavior, with terminal half-lives of 3.2 days at 5 mg/kg and 6.3 days at 40 mg/kg, and demonstrated potent inhibition of rRBC lysis in an *ex-vivo* hemolytic assay. Following single IV administration to monkeys (1, 5 and 20 mg/kg), KP104 showed approximately 5 days terminal half-life at all the doses. The volume of distribution for KP104 in both mice and monkeys showed

typical values of a mAb. Its bioavailability after a single subcutaneous injection (100 mg/kg) to monkeys was calculated to be 93% of that of an IV infusion at the same dose. In the single-dose monkey PK/PD study, KP104 at 20 mg/kg showed complete and prolonged inhibition of rabbit RBC lysis with empirical IC50 of 18 µg/mL. This is consistent with the IC50 of 19.1 µg/mLobtained in an *ex-vivo* PNH patient RBC lysis assay. These data demonstrate promising pharmacokinetics/pharmacodynamics properties of KP104 in relevant animal models and support its development as a potent bi-functional complement inhibitor suitable for both intravenous and subcutaneous administration.

ID: 79

Pitfalls in complement analysis: a systematic literature review of assessing complement activation

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Background: The complement system is an essential component of our innate immune defense and plays a vital role in the pathogenesis of many diseases. Assessment of complement activation is critical in monitoring both disease progression and response to therapy. Complement analysis requires an accurate and standardized sampling and assay procedure, which has proven to be challenging [1].

Objective: We have performed a systematic analysis of the current methods used to assess complement components and reviewed whether the identified studies performed their complement measurements according to the recommended practice regarding pre-analytical sample handling and assay technique.

Methods: A literature search using the MEDLINE database was performed focusing on studies measuring the key complement components C3, C5 and/or their split products and/or the soluble variant of the terminal C5b-9 complement complex in human blood samples that were published between February 2017 and February 2022. The identified studies were reviewed to determine whether the correct sample type and analysis technique were used according to experts in the field of complement analysis [2].

Results: A total of 92 studies were selected for full-text analysis. Forty-five studies (49%) were identified as using the correct sample type and techniques for their complement analyses, while 25 studies (27%) did not use the correct sample type or technique. For 22 studies (24%), it was not specified which sample type was used.

Conclusion: A substantial part of the reviewed studies did not use the appropriate sample type for assessing complement activation or did not mention which sample type was used. This deviation from the standardized procedure, as generally accepted by experts in the field, can lead to misinterpretation of biomarker levels and hampers proper comparison of complement measurements between studies. Therefore, this study underlines the necessity of general guidelines for accurate and standardized complement analysis.

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ID: 28

Plasma concentrations of complement C3 partly explain the associations of measures of adiposity with type 2 diabetes: The Maastricht Study.

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Objective. A wealth of clinical and epidemiological evidence links obesity to type 2 diabetes (T2D), but the underlying causal pathways are not yet fully elucidated. Plasma concentration of complement C3, which is higher in individuals with obesity, has been identified as an independent risk factor for T2D. In this study, we investigated whether and how much of the association between obesity and T2D was explained by C3.
Methods: In 3544 population-based participants (51.7% men, 59.9 ±8.3 years, 28% T2D, oversampled), we measured plasma concentration of C3 by ELISA, adiposity as body mass index (BMI), waist circumference, visceral (VAT), subcutaneous adipose tissue (SAT), the latter by MRI, and a disturbed glucose homeostasis as insulin resistance (HOMA-IR), fasting glucose concentration and the diagnosis T2D. We conducted regression analyses to investigate the mediating effect of C3 in the association between adiposity and disturbed glucose, adjusted for age, sex, socioeconomic status, and smoking habits, with 95% confidence intervals by bootstrapping.
Results.
The measures of adiposity were positively associated with insulin resistance, glucose metabolism and T2D (all P<0.001). Plasma C3 partially and significantly mediated these positive associations and explained 26.2%, 22.3%, 25.3% and 27.5%, respectively, of the associations of BMI, waist, VAT and SAT with T2D. The strongest mediating effects of C3 in the association of adiposity with insulin resistance and glucose metabolism were seen for SAT (C3 explained 28.4% and 44.6% of the association of SAT with HOMA2-IR and fasting glucose, respectively). Additional adjustment for dietary habits and physical activity did not materially alter these observations (n=2951).
Conclusions: In this cohort of middle-aged to elderly participants we observed a significant mediating role for C3 in the association between adiposity and T2D, which suggests direct or indirect effects of C3 on insulin resistance and glucose metabolism contributing to T2D, starting from expanded and dysfunctional fat depots.

ID: 29

Post-translationally modified proteins bind and activate complement leading to increased cellular binding and uptake

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Introduction: Antibodies against post-translationally modified (PTM) proteins such as citrullinated proteins or malondialdehyde-acetaldehyde adduct containing proteins have been described in diseases like rheumatoid arthritis and cardiovascular disease. What triggers the induction of anti-PTM antibodies in these patients is unclear. The complement system is well known for its clearance function and for its role in the induction of antibody responses. We therefore hypothesized that complement could directly bind to these PTMs and in turn target them for either clearance or breach in tolerance through complement receptors.
Materials and Methods: Six PTMs were investigated: nitration (NT), citrullination (Cit), carbamylation (CarP), acetylation (AL), malondialdehyde-acetaldehyde adducts (MAA) and advanced glycation end-products (AGE). Mass spectrometry was used to analyse the binding of serum proteins to the PTM proteins or their unmodified controls in an unbiased way. ELISAs and plate-bound complement activation assays were employed to confirm mass spectrometry data. Finally, PTM-protein-coupled beads were incubated with cells in the presence or absence of complement active serum and binding and uptake was investigated.
Results: Mass spectrometry analysis revealed that complement proteins were highly enriched on PTM proteins as compared to the controls for 4 of the 6 PTMs tested, namely CarP, AL, MAA and AGE. Purified C1q bound directly to CarP, MAA and AGE indicating that C1q binding is independent of (auto)antibodies. On top of that, complement activation was observed for CarP, AL, MAA and AGE at the level of C1q, C3d and C5b9. Lastly, enhanced uptake and binding was observed for CarP, AL, MAA and AGE in the presence of complement for THP-1 macrophages and several leukocyte subsets (B cells, neutrophils and monocytes).
Conclusions: Complement components bind directly to CarP, AL, MAA and AGE modified proteins independently of antibodies, and enhance binding and cellular uptake with major implications for clearance and immune activation.

ID: 18

Predicting Post-Transplant Disease Recurrence in C3 Glomerulopathy

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Background
C3 Glomerulopathy (C3G), defined by dominant C3 deposition on kidney biopsy, is a rare kidney disease

characterized by persistent dysregulation of the alternative complement pathway. More than 50% of patients progress to ESKD within 10 years and post-transplant recurrence is manifest in up to 84%. We sought to examine demographic and pre-transplant complement biomarkers in our cohort in order to identify parameters which may predict disease recurrence.

Methods

Thirty-four C3G transplant patients from the University of Iowa's C3G Natural History Study were included. Demographic data included sex, age at transplantation, time from diagnostic biopsy to transplant, and disease category (DDD vs C3GN). Biomarkers reviewed included C3, C3c/C3 ratio, Bb, C5, C3Nef, C5Nef and sMC5b-9.

Unpaired t-tests and chi-square analyses (95% CI) were applied. ROUT analysis was used to identify definitive outliers (Q = 0.1%). Pretransplant risk of recurrence (PTxRR) scores were calculated for each patient based on the extent of their complement dysregulation, with each abnormal complement biomarker value being assigned one point on a scale of seven.

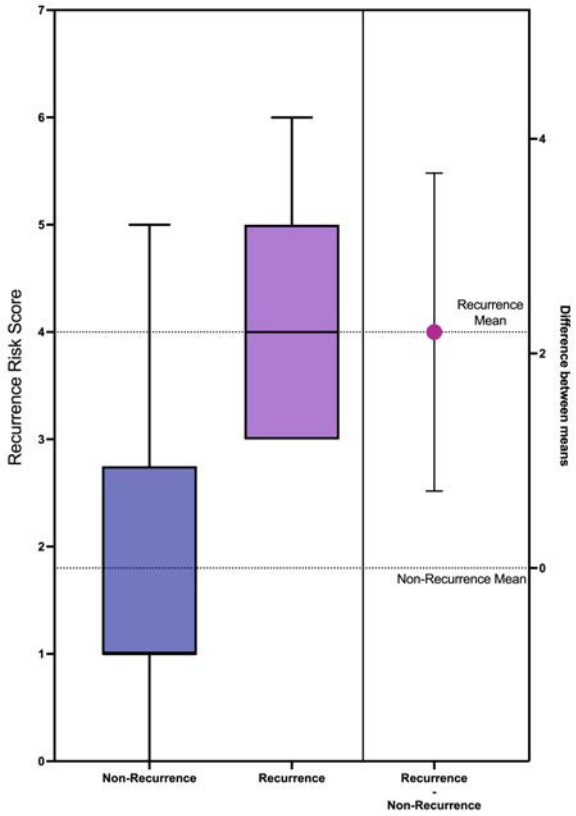
Results

6/34 (18%) of patients had documented recurrence of disease in their renal allograft. There was no significant difference in the demographic data between the groups. C3Nef, C5Nef, and C3c showed no significant differences between groups. In the recurrence group, C3 and C5 were significantly lower, while Bb, sMC5b-9, and the C3c/C3 ratio were significantly higher. PTxRR scores were significantly higher for recurrence patients.

Conclusion

In our cohort, demographic characteristics had no predictive value for determining transplant recurrence of C3G. On the other hand, pre-transplant excess complement activity biomarkers were associated with increased risk for post-transplant recurrence, both in number (PTxRR score) and severity (C3, C5, Bb, sMC5b-9, and C3c/C3 ratio). Our data suggest that the PTxRR score may be useful for identifying patients at higher risk of post-transplant C3G recurrence.

Figure 1: Recurrence Risk Score Estimation Plot



ID: 46

Production of complement regulators factor H and FHL-1 and complement stabilizer properdin by human macrophages

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Most complement factors are produced by the liver, however, also extrahepatic sources of complement factors have been described. Here we investigated the expression and secretion of complement regulator factor H, its splice-variant FHL-1 and the positive regulator of the complement system properdin, by human monocyte-derived macrophages.

CFH and *FHL1* mRNA expression levels in liver tissue were comparable, however, western blot analysis of normal human serum showed only minute amounts of FHL-1 compared to factor H. A possible

explanation for this difference may relate to renal clearance of the ~42kDa FHL-1. To investigate the potential clearance of FHL-1 by the kidneys, human renal tissue was stained for factor H and FHL-1. FHL-1 was detected in healthy kidneys, whereas factor H was not, suggesting renal clearance of the FHL-1 protein.
CFH and *FHL1* expression was not detected in monocytes, but upon differentiation into macrophages, both pro- and anti-inflammatory macrophages expressed *CFH* and *FHL1*. Expression of *FHL1* was higher in both pro- (4.5-fold increase) and anti-inflammatory macrophages (5.2-fold increase) compared to *CFH* expression. Macrophages were able to secrete factor H/FHL-1 and levels were upregulated by IFN-g stimulation. Immunoprecipitation of factor H/FHL-1 from macrophage supernatant showed higher FHL-1 levels when compared to factor H.
Macrophages also secrete properdin and stimulation with IFN-g lowered properdin secretion in both types of macrophages (2.5-fold decrease). Properdin levels were upregulated by various stimuli in pro- (LPS: 5.1-fold; L-CD40L: 2.9-fold) and anti-inflammatory macrophages (LPS: 1.4-fold ; L-CD40L: 3.3-fold).

In conclusion, we demonstrate the production of factor H, FHL-1 and properdin by pro- and anti-inflammatory macrophages, and levels are differentially regulated upon stimulation. Interestingly, both pro- and anti-inflammatory macrophages preferentially express and secrete FHL-1. The production of complement enhancer properdin and complement inhibitor FHL-1 by macrophages suggests a local role for AP activation in the context of innate and adaptive immune responses.

ID: 92

Promoting Fc-Fc interactions between anti-capsular antibodies provides strong immune protection against Streptococcus pneumoniae

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Introduction: Monoclonal antibodies (mAbs) that boost the host immune system have emerged as attractive therapeutic candidates for the treatment of multidrug-resistant pathogens. An important effector function of antibody-dependent immunity is the induction of complement activation, a reaction that triggers a variety of immune responses that induce bacterial phagocytosis and intracellular killing by professional phagocytes. Recent studies have shown that specific point mutations in the Fc domain of antibodies can enhance antibody clustering into hexameric structures, required for a more efficient complement activation. This study examines the efficacy of capsule-specific human mAbs as a therapeutic approach against *S. pneumoniae*, a leading cause of community-acquired pneumonia and an important cause of childhood mortality.

Methods and results: We recombinantly produced human mAbs recognizing pneumococcal capsule polysaccharide (CPS) serogroup 6. Using flow cytometry assays, we showed that CPS6-IgG1 harboring the hexamer-enhancing E430G or E345K point mutations potently increase complement activation and phagocytosis of pneumococcal clinical isolates of this serogroup. Bacterial killing assays demonstrated the strong potency of engineered antibodies to induce neutrophil-dependent killing of *S. pneumoniae* serotype 6B. Moreover, we compared the protective capacity of serogroup-6 specific IgG1 wildtype antibody with its counterpart E345K variant against a mouse model of bacteremic pneumonia. Passive

immunization with the complement-enhancing CPS6-IgG1-E345K mutant protected mice from developing sever pneumonia.

Conclusion: This study demonstrates that when human mAbs against pneumococcal capsule have a poor capacity to induce complement activation, their activity can be strongly improved by hexamer-enhancing mutations. Altogether, the presented work provides an important insights into antibody-dependent immunity and therapies against encapsulated bacteria and represents a first systematic approach to design effective therapeutic antibodies against *S. pneumoniae* with increased potency to activate the human complement system.

ID: 121

Properdin deficiency is protective in the C3 GOF mouse model of aHUS

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Background: We have previously reported the C3 gain-of-function (GOF) mouse model of aHUS (PMID: 30714990)[1]. Homozygote mice display all the hallmarks of aHUS making them an ideal model to test therapeutic strategies for the treatment of the disease. Herein, we investigated the potential for targeting properdin to treat aHUS.

