ON ISSUE NUMBER NINE

<1> THIS “FOCUS ON COMPLEMENT” CONTAINS:

- Flash news on cooperation between MBL and TLRs in signaling within *S.aureus* phagosomes.
- Flash news on the involvement of complement in neuronal synapse remodeling.
- Two Spotlights on complement teams in the USA and The Netherlands.
- A Historical Perspective written by Berhane Gebrehiwet describing the long struggle to identify a C1q Receptor.
- The last report on novel findings described at the 11th European Meeting on Complement in Human Disease that took place last September in Cardiff.

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Mannose-binding lectin (MBL) is a C-type lectin that is well appreciated for its role as a soluble pattern recognition molecule leading to opsonization and lectin pathway activation. This study adds a new aspect to the various functions of MBL which links the complement system to Toll-like receptors (TLR) 2 and 6, a complex of cell-associated pattern recognition receptors that sense gram-positive bacteria. Eddie et al. found that MBL enhances the proinflammatory, TLR2/6-induced cytokine production in response to S. aureus infection. Mechanistically, their data suggest a model in which MBL regulates TLR2/6 heterodimer signaling within the phagosome by binding to S. aureus-derived lipoteichoic acid which also serves as a ligand for TLR2/6. Although the exact nature of this cooperation remains to be determined, this study highlights the importance of cooperation between soluble and cell-bound pattern-recognition molecules, such as the complement system and TLRs, for appropriate innate immune responses.


This study adds to the growing body of data demonstrating critical roles for complement in tissue remodeling. Within the developing CNS, specific neural circuits are formed that require the elimination of inappropriate synapses. The mechanisms underlying such remodeling of synaptic connections in the developing CNS remain obscure. The study by Stevens et al. suggests that C1q-mediated activation of the complement cascade and activation of C3 are required for appropriate neural circuit formation. The authors found that astrocytes trigger neuronal C1q production during murine eye development. Such C1q and subsequently generated C3 cleavage fragments may “flag” CNS synapses for phagocytosis by microglia as one mechanism to prune inappropriate synapses. Importantly, similar upregulation of C1q takes place in the adult retina in a mouse model of glaucoma indicating that the same, complement-dependent mechanisms, that account for synapse remodeling during early postnatal development may also initiate the development of neurodegenerative disorders such as Alzheimer’s disease, ALS and multiple sclerosis.
Jörg Köhl’s Complement lab

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The laboratory has a longstanding interest in the immunochemistry and immunobiology of the anaphylatoxins (AT) C3a and C5a and their corresponding AT receptors. In the 90’s the lab was located at Hannover Medical School in Germany. During these years, we contributed to molecular characterization of AT/AT receptor interactions, the cloning of the C3a receptor, and the development of specific immunoassays to detect ATs. Using such assays, we found that complement activation occurs early in septic- as well as in trauma patients and may serve as a predictive marker for fatal outcome. Later in the 90’s, the focus shifted and we became more interested in the immunobiology of the ATs, i.e. their roles in the inflammatory response of immune complex diseases (cross-talk with IgG receptors), ischemia-reperfusion injury and allergic asthma. As a very helpful tool for such studies, we selected a human C5a mutant from a phage library that blocks the interaction of C5a with the C5aR (CD88) and C5L2. This C5aRA has been successfully used by many labs around the world to target C5aRsi vitro and in vivo.

In 2002, the laboratory moved to Cincinnati Childrens’ Hospital, where we intensified our animal studies on the role of the AT in allergic asthma. In contrast to the well appreciated, proinflammatory role of C3a and C5a in an established inflammatory environment, we found that C5a protects from the development of maladaptive type 2 immune responses during allergen sensitization. A new focus is on the cross-talk between pattern-recognition receptors such as the Toll-like receptors and AT receptors on antigen-presenting cells and its impact on the development of adaptive immune responses.

Within the next couple of months, most parts of laboratory will relocate to Germany and become part of the Institute for Systemic Inflammatory Research at the University of Lübeck, Germany.

The laboratory has hosted and trained many wonderful students and post-docs from all around the world and is very thankful for their outstanding contributions. We are privileged to have several, wonderful collaborators in Cincinnati, the US, Europe and South America who inspired us to seek for connections from the small world of complement to the universe of immunology. Needless to say that the laboratory is open to anybody who likes “chatting” about complement, innate immunity…..

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Jos van Strijp’s research group shows that every pathogenic bacterium scrutinized so far to possess a wide array of small, evolutionary conserved proteins which serve to evade the innate immune system. Many of these proteins are directed against some part of the complement system. The sole identification of novel proteins is the easy part. Figuring out the exact molecular mechanism of action is the most labor-intensive part of the process but also the most exciting.