Methods: Properdin knockout mice (PMID:18292556) were backcrossed with the C3 GOF line[1] and cohorts aged to 6 months. Endpoint plasma was collected for biochemical and cell analysis (BUN/Platelets). FFPE tissues were collected for PAS and MSB staining and frozen tissue for Immunofluorescent staining (Properdin, C3, C9 & Fibrin). Plasma properdin, C3, C5 and FH levels were established via ELISA and alternative pathway haemolysis assay using rabbit blood cells were carried out to evaluate combined effects of C3 mutation and properdin deficiency.

Results: C3GOF mice have low serum properdin but significant deposit of properdin in the kidney in comparison to wild type controls. Over 6 months only 2 of 13 $C3^{D1115N/D1115N}.P^{-/-}$ mice succumbed to disease compared with 14 of 15 homozygote C3GOF mice. $C3^{D1115N/D1115N}.P^{-/-}$ mice had no evidence of haematuria and proteinuria, or thrombotic microangiopathy (TMA). Mice heterozygous for properdin were not protected and succumbed to disease in a similar fashion to properdin sufficient $C3^{D1115N/D1115N.}$ Furthermore, our ex vivo analysis suggests that properdin, even at low levels, can significantly enhance complement activity.

Discussion: In conclusion, circulating levels of properdin in the C3 GOF mouse model of aHUS are already significantly lower than normal. Total knockout of properdin is required to protect against cell lysis and to protect against disease, therefore using antibodies against properdin to block function may be challenging in aHUS treatment. Further studies are ongoing, including using anti-properdin mAb therapy, to assess efficacy in our model of aHUS.

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Smith-Jackson, K., Yang, Y., Denton, H., Pappworth, I.Y., Cooke, K., Barlow, P.N., Atkinson, J.P., Liszewski, M.K., Pickering, M.C., Kavanagh, D., et al. (2019). Hyperfunctional complement C3 promotes C5-dependent atypical hemolytic uremic syndrome in mice. J Clin Invest 129, 1061-1075. 10.1172/JCI99296.

ID: 162

Quantification of the zymogenicity and the substrate-induced activity enhancement of complement factor D

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Complement factor D (FD) is a serine protease present predominantly in the active form in circulation. It is synthesized as a zymogen, pro-FD, but it is continuously converted to FD by circulating active MASP-3. FD is a unique, self-inhibited protease. It has extremely low activity toward synthetic substrates and complement factor B (FB), while it is a highly efficient enzyme toward FB complexed with C3b (C3bB). Structural basis of this phenomenon are known, however the rate enhancement was not yet quantified. It has also been unknown if pro-FD has any enzymatic activity.

In this study, we aimed to measure the activity of human FD and pro-FD toward uncomplexed FB and C3bB to quantitatively characterize its substrate-induced activity enhancement and zymogenicity. Pro-FD was stabilized in the proenzyme form by replacing Arg^{20} (precursor numbering) to Gln (pro-FD-R/Q). Activated MASP-1 and MASP-3 catalytic fragments were also included in the study for comparison.

We found that complex formation with C3b enhanced the cleavage rate of FB by FD about 20 million-fold. Interestingly C3bB was also a better substrate for MASP-1, than FB alone, showing that binding of FB to C3b renders the scissile Arg-Lys bond in FB to become more accessible for proteolysis in general, though the enhancement for MASP-1 was only about 100-fold. Although easily measurable, the cleavage rate of C3bB (or FB) by MASP-1 is not relevant physiologically, whereas MASP-3 does not cleave C3bB (or FB) at an appreciable rate.

Most importantly, pro-FD cleaves C3bB at a rate that might be physiologically relevant. The zymogenicity of FD is about 800, i.e. the cleavage rate of C3bB by pro-FD-R/Q was found to be about 800-fold lower than that by FD. Additionally, pro-FD-R/Q at about 50-fold of the physiological FD concentration could restore half maximal AP activity of FD-depleted human serum on zymosan.

ID: 1

Quantitative Trait Loci Influencing Complement Factor H-related Protein 4A Concentration Are Not Independently Associated With Chromosome 1-directed Age-related Macular Degeneration Risk or Protection

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The complement factor H gene family (*CFH-CFHR5*) on chromosome 1q31 encode the major fluid phase and surface regulators of the alternative pathway (AP). Specific genetic haplotypes/diplotypes within the *CFH-CFHR5* locus associate strongly with protection against or risk for developing age-related macular

degeneration (AMD), a leading cause of blindness worldwide. Common genetic polymorphisms found in Complement Factor H (*CFH*), truncated CFH transcript Complement Factor H-like 1 (*FHL-1*) and the highly homologous Complement Factor H-related gene family (*CFHR1-5*) influence AP activity and dictate the degree of protection or risk in chromosome 1-directed AMD.

Recently, several groups have reported that Complement Factor H-related Protein 4A (FHR-4A) is elevated systemically in individuals with advanced AMD (1,2). Higher concentration of FHR-4A, and potentially other FHR proteins, can alter AP activation via competition with CFH and FHL-1 for critical ligands. These groups also demonstrated that alterations in systemic FHR-4A protein levels are genetically driven. They proposed that quantitative trait loci (QTLs) modulating FHR-4A protein levels are in fact AMD-associated and variants coupled with higher FHR-4A protein levels confer an increased risk for AMD.

To better understand the causal relationship between common chromosome 1-directed AMD polymorphisms, QTLs and FHR-4A protein concentration, we developed and validated an ELISA to detect FHR-4A protein levels in human samples. Genetic stratification on chromosome 1-directed AMD risk, neutral and protection haplotype/diplotype combinations allowed for assessment of FHR-4A protein levels in 486 plasma and 217 ocular samples. Our results demonstrate that FHR-4A protein levels are most significantly influenced by QTLs that are not independently associated with any form of genetic protection or risk for AMD. The consequences of varying levels of FHR-4 protein on AMD disease susceptibility are not obvious and additional studies will be required to disentangle the relationship between altered FHR-4A protein concentration and genetic protection against or risk for AMD.

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ID: 56

Rare genetic variants of the complement system genes identified in patients with fibromyalgia

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Fibromyalgia (FM) is a disease of unknown etiology characterized by generalized musculoskeletal pain, stiffness of the body, sleeping disorders, fatigue, and psychical instability that altogether impair the quality of life and productiveness. Genetic predisposition, especially within loci coding for neurotransmitters and neuronal signaling, was suggested as a possible risk factor. Only recently a link between mediators of inflammation and players of the innate immune system was revealed by the proteomic studies. We performed whole-exome sequencing and analyzed the obtained data for rare

genetic variants of complement genes in three female patients diagnosed with FM (mother, daughter, and the other unrelated woman) who showed abnormal parameters of the complement system. The mother and the unrelated patient had the same SNPs in ten complement genes: CFHR4 p.N209S, CD55 p.K454R, CD55 p.R470H, CR2 p.A1062E, CR1 p.T2060S, C8B p.G117R, CFI p.T300A, FCN1 p.L262S, C8G p.D118G, and C4A p.S1286A. All rare variants but mutation in FCN1 were homozygous. Seven out of ten SNPs were also identified in the daughter. Moreover, in 13 non- complement genes (ADRB1, MRC1, STOX1, KCNJ11, VDR, BIVM-ERCC5, KRT19, CYP2D6, MST1, IL13, OPRM1, CYP21A2, and TAS2R38) the same SNPs were identified in non-related women. Segregation of the same rare variants in non-related individuals with FM may ground certain genetic markers, which need to be confirmed in a bigger group of patients. Relatively high occurrence of mutations in complement system genes compared to all other genes, together with abnormal complement parameters, suggests that innate immunity plays an important role in the pathogenesis of FM.

ID: 111

Recognition and inhibition of SARS-CoV-2 by humoral innate immunity pattern recognition molecules

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The humoral arm of innate immunity includes diverse molecules with antibody-like functions, some of which serve as disease severity biomarkers in COVID-19. We conducted a systematic investigation of the interaction of human humoral fluid phase pattern recognition molecules (PRM) with SARS-CoV-2. Out of 12 PRM tested, the long pentraxin PTX3 and Mannose-Binding Lectin (MBL) bound the viral Nucleocapsid and Spike proteins, respectively. MBL bound trimeric Spike, including that of variants of concern (VoC), in a glycan-dependent way and inhibited SARS-CoV-2 in three *in vitro* models. Moreover, upon binding to Spike, MBL activated the lectin pathway of complement activation. Based on retention of glycosylation sites and modeling, MBL was predicted to recognize the Omicron VoC. Genetic polymorphisms at the *MBL2* locus were associated with disease severity. Thus, in addition to a role as biomarkers of severity, selected humoral fluid phase PRM can play an important role in resistance to, and pathogenesis of, COVID-19, a finding with translational implications.

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ID: 119

Recognition of post-translational lysine modifications by the lectin pathway initiator ficolin-3

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Ficolin-3 is one of the main initiators of the lectin pathway in the complement system. Despite being the quantitatively dominating pattern recognition molecule of the lectin pathway, not much is known about its function and binding specificity. Ficolin-3 has been proven to bind to acetylated proteins in a Ca^{2+}-dependent manner. Therefore, it is also of interest to study the binding of ficolin-3 to other structurally similar post-translational modifications.
In this study, we used model proteins with chemically derivatized lysines to probe the binding specificity of ficolin-3. We show that recombinant ficolin-3 binds strongly to proteins modified by carbamylation. Interestingly, the binding of serum-derived ficolin-3 is significantly reduced while still maintaining a high C3 deposition. This indicates that other serum proteins may conceal or block the carbamylated epitopes. To assess if other complement pathways are involved in this complement activation, different complement depleted sera were analyzed. No significant changes in ficolin-3 binding or C4 and C3 deposition were observed, indicating that the blockage is not induced by either the classical or alternative pathway.
To further characterize the binding specificity of ficolin-3, we systematically derivatized Bovine Serum Albumin (BSA) with acyl groups of different chain lengths. Ficolin-3 binding was highly dependent on the length of the acyl group, with the strongest binding observed for acetylation. Sequence alignment of the ficolin-3 ligand-binding domain with known acetyllysine binding proteins revealed a conserved Asparagine anchor at the base of the binding pocket. Mutation of the Asparagine anchor completely abolished binding, providing a structural basis for acetyllysine recognition by ficolin-3.
These findings give us the ability to further investigate the function of ficolin-3 in humans. Our results also suggest that post-translationally modified proteins constitute important danger signals that are rapidly and specifically recognized by the lectin pathway.

ID: 42

Recombinant human IgG1 Fc hexamer is a potent modulator of classical pathway activation

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The Fc portion of human IgG has been suggested to be critical for many of the therapeutic effects seen for high dose IVIG in treating autoimmune diseases, e.g. by scavenging of complement and antagonism of Fcγ receptors (FcγR). We investigated the inhibitory properties of a rFc hexamer (termed Fc-µTP-L309C), generated by fusion of the IgM µ-tailpiece to the *C*-terminus of human IgG1 Fc, on FcγR effector functions and the complement system.

Fc-µTP-L309C demonstrated high avidity binding to C1q, besides FcγRs. Consequently, Fc-µTP-L309C potently inhibited full activation of the classical complement pathway (CP) and lectin pathway (LP) *in-vitro,* by consumption of C1q and C4, whereas the alternative pathway (AP) was unaffected. Complement deposition on endothelial cells was prevented in 2D as well as 3D cell culture models. In a rat model of acute neuromyelitis optica (NMO), administration of Fc-µTP-L309C reduced C5b-9 in the brain tissue. Similarly, in a mouse model of collagen Ab-induced arthritis (CAIA) Fc-µTP-L309C reduced clinical manifestations along with reduction of C3 and C5a levels in joint washes. Reducing affinity binding to C1q decreased the therapeutic potency of Fc-µTP-L309C in CAIA. Finally, CP activity was inhibited in rats and cynomolgus monkeys *in-vivo,* whereas only a minor effect was observed for the LP. Inhibition of the CP *in-vivo* seemed to be predominantly mediated by consumption of C1q.

We conclude that interference with activation of the CP may be an important therapeutic property of Fc-µTP-L309C in Ab-complex mediated diseases.

ID: 26

Regional and Cell-Specific Imbalance of Complement Gene Expression and Proteins in the Synovial Biopsies from Early Rheumatoid Arthritis Patients

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Rheumatoid arthritis (RA) is characterized histologically by heterogeneous synovial hyperplasia. Synovium in individual patients with Early RA (<12 months of disease) exhibits one of three distinct pathotypes, namely lymphoid, myeloid and fibroid. Here we explored the Pathobiology of Early Arthritis Cohort (PEAC) tissue RNA sequencing database (n = 90) and studied correlations among complement and Fc receptor gene expression and clinical severity, as measured by disease activity score 28 - erythrocyte sedimentation rate (DAS28-ESR), in both blood and synovium. We also evaluated the biodistribution of complement proteins, receptors and inhibitors using Multispectral ImmunoHistoChemical (MIHC) staining on ultrasound-guided synovial biopsies (0.5-3 mm) (n = 23) obtained from Accelerating Medicines

Partnership (AMP) studies. Control synovial biopsies (n = 4) without RA or osteoarthritis were also examined using MIHC. Our analyses revealed that in the synovium, but not in blood, significant positive correlations existed between complement gene expression and DAS28-ESR for *C2, CFB, FCN1, C3AR1, C5AR1,* and *CR1*. In contrast, *MASP1, Colec12, C5* and *C6* expression were inversely correlated with DAS28-ESR. C5AR1 receptors were significantly present more than C3AR1 and CR1 in the synovium and also on the surface of adipocytes. In the synovium, there were also significant positive correlations between DAS28-ESR and *FcγR1A*, *FcγR1B*, *FcγR2A* and *FcγR3A* expression. In Early RA synovium, a significantly ($p < 0.05$) higher number of cells expressed CFH compared with CFB and CFHR4. In the fibroid pathotype, *CFH* and *CFD* expression was significantly higher as compared to *CFB*. CFH, CFB and CFHR4 co-existed in the vicinity of each other in the sub-synovial lining area; however, a substantial regional imbalance existed between C3 and CFH in synovial biopsies, suggesting a means by which uncontrolled activation can proceed in some sites. Early RA synovial studies strongly implicate the complement system in disease pathogenesis through unexpected cell- and region-specific mechanisms.