In the beginning of this millennium we characterized an inhibitory activity in the supernatant of growing staphylococci. This was identified as a protein that blocks the activation of neutrophils via the formyl peptide receptor (FPR) and C5aR. We dubbed it chemotaxis inhibitory protein of staphylococcus aureus or CHIPS. Next in line awaiting discovery was the Staphylococcal Complement Inhibitor or SCIN. SCIN binds to both C3-convertases, and jams the complement system.

In the wake of CHIPS and SCIN, we went on to describe multiple other immune evasive molecules. These immune evasive molecules will have an interesting career ahead. In the first place, the molecules explain why pathogenic bacteria are able to survive and replicate in the human body. Next, they lead researchers directly to key immunological functions. Third, they are leads for anti-inflammatory therapy.

The group now consists of several subgroups. Kok van Kessel heads the group that focuses on evasion strategies for specific receptors on white blood cells that are involved in the process of phagocytosis. Carla de Haas has a group that works on the evasion of G-protein coupled receptors. Suzan Rooijakkers’ group is specialized in identifying molecules that target the human complement system. Others look at intervention of various inflammatory events.

Exciting new molecules have emerged from the group in the last years that will be published in the near future. Examples are: a unique broad Fc-receptor antagonist, evasion of Toll-like receptor signaling, the first bacterial transmigration inhibitor, a highly unexpected complement inhibition strategy and many more.

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**C1q receptors: we have come a long way; but we are not at the end**

A story from the Ghebrehiwet laboratory. Stony Brook University Dept. Medicine, Health Sciences Center, Stony Brook, NY.

Behind the discovery of each complement protein, is a robust history; some of it exciting and some rather polemical and even controversial. This is by and large, due to the methods used, but more often than not, the laboratories and the personalities involved. The history of C1q receptors is therefore no different; and for the sake of our younger colleagues, I will highlight—without making it much of a personal journey although our laboratory has been in the thick of it—some of the key events and personalities, that in my opinion, directly or indirectly contributed to our present understanding of C1q receptors and their functions.

In 1974, I was fortunate enough to have met Dr. H.J. Müller-Eberhard in Paris (Hôpital Necker), who later arranged for me to go to Scripps (La Jolla) to do a Postdoctoral Fellowship with Dr. Viktor Bokisch, and when the latter met an untimely death two years after my arrival, with Dr. Müller-Eberhard himself. My initial project involved the identification and characterization of a C3 fragment that Prof. Dr. Klaus Rother had earlier described as a “leukocyte mobilizing factor” or LMF. This work subsequently led to the identification of a late degradation fragment of C3 with similar activities; and this fragment was later designated C3e.

However, what digressed and indeed attracted my attention during the course of this study, were three, relatively unrelated observations that were made almost concurrently (1975). First, was the demonstration by Alain Sobel and Viktor Bokisch, that human peripheral lymphocytes and lymphoblastoid cell lines possessed C1q receptors as had been predicted by Dickler and Kunkel few years earlier. Furthermore, these studies showed that: (a) $^{125}$IC1q binding was optimal under low ionic strength; (b) the putative receptor was trypsin- but not neuraminidase-sensitive; and (c) $^{125}$IC1q binding was inhibited by C1q but not by IgG or C3. Second was the demonstration by Hedvig and Peter Perlmann—who at the time (1975) were working in the same laboratory as visiting scientists (University of Stockholm)—that the antibody-dependent, lymphocyte-mediated cytotoxicity (ADCC) could be enhanced if the target cells (EA) were coated with suboptimal doses of anti-erythrocyte (target cell) IgG and increasing concentrations of C3b or C3d suggesting that effector cell-target cell interaction via IgG-FcγR was potentiated by the C3b-CR1 interaction. Third was the finding by Conradie, Volanakis and
Stroud (1975) that serum contains a C1q inhibitor that bound to free C1q during purification; and when the inhibitor was removed, the inhibitor-free C1q had a much more enhanced hemolytic activity. Silvestri later (1978) identified the C1q inhibitor to be chondroitin 4-sulfate proteoglycan. These early observations led us to ask two critical questions: (1) Does C1q-like C3-also enhance the ADCC of chicken erythrocyte target
cells? (2) Since cells (mononuclear phagocytes, platelets etc) synthesize and constitutively secrete chondroitin sulfate proteoglycan, could the cell surface C1q receptor be similar or related to the C1q inhibitor in plasma? Whereas, the answer to the
first question is a simple "yes"- in that human lymphoblastoid cells such as Raji, indeed were capable of destroying chicken erythrocytes that carried cell surface bound human
C1q-the second question became a much more intriguing undertaking and became the focus and passion of our laboratory for the past 20 years.