ID: 187

Regulatable complement inhibition of the alternative pathway mitigates age-related macular degeneration pathology

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Background: Age-related macular degeneration (AMD) occurs after age 65. AMD has two forms, dry and wet. AMD is a multifactorial disease; risk factors include aging, smoking, and complement dysregulation. The amplification loop of the complement alternative pathway (AP) is responsible for the majority of complement activation on cell surfaces and extracellular membranes in animal disease models (Harboe et al., 2008). The AP is inhibited naturally by circulating complement protein factor H (fH). Complement component C3 (C3) protein increases and decreases respective to complement activation. In wet AMD, complement effector molecules increase angiogenesis, activate microglia and recruit immune cells, amplifying the complement-microglia inflammatory feedback loop, leading to tissue damage.

Methods: The C3 promoter (−1005 to +251) (Cho et al., 2016), pC3, was cloned into a pTR backbone and plasmids (pC3-mCherry/CR2-fH) were synthesized and utilized to generate adeno-associated virus serotype 5 (AAV5) vectors. pC3 activation was determined in transiently transfected ARPE19 cells stimulated with H_2O_2 and normal human serum (+/- TT30 or NAC). Laser-induced choroidal neovascularization (CNV) was analyzed in mice treated with AAV5-pC3-mCherry/CR2-fH, using imaging (optical coherence tomography), functional (electroretinography, ERG), and molecular (western blotting, complement activation) readouts.

Results: Proof of concept was provided that pC3 is modulated in a complement and oxidative stress-dependent manner in human ARPE19 cells, examining mCherry fluorescence. Safe concentrations of AAV5-pC3-CR2-fH, when injected subretinally, were identified using ERG and OCT (10^{10} - 10^{11} vg/mL). The

expression of CR2-fH significantly reduced CNV in a dose dependent manner, with a maximum reduction of lesion size of ~35% compared to mCherry-treated animals. Injections at 10^{11} viral genomes, CR2-fH expression reduced CNV-associated ocular C3 activation as assessed by western-blotting for C3 protein and breakdown products.

Conclusion: Here we demonstrated that regulating AP inhibition in a complement-dependent manner can ameliorate pathology by reducing activation and the proinflammatory microenvironment.

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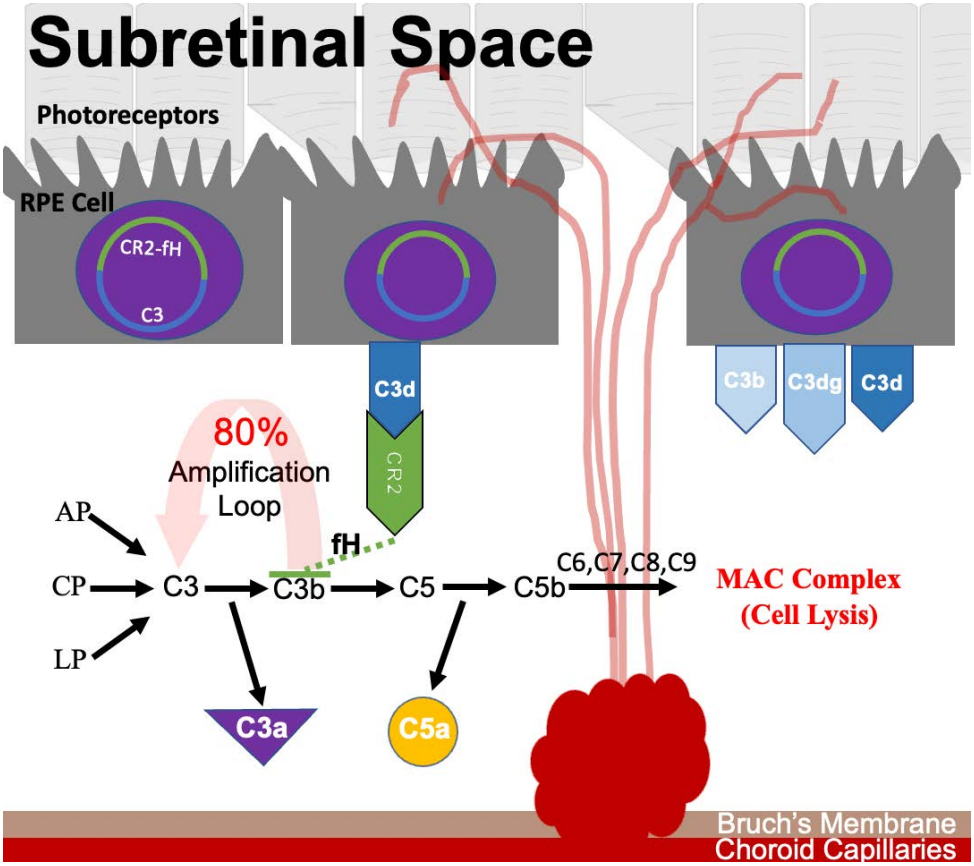

ID: 35

Relevance of complement and mycobiome dysbiosis for progression of pancreatic cancer

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Background: For pancreatic ductal adenocarcinoma (PDAC) exhibits an extremely poor prognosis, there is urgent need for improved pathogenetic understanding. Recent research suggests that the yeast *Malassezia (M.)* migrates from gut into pancreas and subsequently activates complement that promotes PDAC progression. We therefore aim (1) to study the capacity of complement to recognize *Malassezia* with its unusual lipid and polysaccharide surface as foreign; (2) to identify the relevant complement activation pathways; (3) to investigate whether the terminal complement cascade is able to reduce the viability of *Malassezia*; (4) and to compare different *Malassezia* species.
Methods: Various *Malassezia* species were incubated with human serum, subsequent opsonization of the fungal surface was quantified by FACS. Viability of *Malassezia* was checked by measuring the mitochondrial activity or by plating.
Results: Complement could be demonstrated to efficiently recognize and opsonize the surface of all *Malassezia* species. Interestingly, deposition of complement C3 was highest for *M. globosa*, the species previously described to be closely related to PDAC development. *Malassezia*-driven complement activation proceeded throughout the terminal pathway resulting in formation of the lytic C5b-9 complex on the fungal surface. However, no loss of fungal viability was detected after incubation with the human serum, indicating that the thick surface perfectly protects *Malassezia* from efficient lysis. Differentiation of the relevant complement activation pathways revealed that lectin and, even more prominent, the alternative pathway trigger the activation
Conclusion: Our results show that *Malassezia* is able to trigger the complement system without decrease in viability. Consequently, perpetually activated complement can contribute to the pro-inflammatory tumor microenvironment in PDAC that favors tumor progression.

ID: 52

Results from a randomized study of APL-9 in respiratory failure, including mild-to-moderate acute respiratory distress syndrome, (ARDS) due to COVID-19

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Study Objectives and Design
The safety and treatment benefit of APL-9, a compstatin derivative and potent C3/C3b inhibitor, was explored in a study involving 65 subjects with respiratory failure, including mild-to-moderate ARDS, due to COVID-19. Patients were randomized 1:1 to receive either APL-9 (up to 540 mg / day) or vehicle by continuous intravenous infusion in addition to standard of care (SOC) until hospital discharge or Day 21 (whichever occurred earlier).

The primary endpoint was the:
- Cumulative incidence of treatment-emergent serious adverse events (SAEs) and treatment-emergent

adverse events (TEAEs).

Secondary and exploratory endpoints included:
- Hospital length of stay and any-cause mortality
- Sequential Organ Failure Assessment (SOFA) Score
- Total duration of mechanical ventilation
- Total duration of oxygen therapy
- APL-9 serum concentration
- Markers of complement activation

Results
Between treatment groups, no apparent differences were observed in cumulative rates of treatment emergent SAEs and TEAEs. Additionally, no significant differences were observed between overall hospital length of stay and mortality. Changes in SOFA (overall score and individual categories) were comparable between groups.

Consistent with its mechanism of action, APL-9 treatment resulted in a pronounced reduction of circulating C3a and AH50. However, although APL-9 has been shown to bind to C3 and C3b and inhibit alternative C3 and C5 convertases, only marginal differences in circulating C5a concentration were observed between treatment groups in this study. No difference in terminal complement complex (TCC) was observed between treatment groups, suggesting that TCC formation bypasses the commonly regarded cascades involving C3 as the common activator.

Conclusion
APL-9 in combination with SOC was safe in patients with COVID-19, but no incremental treatment benefit over SOC was observed. Analysis of complement degradation products suggest that complement C3 is activated in COVID-19 but C3 inhibition didn't impact clinical outcomes.

ID: 50

Results from the Phase 2 Study of Cemdisiran in Adult Patients with IgA Nephropathy

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INTRODUCTION: Immunoglobulin A nephropathy (IgAN) is the most common primary glomerulonephritis. Proteinuria >1 g/day is a risk factor for progression to kidney failure, and treatment options to slow progression are limited. There is evidence that activation of lectin and alternative pathways of complement lead to complement component 5 (C5) cleavage, and to production of C5a and the membrane attack complex (MAC), with subsequent inflammation and tissue injury occurring in IgA

nephropathy. Cemdisiran is an investigational RNA interference therapeutic that suppresses liver production of C5, reducing C5a and MAC formation regardless of the activating pathway.
METHODS: This Phase 2 study was a randomized, double-blind, placebo-controlled trial (NCT03841448) of cemdisiran in adult patients with IgAN and proteinuria >1 g/day. Patients were randomized (2:1) to receive subcutaneous cemdisiran 600 mg or placebo once every 4 weeks (Q4W) for 32 weeks, alongside standard of care. All patients could then enter an open-label extension to receive subcutaneous cemdisiran 600 mg Q4W for up to an additional 152 weeks. The primary endpoint was percentage change from baseline to Week 32 in urine protein/creatinine ratio (UPCR) measured by 24-hour urine. Secondary endpoints included percentage change from baseline to Week 32 in 24-hour proteinuria, percentage of patients with partial clinical remission (time-averaged proteinuria <1 g/24 hours), percentage of patients with >50% reduction in 24-hour proteinuria, change from baseline UPCR measured by spot urine, and frequency of adverse events.
RESULTS: A total of 31 patients (22 [71%] cemdisiran, 9 [29%] placebo) were randomized across 10 countries. Baseline demographics, primary and secondary endpoints, and safety results will be presented at the meeting.
CONCLUSIONS: Cemdisiran has the potential to address a significant unmet need for patients with IgAN by suppressing hepatic C5 production. The open-label extension continues to investigate the efficacy and safety of cemdisiran.

ID: 22

Role of pentraxin 3 and interaction with complement in immune defence against malaria

Zhong, Hang

Background
The Long pentraxin 3, PTX3, an octameric protein, is part of the humoral arm of innate immunity[1 2]. PTX3 is a soluble pattern recognition molecule that binds to Fc receptors and phagocytes, promoting phagocytosis and acting as an ancestral antibody. Its functions include regulating inflammatory responses and tissue remodeling, and modulating complement-dependent inflammation. The complement system confers protection for malaria patients acting in innate and acquired immunity. Complement binding and opsonization impedes invasion, and mediates lysis and phagocytosis, while its regulation prevents excessive hemolysis and inflammatory responses. However, the exact mechanism on how it balances protection, regulation, variations and the parasite's survival tactics remains to be a myth. PTX3 is known to modulate C1q, Factor H and ficolin-1 binding. On the other hand, elevated PTX3 level is associated with more severe malaria.

Methods
Plasmodium falciparum 3D7 human malaria strain was the experimental model. The study compared the deposition of PTX3 and different complement proteins/complex on iRBCs versus normal RBCs, using techniques including immunofluorescence, Western blot, electron microscopy and flow cytometry, to provide novel perspectives on the interplay between PTX3 and complement on combating malaria.

Results
The results confirmed C1q, C3 and C5b-9 depositions on free merozoites and iRBCs. During parasite cycle, complement proteins (e.g.C1q) first appeared associated to the apicoplast structure, then were

distributed step-by-step onto parasite plasma membrane (PPM), parasitophorous vacuolar membrane (PVN), tubulovesicular network (TVN), and eventually to the RBC membrane. MAC was activated, despite the presence of the regulatory CD59 or Factor H, and treatment with normal human serum led to a reduction of knob-like structures, rich in parasitic proteins such as PfEMP1, central for increased cytoadherence. PTX3 was found associated with RBC membrane, suggesting it may play regulatory roles during the process.

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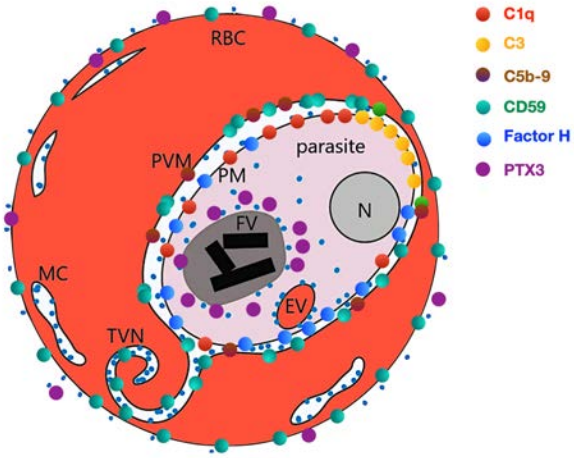

ID: 65

SciFiMed - Screening of inFlammation to enable personalized Medicine

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This transdisciplinary, EU-funded project combines fundamental immunological research with novel nanomaterial-based biosensor development translated into proof-of-principle diagnostics to revolutionise doctors' ability to diagnose and treat severe diseases that are associated with the dysregulation of the complement system.

Complement factor H (FH) and FH-related (FHR) proteins are strongly associated with systemic and organ-specific pathologies like infections (IMD), renal (IgAN, aHUS, C3G), eye (AMD) and autoimmune

diseases (SLE). In contrast to FH, the function of the remaining five homologous FHR proteins is largely unknown and, hence, their role in the different disease-specific pathogenic mechanisms remain elusive.

Our EU consortium (eight partners from four countries) aims to unravel the mysteries of the FH-protein family's contributions to diseases and will develop a multiplex detection system to simultaneously quantify and analyse the functional activity of all seven FH-protein family members in patient samples. This novel detection system will have added value for diagnosis in a daily clinical setting.