Our first attempt was therefore to purify a "C1q inhibitor' from cell membranes of
peripheral blood lymphocytes. Using a C1q-affinity column, a cell membrane component
with properties similar to the plasma C1q inhibitor (binding to the collagen region, inhibition of hemolytic activity of C1q, etc) was isolated. Encouraged by this, we proceeded to make a large-scale purification using Raji cells as a source of membranes. Immunochemical and functional analyses of the purified membrane protein, led us to
conclude that the 60-70 kDa membrane component, which bound to the collagen tail of
C1q, was a receptor for human C1q. Although its significance was not appreciated at that
time, a proteoglycan component was also identified to be a component of the receptor.

Few years later, Rajneesh Malhotra and Bob Sim at the MRC (Oxford) used a
combination of FPLC and HPLC to purify a 53-
kDa protein that bound not only to the
collagen tail of C1q, but also to some members
of the collectins. Rightfully, many members
of our complement community raised several
questions, with regards to our findings. There
are two molecules, which bind to the collagen
like region of C1q: how are these molecules
related? Is the smaller mol.wt protein a
degradation fragment of the larger protein?
If they are not related, are they part of a
"C1q receptor complex"?

Therefore, when Ellinor Peerschke and
I decided to spend a sabbatical at the MRC in
Oxford (1991), with Bob Sim and Ken Reid
respectively, we had two specific objectives.
One was to resolve the molecular weight
difference between the two molecules; and
the other was to clone the 60-70 kDa
molecule. The sabbatical proved to be very productive in many ways. First Peerschke and Malhotra, showed that the N-terminal sequence of the C1qR purified from endothelial cells was identical to the 53-kDa protein that Malhotra described and that the difference in molecular weight was an artifact of the SDS-PAGE as both molecules run at approximately 60 kDa under the same conditions. Secondly, the N-terminal sequence was shown to be identical to calreticulin (CR). However, since CR was at that time already cloned, our second objective, which was to clone this molecule, became redundant. Regardless, we proceeded to purify a substantial amount of 60-kDa protein from Raji cell membranes suitable for N-terminal sequencing and immunochemical and functional characterization. This generated not only a high yield of purified 60-kDa protein, but also revealed a conspicuous, highly negatively charged peak (previously unnoticed), at the end of the HPLC-DEAE profile, which eluted with very high salt. When this protein was sequenced, it generated a highly negatively sequence of 22 amino acid residues that was wholly unrelated to the 60 kDa molecule. Immunochemical and functional characterization of this molecule revealed that it was a 33-kDa protein that bound to the globular heads of C1q with high affinity. Together with Wallace Lim, who was a graduate student in Ken Reid’s laboratory, the 33-kDa molecule was cloned and sequenced. In order to differentiate between the two receptors, the designation of “gC1qR” was coined to describe the 33-kDa protein, whereas the term cC1qR/CR (calreticulin) was used to describe the 60-kDa protein. Incidentally, although the identity of the binding sites was not known, Sandra Bordin (University of Washington) had previously shown that gingival fibroblasts could be separated into two distinct subpopulations; one enriched in surface molecule(s) that preferentially bind the collagen tail of C1q, while the other bound the globular heads.

Although these proteins are now generally accepted as receptors for C1q; the lack of a transmembrane domain in their respective sequences had nevertheless been the subject of great debate. However, we are now convinced that both molecules transmit downstream signaling by recruiting transmembrane proteins such as β1-integrins, CD44, and CD91. More to follow....
Session 5: Complement, aHUS and other renal diseases.

For reasons related to its anatomy and physiological roles, the kidney is particularly susceptible to complement-mediated injury. Both atypical haemolytic uraemic syndrome (aHUS) and Type II membranoproliferative glomerulonephritis (MPGN-II) are associated with defective complement regulation. This session focussed on these defects and the mechanisms by which they cause disease.

In some patients with aHUS, autoantibodies against factor H have been demonstrated and implicated in the disease process. Dragon-Durey et al. have now gathered a cohort of 20 aHUS patients with anti-factor H autoantibodies and looked for evidence of predisposing genetic factors. They show an association with specific haplotypes in HLA and in the factor H gene cluster and, remarkably, that almost all anti-factor H positive patients are homozygous for the deletion CFH3-CFH1 in the factor H gene cluster (found in about 2% of normal controls and 10% of all aHUS patients). The data demonstrate that these genetic features predispose to the development of the anti-factor H autoantibodies.

The association of the CFH3-CFH1 deletion with anti-factor H autoantibodies was independently replicated in a separate patient cohort by Skerka et al. They also showed that the autoantibodies bound SCRs 19/20 of factor H and inhibited the binding of factor H to surfaces.