During the first year of the project, SciFiMed identified novel binding partners for two FH- family members, generated specific antibodies against three FH-family members and performed feasibility studies for two sandwich ELISA. A novel liposome-based assay to determine functional activity of the complement system is being developed and an innovative multiplex lateral flow assay using liposomes was designed. SciFiMed validated recombinant FH protein family members and assisted in quantifying FH and FHR levels in Covid-19 cohorts.

In the future, SciFiMed aims to propel immunological research in Europe, enable new product development of bioanalytical companies, modernise the EU-diagnostic market and provide new perspectives for patient treatment, which in turn will pave the way for novel approaches for drug development.

ID: 83

Selective acquired absence of C1r in serum

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Background
Over a number of years, we have in several patients with different inflammatory conditions observed absence of C1r combined with non-functioning classical pathway (CP). There are to our knowledge only very scarce previous reports about similar findings. We describe three cases of acquired C1r deficiency in serum, with associated clinical findings.

Samples & analyses
Serum and EDTA plasma were available from patients 1 and 3, only serum from patient 2. Concentrations

of complement components as well as CP and C1 inhibitor (C1INH) function (the latter only in patient 3) were determined. Samples were substituted with external C1, C4 and C2 (Complement Technology ®) in different combinations, followed by analysis of CP function. Serum samples were also subjected to crossed immunoelectrophoresis.

Results
Patient 1 (age 78) had MPO-ANCA-positive microscopic polyangiitis, patient 2 (age 76) had PR3-ANCA-positive granulomatosis with polyangiitis, and patient 3 (age 73) had HCV infection and cryoglobulinemia. C1r was undetectable in serum, CP was non-functional in serum and concentrations of C1q, C1s, C4, C2 and C1INH were normal in all patients. Follow-up samples from patients 1 and 3 showed normal CP function in EDTA plasma, but no function in serum. C1INH function was normal in patient 3. Substitution of serum from patients 1 and 3 with external C1, C4 and C2 showed restoration of CP function in serum from patient 1, but not in serum from patient 3. Crossed immunoelectrophoresis showed an increased amount of C1s-containing complexes in initial samples from all patients.

Conclusion
Undetectable C1r and absent CP function was associated with ANCA-positive vasculitis in two out of three patients. C1r was absent only in serum and not in EDTA plasma, suggesting a possible association with coagulation or with cryoglobulinemia. Acquired selective absence of C1r in serum may be observed in cases of unexplained defective classical pathway function.

ID: 59

Silencing Hepatic Complement C3 by RNA Interference Alleviates the Progression of Renal Histopathological Lesions in a Mouse Model of Nephropathy

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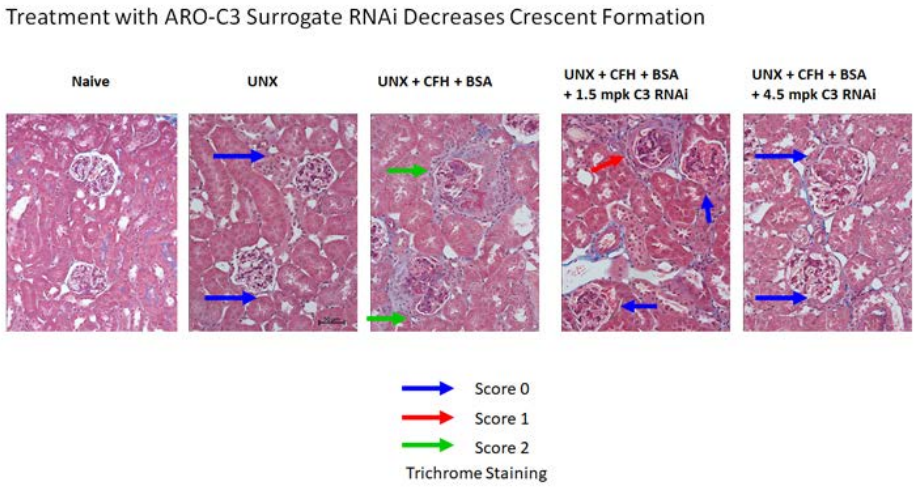
Dysregulation of the complement system is a cause of lesion progression in nephropathies of a variety of etiologies. Complement C3, centralizes signals from all 3 complement pathways and plays a critical role in complement activation and therefore is an attractive drug target. Approval of Pegcetacoplan for PNH exemplified the therapeutic value of C3 inhibition. RNAi as a therapeutic approach has demonstrated specificity, efficacy, and safety with a long duration of effect for hepatic gene targets. We therefore developed a surrogate RNAi drug of ARO-C3, which targets rodent hepatic C3 transcripts for preclinical proof of concept. Subcutaneous administration of the surrogate of ARO-C3 to C57BL/6J mice caused a dose-dependent decrease in hepatic C3 mRNA transcripts. To induce nephrotic lesions in mice, male C57BL/6J mice received unilateral nephrectomy and complement factor H mRNA silencing by RNAi. These mice were then challenged by intraperitoneal injection of BSA. The challenged mice showed >60% decrease in serum C3 levels due to the increased glomerular C3 deposition. They also showed a transient increase in proteinuria. Histopathological analysis revealed glomerular lesions consistent with glomerulonephritis including crescents and mesangial deposition of complement C3 and other proteins. Subcutaneous administration of ARO-C3 surrogate RNAi in this model of nephropathy caused a dose-dependent decrease in hepatic C3 mRNA transcripts, serum C3 protein and serum BUN. Furthermore, hepatic C3 silencing by RNAi substantially reduced crescents, renal C3 deposits and mesangial PAS deposition. Evidence provided by this preclinical study suggests that hepatic C3 silencing by RNAi is a

promising therapeutic approach for treating complement-mediated glomerular injury and nephropathy.



ID: 106

Storage of transfusion platelet concentrates is associated with complement activation and reduced ability of platelets to respond to PAR-1 and TXA2R-activation

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Background: Platelets are transfused to patients for their hemostatic properties, and considerations regarding the quality of platelet concentrates are primarily judged from that perspective. However, the role of platelets in circulation is far more complex than hemostasis, including their immunologic function. Platelets can respond to complement activation and activated platelets can modulate inflammatory responses (1, 2). Here, we investigated complement activation and platelet function in routine clinically produced platelet concentrates related to the storage time.

Methods: Ten transfusion platelet concentrates were stored at 22°C for seven days. The platelet concentrates were analyzed daily for complement activation and platelet activation markers using immunoassays. In addition, platelet function was assessed as the ability to respond to activation, and hemostasis as the ability to adhere to collagen.

Results: Complement activation in the platelet concentrates was increased as a function of the seven days storage time for all analyzed markers, i.e., C1s/C1INH complex (fold change (FC)=1.9; p<0.001), MASP-1/C1-INH complex (FC=2.0; p<0.001), C3bc (FC=5.3; p<0.01), and sC5b-9 (FC=1.7, p<0.001). Also, the soluble platelet activation markers increased in the concentrates over time, TSP-1 (FC=1.4; p<0.01), TGF-β1 (FC=1.9; p<0.001), and PF4 (FC=2.5; p<0.001). The ability of platelets to respond to activation via PAR-1 and TXA_2R, as evaluated by surface expression of CD62P, had declined by 20% p<0.01) and 65% (p<0.01), respectively, at day 7 compared to day 1. The degree of platelet-binding to collagen was decreased by 31% at day 7 compared to day 1 (p=0.068).

Conclusion: In conclusion, we show that complement activation is increased during platelet storage, which correlates with reduced ability of platelets to respond to, primarily, TXA_2R activation. If complement activation influences the function of platelets stored in concentrates remains indefinite, as well as potential effects when transfused to patients. However, complement might be a target for inhibition in the platelet preservation solution.

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ID: 63

Strong association between complement C7 and clusterin reveals a potential C7-clusterin complex in circulation

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Complement component C7 is an important complement protein that acts as a driving or limiting factor for the assembly of the membrane attack complex on local surfaces. However, its precise regulatory role in complement is poorly understood and may therefore be underappreciated. The aim of this study was to investigate the potential regulatory role of C7 isoforms and/or complexes in complement activation and function.

It has been shown by colleagues Reinhard Würzner and Otto Götze, through Western blot analysis, that C7 produces two distinct bands when probed with an allotype-specific C7 antibody, one band detected at around 100kDa, compatible with the molecular weight of mature C7, and another unknown band at around 75kDa (unpublished). It was hypothesized that the 75kDa band points towards a C7 isoform which could potentially harbor regulatory functions.

To validate this hypothesis, C7 was purified from normal human serum (NHS) using a co-immunoprecipitation technique and subsequently analyzed by Western blot. Similar to the pattern observed by Würzner and Götze, our Western blot analysis of purified C7 revealed two distinct bands, at around 100kDa and 75kDa, when probed with a monoclonal anti-C7 antibody. Unexpectedly, mass spectrometry analysis of the 75kDa band did not point towards a variant form of C7. However, the protein composition of the 75kDa band uncovered an abundance of clusterin, a known complement inhibitor. Upon purification of clusterin from NHS and its assessment with Western blot, we further observed a strong presence of C7 in purified clusterin. Consequently, an in-house C7-clusterin sandwich ELISA was established to investigate the relationship between C7 and clusterin. The initial findings suggest a prominent and direct association between these two proteins, thereby revealing a potential C7-clusterin complex present in circulation. Further characterization of this complex is currently underway to examine its functional role in the complement system.

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ID: 75

Structural insights into the hexameric form of the IgM Fc-core domain and its binding characterization to C1q

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Background:
Immunoglobulins type-M (IgM) are one of the first antibody isoforms that are produced by vertebrates in response to a range of antigens from viruses to tumor cells, making them suitable therapeutic targets. The antigen bound IgMs are known to strongly activate the immunological complement system, in particular the classical pathway, by binding to its first recognition molecule, C1q. Despite their essential role and longstanding studies for several decades, the structural mechanisms of the complement activation by the different pentameric or hexameric oligomeric forms of IgMs are only started to be elucidated in fine details thanks to the newly developed methods in protein engineering, biophysics and structural biology (Chouquet et al., 2021; Sharp et al., 2019).
Materials and Methods:
Here in this study, we used Cryo-Electron Microscopy (Cryo-EM) to determine the structure of the hexameric form of the IgMs produced in HEK293F cells devoid of the Fab domains (Fc-core). Bio-Layer Interferometry and in-house ELISA-like assays were used respectively to characterize the binding to C1q of IgM Fc-core forms and complement activation.

Results:
The structure of the hexameric IgM Fc-core was successfully reconstructed at about 5Å resolution, providing insights into the overall symmetry and the assembly of the complex. A comparison with the pentameric forms revealed further information on the amyloid-like tail piece assembly to accomodate the sixth protomer in the hexamer. In addition, the results from the Bio-Layer Interferometry still showed kinetic binding to C1q of the pentameric or hexameric Fc-core, although with lower affinities than full IgMs. Finally, and surprisingly, both oligomeric truncated IgM forms are also able to activate *in vitro* the complement system.

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ID: 108

Study of the complement-regulating and antimicrobial properties of C3f peptide

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Background. Complement system contributes to immune defense of organism but also participates in pathological processes. Complement regulation involves many mechanisms, one of which is the cleavage of C3b protein by factor I resulting in the release of the 2 kDa peptide, C3f. The physiological role of C3f is obscure. We hypothesized that C3f may participate in a feedback loop in complement activation or exhibit antimicrobial activity.
Materials and methods. Human C3f and C3f-desArg were synthesized by solid-phase method according to the Fmoc/tBu strategy. Normal human serum was used as the source of complement. *In vitro* models of the classical (CP) and the alternative pathways (AP) of complement activation contained antibody-sensitized sheep erythrocytes or rabbit erythrocytes, respectively. Complement activity was measured in hemolytic assays and by ELISA for assessment of C3a and C5a anaphylatoxins. Antimicrobial activity of C3f was evaluated by radial diffusion assay.
Results. In the CP model, C3a accumulation was significantly increased in the presence of 8-64 μM of C3f (up to 5-6-fold), and augmentation of C5a accumulation was less pronounced but significant in the presence of 64 μM of the peptide. Both effects were abolished in heat- and EDTA-inhibited serum suggesting specific action on complement. C3f-desArg acted similarly to C3f. C3f had no effects in the AP model. C3f demonstrated weak antimicrobial activity against some Gram-positive bacteria. *Listeria monocytogenes* EGD was the most sensitive (MIC = 70 μM).
Conclusions. Despite being a product of complement inactivation, C3f exerts a potentiating action on complement by enhancing its activation via the CP. Therefore, C3f can be considered as an endogenous complement regulator. Probably, C3f may act as an internal switch between the complement pathways, making the complement attack more targeted. C3f possesses low antimicrobial activity. Our findings suggest that complement self-regulation may be a primary function of C3f.

ID: 122

Sushi domain-containing protein 4 binds to Epidermal Growth Factor Receptor and initiates autophagy

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With merely a few publications pertaining to its function, the sushi domain-containing protein 4 (SUSD4) remains a poorly understood protein with largely unknown functions. In addition to its ability to inhibit both the classical- and alternative complement pathways, SUSD4 has previously been described as a potential tumor suppressor. In the current study, expression databases and online analysis tools employed, revealed a better prognosis for breast cancer patients with SUSD4 expression in the tumor tissue. In concurrence with a tumor suppressive role for SUSD4, smaller tumors were formed in a syngeneic mouse model of breast cancer following transplantation with SUSD4-expressing cells compared to mock control cells.

In vitro work using breast cancer cell lines stably expressing SUSD4, revealed higher protein levels of the epidermal growth factor receptor (EGFR) in SUSD4-expressing cells compared to mock control cells. Furthermore, SUSD4 was found to interact with the EGFR, leading to receptor inactivation and reduced signaling. Consequently, SUSD4-expressing cells displayed increased activation of AMPKα1 and an altered phosphorylation status of proteins promoting autophagy, including LKB1, ULK1 and Beclin-1. Additionally, cells expressing SUSD4 had higher levels of the well-characterized marker for autophagy LC3B-II and a higher number of LC3B puncta per cell, without any impairment in autophagosome fusion with lysosomes. Furthermore, the higher LC3B-II levels in SUSD4-expressing cells could not be seen in CRISPR/cas9-mediated EGFR knockout cells.

The results show that SUSD4 expression leads to AMPKα1 activation and higher levels of autophagy in breast cancer cells. An effect dependent on its interaction with the EGFR. The study thus provides further mechanistic insight into the unexplored complement inhibitor SUSD4 and its role as a tumor suppressor.

ID: 155

Synergism between complement MASP-1 and other pro-inflammatory factors in the activation of endothelial cells

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Background
Complement MASP-1 is the first serine protease in the cascade of lectin pathway activation. It is activated when a lectin pathway pattern recognition molecule (MBL, ficolin-1/2/3 or collectin-10/11) binds its target, such as bacterial- or fungal surface, or damaged self-macromolecules. Besides activated MASP-1 carries on the lectin pathway cascade, we demonstrated that it can stimulate endothelial cells driving them into a pro-inflammatory phenotype (Ref1,2). Since MASP-1 activation can only happen when other pro-inflammatory stimuli are also present, we asked whether MASP-1 can synergize with these factors to induce endothelial cell activation even more efficiently.
Materials and Methods
We used HUVEC in vitro culture as endothelial cell model system. Both MASP-1 and the other pro-inflammatory factors (LPS, lipoteichoic acid - LTA, histamine, bradykinin, IFNgamma) were applied in suboptimal doses. Sandwich ELISA, immunofluorescence microscopy, XperT permeability test and qPCR were utilized to detect specific endothelial cell responses.
Results
We observed that LPS synergized with MASP-1 in the induction of E-Selectin expression and IL-8 production. IL-8 production was also significantly elevated when MASP-1 was applied together with histamine, IFNgamma or LTA. Bradykinin and LTA synergized with MASP-1 in VCAM-1 expression. MASP-1 applied after LPS pretreatment induced significantly higher E-Selectin expression than either compound alone. Finally, MASP-1 pretreatment induced elevated bradykinin receptor 2 expression, and in concert with this, elevated Ca-mobilization in response to bradykinin.
Conclusion
MASP-1 can synergize with several pro-inflammatory factors inducing phenotypical changes in endothelial cells. Moreover, the synergy of MASP-1 with distinct pro-inflammatory factors results in different endothelial activation outcomes, which suggests that this non-canonical function of complement activation may act as an input-sensitive regulator of the immediate immune response.

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ID: 149

Synergy of protease binding sites within the ecotin homodimer is crucial for inhibition of MASP enzymes and for blocking lectin pathway activation

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Ecotin is a homodimeric serine protease inhibitor produced by several commensal and pathogenic microbes. It functions as a virulence factor, enabling survival of various pathogens in the blood (1). The ecotin dimer binds two protease molecules and each ecotin protomer has two protease binding sites: Site1 occupies the substrate binding groove, while Site2 engages a distinct secondary region. Owing to the two-fold rotational symmetry within the ecotin dimer, Sites 1 and 2 of a protomer bind to different protease molecules within the tetrameric complex (2). *E. coli* ecotin inhibits trypsin-, chymotrypsin- and elastase-like enzymes, including pancreatic proteases, leukocyte elastase, key enzymes of blood coagulation, the contact and complement systems, and other antimicrobial cascades. Here, we show that mannan-binding lectin-associated serine protease-1 (MASP-1) and -2, essential activators of the complement lectin pathway, and MASP-3, an essential alternative pathway activator, are all inhibited by ecotin. We decipher in detail how the preorganization of Site1 and Site2 within the ecotin dimer contributes to the inhibition of each MASP enzyme. In addition, using mutated and monomeric ecotin variants, we show that Site1, Site2, and dimerization contribute to inhibition in a surprisingly target-dependent manner. We present the first ecotin : MASP-1 and ecotin:MASP-2 crystal structures, which provide additional insights and permit structural interpretation of the observed functional results. Importantly, we reveal that monomerization completely disables the MASP-2-, MASP-3- and lectin pathway-inhibitory capacity of ecotin. These findings provide new opportunities to combat dangerous multidrug-resistant pathogens through development of compounds capable of blocking ecotin dimer formation.

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ID: 6

Terminal Complement Pathway Activation Drives Synaptic Loss in Alzheimer's Disease Models

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Background: Complement is involved in developmental synaptic pruning and pathological synapse loss in Alzheimer's disease. It is posited that C1 binding initiates complement activation on synapses; C3

fragments then tag them for microglial phagocytosis. However, the precise mechanisms of complement-mediated synaptic loss remain unclear, and the role of the lytic membrane attack complex (MAC) is unexplored. We here address several knowledge gaps: (i) is complement activated through to MAC at the synapse? (ii) does MAC contribute to synaptic loss? (iii) can MAC inhibition prevent synaptic loss?
Methods: Novel methods were developed and optimised to quantify C1q, C3 fragments and MAC in total and regional brain homogenates and synaptoneurosomes from WT and *App*$^{NL-G-F}$ Alzheimer's disease model mouse brains at 3, 6, 9 and 12 months of age. The impact on synapse loss of systemic treatment with a MAC blocking antibody and gene knockout of a MAC component was assessed in Alzheimer's disease model mice.
Results: A significant increase in C1q, C3 fragments and MAC was observed in *App*$^{NL-G-F}$ mice compared to controls, increasing with age and severity. Administration of anti-C7 antibody to *App*$^{NL-G-F}$ mice modulated synapse loss, reflected by the density of dendritic spines in the vicinity of plaques. Constitutive knockout of C6 significantly reduced synapse loss in 3xTg-AD mice.
Conclusions: We demonstrate that complement dysregulation occurs in Alzheimer's disease mice involving the activation (C1q; C3b/iC3b) and terminal (MAC) pathways in brain areas associated with pathology. Inhibition or ablation of MAC formation reduced synapse loss in two Alzheimer's disease mouse models, demonstrating that MAC formation is a driver of synapse loss. We suggest that MAC directly damages synapses, analogous to neuromuscular junction destruction in myasthenia gravis.

ID: 47

The C5a/C5ar1 axis controls the early IgM to IgG switch of collagen type VII autoantibodies in experimental Epidermoliysis Bullosa Acquisita

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Epidermolysis bullosa acquisita (EBA) is an autoimmune skin blistering disease, characterized by strong production of type VII collagen (COL7)-specific IgM/IgG autoantibodies (AAb). The AAb-driven complement activation at the dermal-epidermal-junction drives neutrophil infiltration and reactive oxygen species release promoting skin blisters and erosions. The mechanisms underlying the generation of pathogenic IgM/IgG AAb in EBA are poorly understood. Here, we determined the impact of C5a/C5aR1 axis activation on IgM/IgG AAb production in experimental EBA. Immunization of B6.s (B6.SJL-H2s C3c/1CyJ) mice with COL7 resulted in a strong clinical phenotype with blisters and erosions, which was absent in B6.sC5ar1$^{-/-}$ mice. B6.sC5ar1$^{-/-}$ mice showed significantly lower anti-COL7 IgG AAb production but significantly higher IgM AAb production as compared with B6.s mice during the early phase post EBA immunization (Day10). To delineate the impact of C5a/C5ar1 axis on the delayed IgM/IgG switch and the GCB formation in B6.sC5ar1$^{-/-}$ mice, we used an ex vivo cell culture system comprising 3T3 cells, overexpressing CD40-ligand and B-cell activating factor (Haniuda and Kitamura 2019) co-cultured with naïve B cells from B6.s and B6.sC5ar1$^{-/-}$ in the presence of IL-4. This system induces strong B cell proliferation, differentiation towards GCB cells and IgM to IgG switch after 4/6 days of co-culture. FACS analysis of IgG1 and IgM production in GCB cells from B6.s and B6.sC5ar1$^{-/-}$ confirmed our *in-vivo* data showing a delayed IgM/IgG switch in B6.sC5ar1$^{-/-}$ at Day-4/6. Further, the markers of GC formation, i.e. GL7 and Fas were downregulated in B6.sC5ar1$^{-/-}$ compared to B6.s mice. Our findings identify C5aR1 as a critical regulator of the IgM/IgG switch

in GCB cells and the GC formation. Our data demonstrate that C5aR1 is not only a driver of the immunopathology during the effector phase but serves as a rheostat of early IgG AAb formation in GCB cells in EBA.

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ID: 60

The clinical-laboratory features of multisystem inflammatory syndrome in children (MIS-C) in newborns COVID-19-infected from mother: A case series.

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Introduction: SARS-CoV-2 is the etiological agent of COVID-19 that has caused a worldwide pandemic. Even though the course of COVID-19 in children is in the most cases easier, 12% of children with COVID-19 develop multisystem inflammation (MIS-C). MIS-C associated with COVID-19 is characterized as shock and multiple organ failure. This is still a lack of information pathogenesis and clinical spectrum of MIS-C. This study aimed to discuss clinical manifestations, laboratory features of neonates diagnosed with MIS-C.
Methods: The study was conducted in two maternity hospitals in Karaganda, Kazakhstan. The study included 13 cases of newborns with MIS-C associated with high level of IgG, IgM for SARS-CoV-2 from mothers who had a positive PCR during pregnancy. Blood tests, neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), and C-reactive protein (CRP) of 13 patients were investigated and compared. Statistical analysis was done by evaluating mean and standard deviation values.
Results: The median gestation age was 32 [28; 35] and 85% of newborns were premature, the median of body weight was 1750 [925; 2348]. Neonates discharged home were 10 (77%) and died 3 (23%). All newborns had an encephalopathy, 85% a respiratory distress, 69% a congenital pneumonia, 30% disseminated intravascular coagulation. All died newborns had leukocytosis, mean 35 while survived group 15 ±6; high level of CRP 20 while 1.8 ±3.8 in the survived group; NLR 5.7 against 1.8 ±1.3 and PLR 19.2 against 6.9 ±4.1.
Conclusion: Newborns, which were born from mothers with COVID-19 during pregnancy, are in the high suspicion group of MIS-C development. Transmitted COVID-19 infection might lead to early birth and low birth weight and newborn immaturity. MIS-C associated with COVID-19 leads to more than two systems involvement. Elevated biomarkers such as CRP, NLR and PLR can be considered as an inflammatory stress reaction and as indicating poor clinical outcomes.

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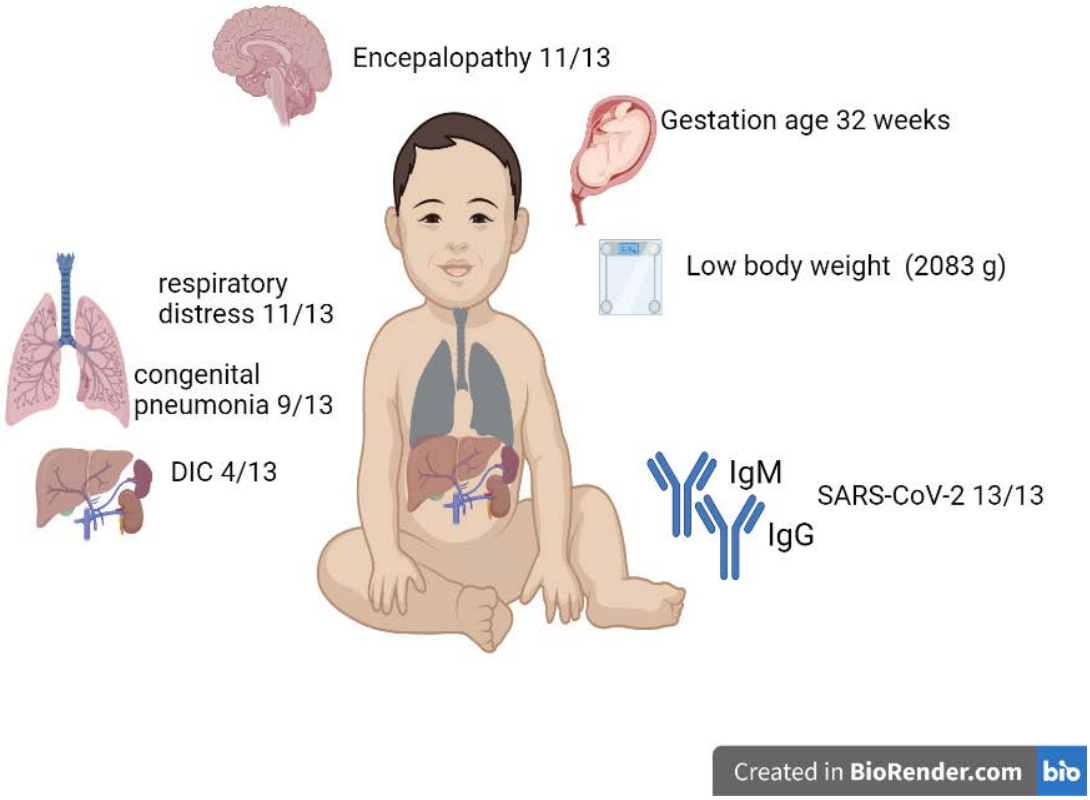

ID: 129

The Complement System in Peritoneal dialysis patients: independent pathway activation occurring at local and systemic levels.

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Background: Despite its many advantages, Peritoneal dialysis (PD) remains an underused dialysis technique due to a number of pitfalls, of which peritoneal membrane inflammation and fibrosis remains an important one contributing to technique failure. Previous work has implicated the complement system in these processes, highlighting the need for a more comprehensive examination of this vital part of innate immunity in PD .
Methods: Plasma levels of C1q, mannose-binding lectin (MBL), properdin, Factor D, C4d, C3d/C3-ratios, and sC5b-9 were determined in PD patients (n=55), HD patients (n=41), non-dialysis chronic kidney disease (CKD) patients (n=15) and healthy controls (n=14). Additionally, C1q, MBL, properdin, Factor D, and sC5b-9, levels were assessed in the peritoneal dialysis fluid (PDF) of PD patients. In a subgroup,

interleukin-6, matrix metalloproteinase-2 (MMP-2), myeloperoxidase (MPO) and elastase were measured in the PDF.
Results: PD patients had significantly higher systemic levels of sC5b-9 compared to healthy controls, CKD and HD patients (P<0.001). Only plasma C1q levels and C3d/C3-ratio were associated with systemic C5b-9 levels (P<0.001). Locally, sC5b-9 was detected in the PDF of all PD patients and levels were approximately 33% of those in matched plasma, but didn't correlate (r=0.03, P=0.83). In the PDF, only properdin levels remained significantly associated with local sC5b-9 levels in the multivariate analysis (P<0.001). Additionally, PDF levels of sC5b-9 levels positively correlated with elastase (r=0.59, P<0.001), MPO (r=0.45, P=0.009) and MMP-2 (r=0.46, P=0.005) in the PDF.
Conclusions: PD seems to induce both systemic and local complement activation. Our data suggests that these two processes are independent. Specifically, the classical pathway seems to be predominantly activated systemically in PD patients, whereas at the peritoneal level, the alternative pathway plays a dominant role. The pathophysiology behind these different processes remains to be elucidated.

ID: 70

The contribution of the alternative pathway to complement activation depends on the strength of classical pathway initiation

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Introduction
The complement system is part of innate immunity and can be activated via three pathways. Of these pathways, the alternative pathway (AP) can amplify activation from the other two. The precise contribution of this amplification loop to complement-mediated damage on cells is not well-established. This is crucial knowledge for future drug development when looking for potential targets to inhibit complement-mediated disease. Here, we aim to investigate under which conditions amplification via the AP contributes to complement activation.

Methods
We evaluated the contribution of the AP to complement responses initiated through the classical pathway (CP) on human red blood cells (RBCs) by serum of AIHA patients and recombinant antibodies. Moreover, we studied complement activation on Neisseria meningitidis and Escherichia coli. The effect of the AP was

examined using serum depleted of or with antibodies against factor B and factor D (FD), as well as genetically FD-deficient serum both before and after vaccination.

Results
Our results indicate that the contribution of the AP depends on antibody levels and (sub)class and thus on the strength of initial CP activation. When activation of the CP is low, the AP is of importance, both in an ELISA setting, on healthy RBCs and on N. meningitidis. Using AIHA serum to induce complement deposition on healthy RBCs, we saw no contribution of the AP. Overall, when antibody levels are high, we show that the contribution of the AP is not detectable.

Conclusion
In conclusion, we have indications that the importance of the AP depends on the degree to which the CP is activated. This means that the AP can have a key role in certain conditions, but may be redundant if CP activation is strong. Thus, the AP could be a target of interest in disease that is not antibody-mediated or has low antibody levels.

ID: 171

The development of glycomimetic collectin-11 antagonists in efforts to limit ischemia-reperfusion injury

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Collectin-11 (CL-11, CL-K1) is an initiatory protein of the complement pathway that is highly relevant in the context of ischemia-reperfusion injury in renal transplantation, having recently been demonstrated to play an important role in both acute kidney injury and late-stage/chronic renal inflammation. In a murine renal transplantation model, preventing CL-11 binding to carbohydrate structures at the renal cell surface reduced complement-mediated tissue damage and neutrophil infiltration while improving overall renal function [1].

Glycans are ubiquitous in nature, decorating the extracellular surface of cells and acting as a first point of contact with neighbouring cells and biomolecules. They play a critical role in numerous biological processes, including inflammation, cell adhesion, and host-pathogen recognition, and as a result carbohydrate-binding proteins have been rapidly gaining interest as targets in drug development programs. Although carbohydrate structures have extensive structural diversity, they suffer from weak binding affinities and inherently poor pharmacokinetic properties. In order to improve their drug-like properties, 'glycomimetic' compounds can be designed which mimic the structure and function of the native glycan, yet show improved affinity, specificity, and bioavailability [2].

We therefore explored the option to design 'glycomimetic' antagonists of CL-11. Molecular dynamics simulations and docking studies were performed to better characterize the ligand binding mode of CL-11 and identify potential extended binding site motifs. Based on these results, different glycomimetic libraries were constructed and evaluated *in vitro* using a thermal denaturation-based nano-differential

scanning fluorimetry assay, and a polymer-based competitive binding assay. Early lead structures have demonstrated nearly 100-fold improvements in affinity, advancing our efforts at developing a therapeutic entity against ischemia-reperfusion injuries and further evaluating the physiological role of CL-11.

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ID: 7

The impact of MBL on the outcome of infection with representative mycobacterial strains of the Mycobacterium tuberculosis complex

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Tuberculosis (TB) infectious disease in humans 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.*

Host factors have a significant influence on the pathogenesis of TB. However, the relevance of the complement system and in particular complement evasion strategies of MTBC strains have been inadequately investigated so far. 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 of the complement cascade and thereby modulate the innate and adaptive immune response. Human epidemiological studies investigating an association between genetic variants of MBL and susceptibility to TB have yielded inconsistent results. We could previously demonstrate that strains of the ancient lineage *Mycobacterium africanum* bind MBL to a higher extent than modern stains and that a specific *MBL2* variant confers protection against TB caused by *Mycobacterium africanum*[1].

The current project aims to comprehensively investigate the MBL-mediated modulation of host-pathogen interaction during infection with selected lineages of the MTBC. Different lineages were chosen. Subsequently, the binding of MBL to different MTBC isolates are compared by flow cytometry. Since macrophages take up mycobacteria after infection, macrophages are infected with preselected MTBC isolates in the presence or absence MBL and the uptake, intracellular replication and production of proinflammatory cytokines are compared[2]. Furthermore, by exploiting MBL-null mice, host-pathogen interactions which are influenced by MBL will be elucidated in detail. Wild-type and MBL-deficient mice will be infected with preselected MTBC isolates and the bacterial burden, lung histopathology, pulmonary chemokine, cytokine expression and phenotypic characterization of innate and adaptive immune cells compared.

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ID: 71

The Importance of the Mg2+ Ion for Nephritic Factor Function

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Introduction:
C3-convertase (C3bBb) is formed from C3bB (proconvertase) by Factor D-dependent cleavage of Factor B (FB) to release Ba. The divalent-ion Mg^{2+} (or Ni^{2+}) influences the interaction between FB and C3b and is necessary for FD cleavage.[1] C3-Nephritic Factors (Nefs) are autoantibodies that bind to and stabilize C3bBb. We hypothesized that Nef-stabilization is also ion-dependent.

Methods:
Using surface plasmon resonance (SPR), we measured the binding of FB, FD, and Nef+ IgG and the decay of the resulting complexes in various conditions:

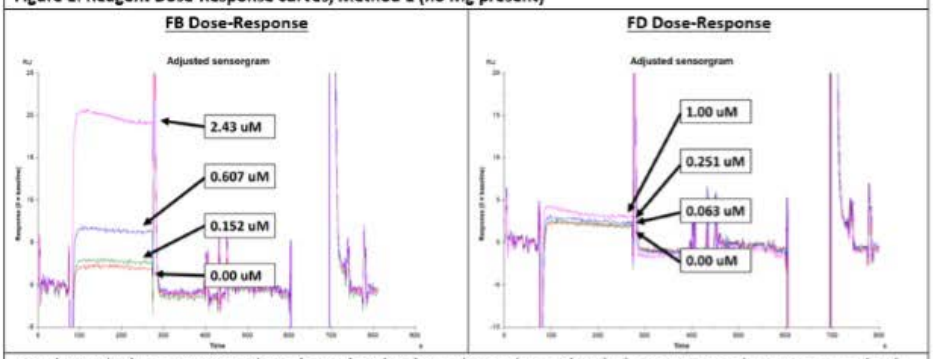
1. FB and FD were tested for C3b binding at 0mM Mg^{2+} using Dose-Response curves; (Fig 1)
2. Nef samples were tested for specificity to Convertase and Proconvertase in the presence of 10mM Mg^{2+}; (Fig 2)
3. Nef samples were tested for stabilizing function at 0mM Mg^{2+}; (Fig 3)

Results:
C3b-FB association is detectable on SPR in the absence of Mg^{2+} (Fig 1). A similar association with FD was not observed. All Nef+ samples were able to selectively recognize and stabilize C3bB and C3bBb in the presence of Mg^{2+} as seen by the increased binding and slower decay specifically in samples containing both FB and Nefs (Fig 2). No Nef stabilizing activity was observed in any sample when Mg^{2+} was absent as indicated by the rapid decay (Fig 3).

Discussion:
The selective C3b-FB association in Figure 1 suggests that subsequent Nef testing (method 2 and 3) was not limited by FB association. Our data in Figure 2 suggest that Nefs stabilize the proconvertase, C3bB, in addition to the convertase. However, as seen in Figure 3, the divalent-ion Mg^{2+} was necessary for stabilizing both complexes. Whether the C3b-Mg-Bb interaction is functionally enhanced by Nefs or Mg^{2+} promotes necessary conformational changes for Nef binding is being investigated further.

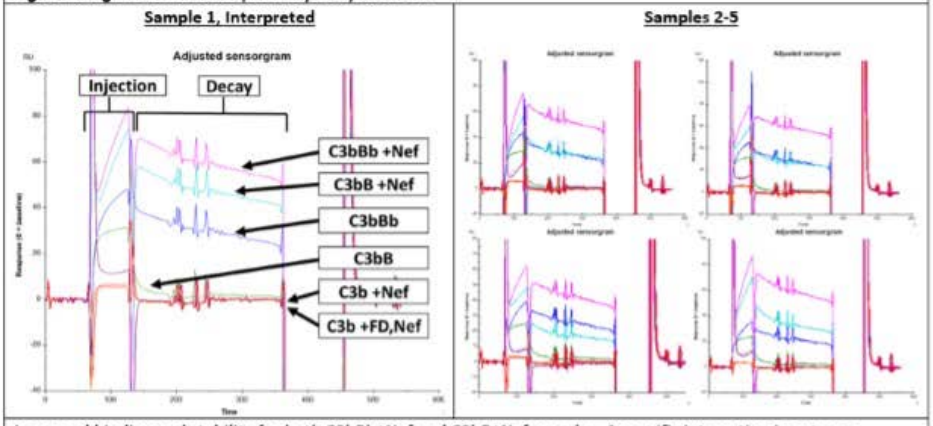
[1]*Rooijakkers, S., Wu, J., Ruyken, M. et al. Structural and functional implications of the alternative complement pathway C3 convertase stabilized by a staphylococcal inhibitor. Nat Immunol 10, 721–727 (2009). https://doi.org/10.1038/ni.1756*

Figure 1: Reagent Dose-Response curves, Method 1 (no Mg present)



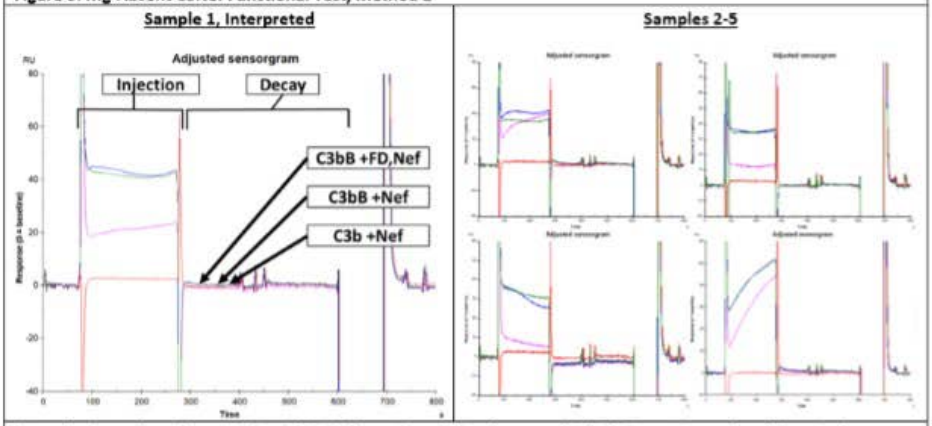
FB selectively demonstrates a dose-dependent binding relationship with C3b that is not seen between FD and C3b. This holds true despite the absence of Mg ions.

Figure 2: Mg-Present C3Nef Specificity Test, Method 2



Increased binding and stability for both C3bBb+Nef and C3bB+Nef samples. A specific interaction is not seen between C3b+Nefs or C3b+FD+Nef. This observation was consistent across five Nef-positive samples.

Figure 3: Mg-Absent C3Nef Functional Test, Method 2



Note the immediate decay of the C3bB+Nef sample and the absence of C3bBb formation and stabilization by C3bB+FD+Nefs. The stabilization of both complexes was lost in all five samples tested.

ID: 105

The influence of C5aR2 signaling for B cell activation in early autoimmunity

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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 diseases is well appreciated, the function of C5aR2 is still elusive. Initially considered a decoy receptor negatively regulating the functions of C5aR1, C5aR2 was recently shown to exert both pro- and anti-inflammatory properties even independent of C5aR1. Recent experiments of our group using C5aR2-reporter-mice revealed that B cells expressed C5aR2, but not C5aR1. Also, we observed 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, assessing the role of C5aR2 for B cells under homeostatic conditions, we found significantly increased frequencies of memory B cells in the spleen of naïve *C5aR2*$^{-/-}$ mice, which was accompanied by increased expression of decay accelerating factor (DAF, CD55) in the B cell compartment. When B cells were stimulated with LPS for 96h there was no difference between wild-type (wt) and *C5aR2*$^{-/-}$ B cells. However, when stimulating B cells in a T-dependent manner we found attenuated B cell differentiation in *C5aR2*$^{-/-}$ mice. Altogether, we observed an altered maturation and differentiation of B cells in *C5aR2*$^{-/-}$ mice, possibly highlighting an important function of C5aR2 as a novel checkpoint for B cell development.

ID: 14

The role of Pf92 in complement evasion during the intraerythrocytic replication phase of the malaria parasite Plasmodium falciparum

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The tropical disease malaria leads to more than 445,000 deaths each year and is caused by an infection with parasites of the species *Plasmodium*, which are transmitted by the bite of an *Anopheles* mosquito. Characteristic symptoms of malaria are fever, head- and body ache, and nausea, and in malaria tropica, which is caused by *P. falciparum*, may further include anemia, respiratory distress or cerebral malaria. The symptomatic phase of malaria tropica is caused by the blood stages of *P. falciparum*, which develop inside erythrocytes. During intraerythrocytic growth, the parasite matures via the ring and trophozoite stages into a schizont, which forms up to 32 merozoites. After erythrocyte rupture, the merozoites are released into the bloodstream and here invade other red blood cells. The blood stages of *P. falciparum* are exposed to the human complement system and have therefore evolved various evasion strategies to combat complement-mediated destruction. In particular, both the intraerythrocytic stages and the merozoites bind the complement factor H (FH) to inhibit the alternative complement pathway. Previously, the 6 □□cysteine protein Pf92 has been identified as a potential FH-receptor of merozoites. Here we aim

to characterize Pf92 in more detail. Therefore, we are generating a Pf92 knockout cell line using the recently described pSLI-TGD vector system. The cell line is used to phenotypically characterize Pf92 and to analyze its role in complement evasion using FH and factor H-related protein 1 (FHR-1) binding assays. In addition, we aim to identify further putative plasmodial receptors of FH and FHR-1 by co-immunoprecipitation and mass spectrometric analysis.

ID: 11

The role of plasminogen for red blood cell invasion by Plasmodium falciparum merozoites

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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 regions. With over 445,000 deaths each year, malaria is still one of the deadliest infectious diseases in the world. The symptomatic phase of malaria, which is characterized by e.g. fever, head- and body aches, and diarrhea, is caused by the intraerythrocytic stages of the parasite. Inside erythrocytes, the parasite develops over a course of 48 h via the ring and trophozoite stages into a mature schizont, which forms up to 32 daughter merozoites. After rupture of the erythrocyte, the merozoites are released into the bloodstream where they invade other red blood cells. Outside of the host erythrocytes, merozoites are rapidly targeted by the human complement system and hence require effective complement evasion mechanisms while keeping the extracellular phase as short as possible. We recently discovered that in addition to typical complement regulators like factor H the serum zymogen plasminogen, a component of fibrinolysis, impacts merozoite infectivity. We show that the extracellular merozoites effectively bind plasminogen from human serum. However, in contrast to plasminogen bound by the intraerythrocytic parasite stages, merozoite-bound plasminogen is not actively converted into the serine protease plasmin, but requires host plasminogen activators such as tPA (tissue-specific plasminogen activator) for conversion. We further discovered that plasminogen, but not plasmin, leads to increased invasion of erythrocytes by merozoites independent of active complement. Finally, we identified putative plasminogen receptors of the parasite blood stages using co-immunoprecipitation followed by mass spectrometry. The hits suggest that the binding of plasminogen involves several known merozoite surface proteins linked to invasion as well as parasite proteins with PEXEL motifs.

Reiss, T., Theis, H.I., Gonzalez-Delgado, A., Vega-Rodriguez, J., Zipfel, P.F., Skerka, C., Pradel, G., 2021. Acquisition of human plasminogen facilitates complement evasion by the malaria parasite Plasmodium falciparum. Eur. J. Immunol. 51, 490–493. https://doi.org/10.1002/eji.202048718

ID: 12

The role of the complement system in a murine model of disseminated mucormycosis

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Background: Mucormycetes, a rather heterogeneous group of fungi, induce a life-threatening disease called mucormycosis. The prevalence of this disease, which shows high morbidity and mortality, increased within the last decade. Immune deficiencies represent a major risk factor for mucormycosis. The purpose of our study is to enlighten the responsibility of the complement system, in the defense against mucormycosis.
Methods: Mice with a deficiency in complement C3 (ΔC3) or C6 (ΔC6) were intravenously infected with *Lichtheimia corymbifera* (LC), *Lichtheimia ramosa* (LR), *Rhizopus arrhizus* (RO), *Rhizopus microsporus* (RM), *Rhizomucor pusillus* (RmP) or *Mucor circinelloides* (M). Survival, clinical status, and immunological parameters were monitored over 14 days and compared to that of immunocompetent or neutropenic mice. Additionally, serum from healthy immunocompetent mice was analyzed for capacity to opsonize the fungi.
Results: When intravenously challenged with M or RO, there is no difference between immunodeficient and immunocompetent mice. Complement deficiencies represent a risk factor for a lethal outcome in LC, LR, RM, and RmP. LC and RM lead to higher mortality in complement deficient mice, compared to neutropenic animals. There is no significant difference between the lethality of ΔC3 and ΔC6 mice challenged with LC, M, RO, and RM. ΔC3 mice exhibited higher mortality than ΔC6 mice when infected with LR, contrary to RmP infections. Complement opsonization of LC, LR, RO, RM, and M spores inversely correlate to the virulence of these species in immunocompetent mice, showing low complement deposition on RM and RO spores, moderate deposition on LC, and high deposition on M and LR.
Conclusions: Complement plays an important role in the murine model of disseminated mucormycosis. Mortality of the complement-deficient animals varies between the species. Further investigations have to be performed to fully understand the immunopathogenesis of mucormycosis and help to fight the high morbidity and mortality of this disease.

ID: 120

The RPE-specific complement system correlates with EMT during disease development

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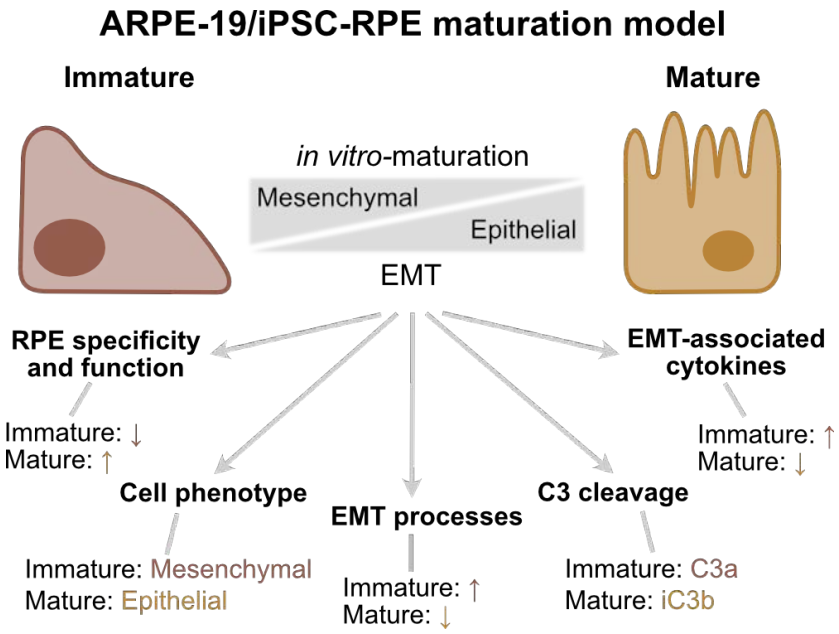
Background:
The cellular and specific complement system of the retinal pigment epithelium (RPE) is involved in the development of retinopathies. These conditions are further associated with a de-maturation and epithelial-to-mesenchymal transition (EMT) of RPE cells. Here, we describe a cell-based maturation model to compare immature and mature RPE cells and elucidate the role of the RPE-specific complement components in EMT.

Methods and Results:



Conclusion:
The analysis of the RPE maturation model resulted in the identification of different complement secretion profiles of immature and mature RPE cells. On the one hand, immature RPE cells displayed a pathological environment that was accompanied by a cell-based complement activation and inflammation. This was underlined by increased C3a and pro-inflammatory cytokine profiles. On the other hand, mature RPE cells mirrored the physiological *in vivo*-RPE. They further displayed a dampened complement activity while showing an increase of inactive, cellular iC3b fragments. Prospectively, this project lays the groundwork for a more precise elucidation of the relationship of the cellular RPE-specific complement system and its role in EMT-associated retinal diseases.

ID: 188

The Sulphated glycolipid Su7Malt-Sp6-C2HN3-CMG2-Ad-DE attenuates complement activation in an in vitro xenotransplantation model.

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Background:
The complement system consists of soluble and membrane-bound protein which, when activated, leads to inflammation, phagocytosis, and cell lysis. Uncontrolled complement activation is observed in several conditions, for exampla in cardiac xeno- or ABO-incompatible allotransplantation, cardiac ischemia reperfusion injuries and autoimmune diseases. In our study, we investigated the effect of a novel synthetic sulfated glycolipid (LipoSulf) obtained by conjugation of hepta-O-sulphomaltose with dioleoyl

phosphatidyletanolamine (LipoSulf), on complement activation and deposition on endothelial cells.

Method:
We first determined the toxicity of the compound on wildtype porcine aortic endothelial cells (PAECs) with concentration series ranging from 5 µg/ml-50mg/ml. Subsequently, PAECs were exposed to 10% pooled normal human serum (NHS) to mimic a xenotransplantation setting. Four concentrations of LipoSulf, ranging from 0-15 µg/ml were added to NHS, which were then incubated with PAECs for 1h. Deposition of the complemnt proteins C3b/c, C4b/c abd C5b-9 was then assessed by immunofluorescence staining and flowcytometry. The release of soluble complement factor C3a was quantified in the supernatant by ELISA.

Results:
We observed that PAECs were viable up to a LipoSulf concentration of 1mg/ml. When added to NHS, LipoSulf led to a significant reduction of complement deposition on PAECs dose-dependent manner. Deposition of all tested complement proteins, i.e. C3b/c, C4b/c, as well as the terminal complement complex C5b-9, was inhibited significantly with administration of 10 µg/ml and 15 µg/ml of LipoSulf. Incubation of PAECs with 10 µg/ml and 15 µg/ml of LipoSulf reduced complement deposition marker C3b/c by 56% and 71%, respectively. Interestingly, this synthetic glycolipid did not affect the concentration of soluble anaphylatoxin C3a in the cell supernatant.

ID: 174

Therapeutic efficacy of a bi-functional anti-C5 mAb/FH1-5 fusion protein in a mouse model of rapidly progressing lethal C3 glomerulopathy

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C3 glomerulopathy (C3G) is a rare kidney disease caused by dysregulated alternative pathway (AP) complement activation. It is characterized by glomerular C3 deposition, proteinuria, crescentic glomerulonephritis and renal failure. The anti-C5 mAb drug Eculizumab has shown therapeutic effect in some but not all C3G patients and no approved therapy is currently available. We previously characterized a lethal murine model of C3G ($FH^{m/m}P^{-/-}$ mice)[1] and demonstrated an anti-C5 mAb to be completely effective in preventing disease but only partially effective in treating established disease[2]. To study if inhibition of proximal AP complement components such as factor D (FD) might be more efficacious than C5 inhibition in C3G, we have humanized FD in $FH^{m/m}P^{-/-}$ mice by crossing with a human FD knock-in mouse (hFD). Unexpectedly, $FH^{m/m}P^{-/-}$hFD mice not only recapitulated the C3G phenotype but also developed a more aggressive form of C3G, with 100% mortality occurring before 7 (instead of 12-14) weeks of age. In this studay, we tested the therapeutic efficacy of an anti-mouse C5 mAb and a bi-functional anti-mouse C5 mAb/mouse FH1-5 fusion protein in $FH^{m/m}P^{-/-}$hFD mice. Mice with high (3+) proteinuria and hematuria were selected and treated with either drug (n=6 each group, 40 mg/kg, i.p., every 3 days). After 30 days of treatment, all mice treated with anti-C5 mAb/FH1-5 survived, whereas 3 of 6 mice treated with anti-C5 mAb did not. Mice in the former group normalized AP complement control, and showed less glomerular disease, as assessed by proteinuria, hematuria, glomerular C3 and fibrin

deposition and % of normal glomeruli, than those in the latter group. These data suggest that bi-functional anti-C5 mAb/FH1-5 fusion protein is more efficacious than anti-C5 mAb in treating established C3G. The $FH^{m/m}P^{-/-}$hFD mouse provides a new rapidly progressing lethal C3G animal model in the study of anti-FD and other AP complement inhibitors.

References:

Lesher AM, Zhou L, Kimura Y, Sato S, Gullipalli D, Herbert AP, Barlow PN, Eberhardt HU, Skerka C, Zipfel PF, Hamano T, Miwa T, Tung KS, Song WC. Combination of factor H mutation and properdin deficiency causes severe C3 glomerulonephritis. J Am Soc Nephrol. 2013 Jan;24(1):53-65. doi: 10.1681/ASN.2012060570.

Williams AL, Gullipalli D, Ueda Y, Sato S, Zhou L, Miwa T, Tung KS, Song WC. C5 inhibition prevents renal failure in a mouse model of lethal C3 glomerulopathy. Kidney Int. 2017 Jun;91(6):1386-1397. doi: 10.1016/j.kint.2016.11.018.

ID: 130

Therapeutic Targeting of Factor H to Injured Tissues Through Linkage with Anti-C3d mAb Delivers Potent and Durable Complement Inhibition in Skin Without Inhibiting Systemic Complement

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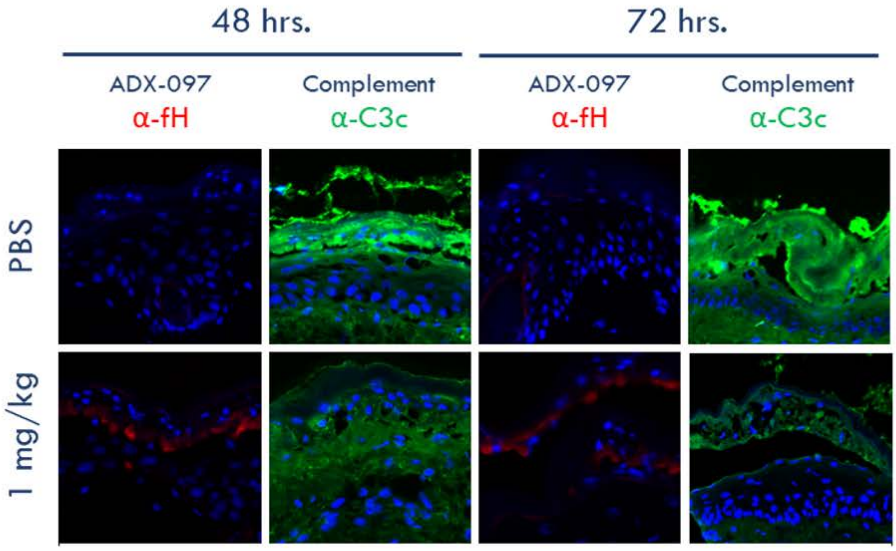
Background:
While systemic complement inhibition is a therapeutic strategy for complement-driven diseases, potency and durability of inhibition are limited by rapid protein turnover and high circulating concentrations of complement proteins. Because of the essential role of complement in innate and adaptive immunity, systemic blockade also raises infection risk in patients. Thus, substantial unmet need remains for safer and more effective anti-complement therapies.
Methods:
ADX-097, a humanized anti-C3d antibody-derived fusion protein containing the first five SCRs of Factor H (FH), was designed to inhibit local complement alternative pathway (AP) activation and amplification loop engagement while minimizing systemic blockade. To assess tissue targeting in skin, we characterized circulating and tissue PK/PD of ADX-097 in two models of skin complement activation: a non-human primate (NHP) model of UVB-induced skin complement activation and an antibody-transfer mouse model of epidermolysis bullosa acquisita (EBA). We have further explored the utility of C3d-targeted therapies by demonstrating complement activation and C3d deposition in human skin disease biopsies by immunostaining.
Results:
In the NHP UVB model, we show that ADX-097 binds to C3d deposits in the epidermis and inhibits local complement activation. In a dose-response study, subcutaneous delivery of 1 mg/kg ADX-097 achieves 60% inhibition of epidermal complement for 6 days without systemic complement blockade. In the mouse EBA model, subcutaneous delivery of 50 mg/kg ADX-097 achieves up to 70% inhibition of skin

complement activation. Finally, immunostaining of skin biopsies from patients across several human skin disorders, including cutaneous lupus, bullous pemphigoid, pemphigus and cutaneous vasculitis, demonstrates C3d deposition and C3 complement activation, indicating potential utility for a C3d-targeted delivery in dermatological diseases.

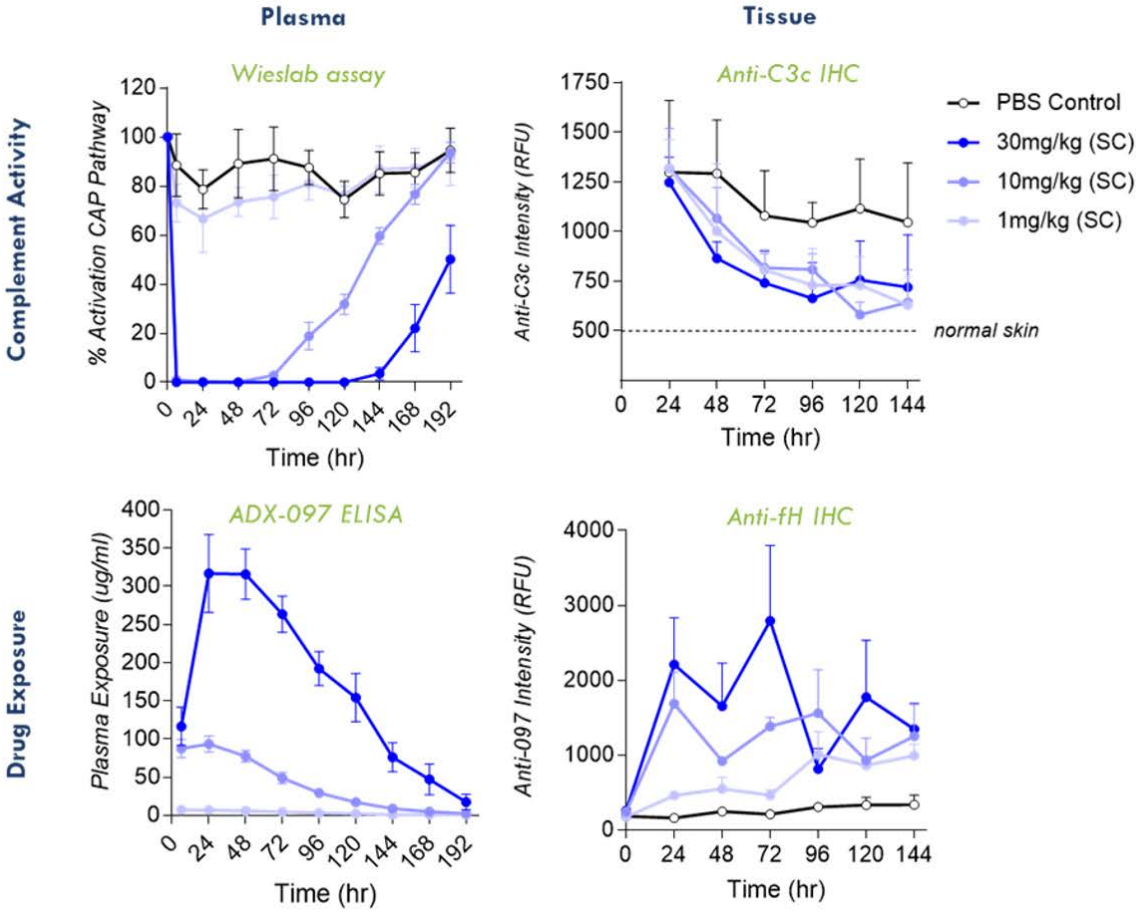
Conclusions:

These data demonstrate the therapeutic potential of ADX-097, showing that C3d-mediated tissue targeting in preclinical models results in potent and durable local AP complement blockade in skin without substantially affecting systemic complement inhibition.

Immunostaining of ADX-097 in skin and inhibition of complement activation (Tissue PK/PD)



Quantitation of Circulating vs Tissue PK/PD



ID: 96

Therapy monitoring of multisystem inflammatory syndrome in children using macrophage- and complement activation markers

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Multisystem inflammatory syndrome in children and adolescents (MIS-C) is a rare, potentially life-threatening complication of respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Standard therapy of MIS-C includes glucocorticoids, immunomodulatory and antithrombotic modalities, intravenous immunoglobulin (IVIG) and aspirin treatment in the first line. Complement and macrophage activation has been shown to play a role in the pathogenesis of MIS-C, but data on the changes of its biomarkers and therapy responses are scarce.
Therefore, we aimed to investigate if levels of complement and macrophage activation biomarkers are appropriate to follow therapy efficacy in MIS-C patients.

Concentrations of multiple complement and macrophage activation markers were measured in 34 MIS-C cases (23 males), who had available samples during disease course. Age distribution of the cohort was between 1 and 21 years, with peak around 10 years (median 9.5, 25th-75th percentile 7-13).

Macrophage activation markers ferritin and neopterin showed very high elevation and close association with acute MIS-C, similarly to classical- (C1s/C1-INH complex and C4d), alternative- (Bb) and terminal pathway (sC5b-9) complement activation markers. There was positive correlation between macrophage- (ferritin) and complement alternative pathway (Bb) activation. All of the macrophage and complement activation markers (except C4d) showed a rapid and sharp decline in parallel to a positive clinical response to IVIG therapy.

In conclusion, we describe here that neopterin, a small molecule belonging to the pteridine family, ferritin, a protein involved in iron storage and metabolism, and multiple activation markers of complement classical-, alternative- and terminal pathways are useful activity markers in MIS-C, and seem to be suitable to follow therapy efficacy in this condition.

ID: 25

Treatment with TRF-budesonide (Nefecon) modulates the complement system in patients with IgA nephropathy

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Background: The NEFIGAN trial (NCT01738035) evaluated the effect of a novel targeted-release investigational formulation of budesonide (TRF-budesonide [Nefecon]) in the treatment of IgA nephropathy (IgAN). Participants in this Phase 2b trial were randomly assigned placebo, Nefecon 8 mg or 16 mg/day, and samples were collected at baseline (0 months), at the end of the treatment (9 months) and at the end of the study (12 months). In this exploratory study we evaluated the effect of Nefecon treatment on the circulating levels and urinary excretion of a panel of complement components.

Methods: Plasma and urine levels of complement proteins (C4c, C3bc and soluble C5b-9) and ficolin-1, -2 and -3, MBL, CL-11, MASP-3, MAP-1 and PTX-3 were measured using in-house sandwich-ELISAs. Treatment differences between baseline, end of treatment and end of study were assessed by mixed-effects model analysis. The effect of the treatment correcting for the baseline was assessed by multiple linear regression. Significance: $p<0.05$. Urinary proteins were adjusted for creatinine excretion.

Results: Circulating levels of MASP-3 were decreased in a dose-dependent manner after treatment ($p=0.0313$ Nefecon 8 mg/day, $p=0.0080$ Nefecon 16 mg/day). MBL/creatinine was significantly reduced in the urine by both doses of Nefecon compared with placebo ($p<0.0001$ Nefecon 8 mg and 16 mg/day). CL-11/creatinine levels were also significantly reduced in a dose-dependent manner after Nefecon administration ($p<0.0411$ Nefecon 8 mg/day, $p<0.0095$ Nefecon 16 mg/day). Complement activation markers and ficolin-3 levels were detectable in urine but levels remained unaltered.

Conclusions: Treatment with Nefecon modulates components of both the alternative (MASP-3) and lectin (MBL and CL-11) pathways of complement, two pathways known to be important in mediating kidney damage in IgAN. These initial observations warrant further investigation.

ID: 23

Tumor Suppressor Role of CSMD1 in Gliomas

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Background: Gliomas are brain tumors originating from glial cells. They make up 20-30% of brain tumors and 80% of malignant brain tumors. Tumor suppressor role of complement inhibitor CSMD1 has been elucidated in several cancers but not in gliomas, even though it is highly expressed in the brain. To this end, we have used large number of patients with glioma, *in vitro* and *in vivo* approaches to determine both tumor suppressive and prognostic roles of CSMD1 in gliomas.

Materials and Methods: A total of 1507 patients with glioma from three different datasets were used to assess prognostic role of CSMD1 expression in patient cohorts. C-bioportal and EPIC databases were used to detect differentially expressed genes, proteins and signaling pathways, as well as immune cell

infiltration between CSMD1-high vs. CSMD1-low groups. In addition, we overexpressed CSMD1 in three glioma cell lines and functional studies were performed. Thereafter, $CD4^+$ or $CD8^+$ T cells were co-cultured with CTRL or CSMD1-expressing clones of glioma cell lines. Lastly, CSMD1 expression was monitored by immunofluorescence microscopy in cryosections of PDGF induced murine gliomas using the RCAS/tv-a model.

Results: Our bioinformatical analysis suggested that high expression of CSMD1 was associated with increased overall survival and disease-free survival, as well as lower tumor grade. CSMD1 expression was positively correlated with $CD4^+$, $CD8^+$ and B cell infiltration, it was negatively correlated with macrophage infiltration. *In vitro*, overexpression of CSMD1 in glioma cell lines inhibited proliferation, invasion, migration and tumorosphere formation, whereas promoted apoptosis. Moreover, CSMD1 overexpression increased the activation of $CD4^+$ and $CD8^+$ T cells during co-culture experiments. Next, in the mouse brain tumor model, CSMD1 expression was downregulated in tumor area compared to healthy brain tissue area.

Conclusions: Our study demonstrated that CSMD1 acts as a tumor suppressor in gliomas and loss of CSMD1 expression is associated with poor prognosis.

ID: 123

Vastly different alternative complement pathway activation on seemingly homogeneous eukaryotic cell surfaces

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The complement system is pivotal for the elimination of pathogens and divided into three distinct pathways: classical (CP), lectin (LP) and alternative pathway (AP). Pattern recognition is essential to initiate the complement cascade but more importantly restrict complement activation to unwanted surfaces. Inappropriate activation of the complement system can have devastating effects on human health and lead to various diseases such as age-related macular degeneration (AMD), a progressive disease causing the deterioration of the central vision. The chemical changes to the macromolecular system in ocular tissue involving photodegradation during the aging process may present a breeding ground for pathological complement activation. Paired with the polymorphic variants in complement genes (FH, FB, C3) strongly associated with altered AMD risk suggest that the AP plays a prominent role in disease etiology.1
To gain insight into the pathomechanism, we investigated the mechanism of AP activation on various eukaryotic cell surfaces. Rabbit erythrocytes have traditionally been used to assess the activity of AP, because their membrane proteins are ineffective in providing protection against human complement. We found striking differences among red blood cells in terms of their capacity to fix C3 even in the absence of properdin. Heat inactivation as well as the addition of complement inhibitors prevented complement

deposition. Similar heterogeneous complement deposition was found using a mutant human erythroblast cell line lacking protective membrane proteins.
In summary, these two model systems suggest that the AP exhibits certain surface preferences even at the very early stage of fluid-phase activation. C3b-acceptor molecules may become more available on deteriorated or end-of-life cells, and if so, these scavengers can divert an emerging AP activation and preserve cells with greater fitness. A better understanding of the selectivity of the AP will pave the way for therapies with a new mode of action that protects worn membranes/surfaces.

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ID: 180

α-MSH modulates complement activation and inflammatory state of senescent human osteoarthritic chondrocytes

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Background: Osteoarthritis (OA) is a degenerative joint disease, which is closely associated with age. OA causes cartilage loss, structural damage, and senescence in all joint tissues. Inflammatory mediators, especially the alternative complement pathway seems to play a crucial role already in early stages of OA. Moreover, cartilage is a tissue with only minimal intrinsic regenerative capacity. The α-melanocyte–stimulating hormone (α-MSH) is a melanocortin peptide. Beyond pigment induction, α-MSH has anti-inflammatory and chondro-protective effects dampening inflammatory and presumably age-related processes. The underlying molecular mechanisms are mostly unknown yet. Therefore, the aim of this study was to analyse the effect of α-MSH on complement activation and inflammatory state of senescent human OA-chondrocytes.
Material & Methods: Chondrocytes and synovial fluid (SF) were obtained from OA and non-OA patients. Chondrocytes, cultivated in 2D monolayers, were incubated with α-MSH and treated with doxorubicin, to induce a senescent phenotype. Induction of senescence was verified by senescence-associated ß-galactosidase (SA-ß-Gal) activity, caspase 3/7 activity, mitochondrial membrane potential (JC-1 assay) and senescence-marker expression. Expression and secretion of complement components, pro-inflammatory cytokines and Matrix metalloproteinases were analysed in SF, cell lysates and supernatants by digital PCR (dPCR), Western Blot and Multiplex-ELISA.
Results: SA-ß-Gal expression was induced in all doxorubicin-treated human chondrocytes, and no changes in apoptosis rate were observed. CDK-inhibitors p16 and p21 were increased in senescent OA-chondrocytes. IL-6 and MMP-13 RNA expression was time-dependently upregulated in doxorubicin-treated OA-chondrocytes. Secretion levels of C3b, CFB, CFD, as well as MMP-13 were increased in supernatants of senescent OA-chondrocytes, whereas α-MSH diminished these effects. Higher levels of C3b, C3a, CFD, CFH, as well as α-MSH were detected in SF of OA patients compared to SF of non-OA

patients.
Conclusion: These *in vitro* data revealed increased complement activation in senescent human OA-chondrocytes and an anti-inflammatory effect of α-MSH. A correlation between the effect of α-MSH and complement components on OA-chondrocyte metabolism needs to be further investigated.