Mutations in CD46 have more recently been associated with aHUS. More than 20 different mutations have now been described, most of which cause decreased expression of CD46 on cells. Fang et al. described a case of aHUS in a young girl who had heterozygous mutations in factor I and CD46, and a second, unrelated case with the same mutation in CD46 and a different mutation in factor I, both heterozygous. The functional defect in this mutant CD46 (and for the same mutation found in 6 aHUS cases) was only revealed when tested in a cell-based assay; this showed that both mutant proteins were poor regulators of the alternative pathway.

Gain-of-function mutations in factor B that create a more stable C3 convertase were recently reported in aHUS. Here, Fremeaux-Bacchi et al. described similar mutations in C3 that again create a more stable enzyme. They identified a total of 9 mutations in 14 patients. Four of the mutations influenced the capacity to interact with CD46. The functional basis for the effects of the other mutant proteins is under investigation.

Goicoechea de Jorge reviewed the current status of the Spanish aHUS registry, comprising 98 unrelated patients. The distribution of mutations is: factor H, 16%; CD46, 12%, factor I, 5%; factor B, 2%. This leaves 64% with no defined mutation. Within the families of the aHUS patients numerous healthy carriers were identified, indicating that the penetrance of most of the mutations was around 50-60%. Patients tended to have additional risk factors absent in healthy carriers, suggesting that multiple hits are needed for disease to manifest.

Factor H deficiency is associated with MPGN II; however, deficiency of factor I, which also causes uncontrolled complement activation, is not. To explore the reasons for this dichotomy, Rose et al. generated factor I deficient mice. The mice were hypocomplementaemic and had C3 deposed in the kidney; however, no MPGN-like changes were seen. To test whether factor H was preventing damage, mice deficient in both factor I and factor H were tested; again, no MPGN-like
pathology was seen. The data show that the presence of factor I is necessary for the development of MPGN and suggest that C3 cleavage products generated by factor I are critical for the development of this pathology.

The association noted above of a common polymorphism in factor H with AMD raises the prospect of using polymorphic status as a predictor of disease risk. To this end, Tortajada and colleagues have generated monoclonal antibodies that discriminate the H402 and Y402 variants of factor H, enabling the polymorphic status to be assigned and the two variants quantified in a simple assay from serum. An unanticipated outcome was that these assays enabled the identification in aHUS patients of low expression alleles that may be independent risk factors for the disease.

Session 6; Complement and infection

The principle role of complement is in defence against bacterial infection. Brekke et al. used the whole blood system described above to investigate the contribution of complement to leukocyte activation in the presence of bacteria. They found that complement activation products, particularly C5a, collaborate with bacterial products, principally LPS, to induce leukocyte activation and inflammation. Blocking the LPS receptor (CD14) and the C5a receptor markedly inhibited activation in the model.

Numerous pathogens bind factor H in order to resist killing by complement. Work by Hallstrom et al. adds another, Haemophilus influenzae. The binding sites in factor H were localised to SCR6-7 and 18-20 and binding conferred serum resistance on the bacterium.

Pathogenic fungi have also been shown to acquire factor H as part of their defence against complement attack. Vogl et al. investigated whether moulds of the genus Aspergillus, important pathogens in immunocompromised patients, acquired factor H, factor H-like 1 or C4 binding protein to protect from complement. All three fluid-phase inhibitors bound strongly and this binding reduced the susceptibility of the organism to opsonisation and killing by complement. The factor H binding moiety on Aspergillus was identified as CaHGT-1, the high affinity glucose transporter.

Staphylococcus is a skin commensal and opportunistic pathogen. Multiple complement evasion molecules have been previously identified in S. aureus. Lambris showed that the C3-inhibiting secreted protein Efb-C binds the C3d portion of C3 and alters its conformation rendering it susceptible to proteolytic degradation. A second S. aureus product, Ehp, also binds C3d in a similar manner but more tightly, and directly blocked X3 cleavage and formation of C3b.

Malaria is associated with red cell haemolysis in vivo, a consequence of rupture of infected red cells. Taylor tested whether non-infected red cells might also be prone to lysis in malaria, a consequence of binding haematin. Red cells coated with haematin activated the alternative pathway of complement but this activation was dependent on the presence of CR1 and therefore selected “younger” red cells. The data suggest that complement inhibition might protect from the severe anaemia that often accompanies malaria infections.
Session 7; Complement structure-function.

Structural analyses have in recent years revolutionised our understanding of the mechanisms of complement activation and regulation and this revolution continues. Allen and colleagues used point mutagenesis to study the binding between the relevant domains in CR1 and C3b/C4b. Residues to be mutated were chosen based upon structural predictions. The data were used to build a model of the binding events which predicted a major conformational change in C3b, and a large contact area between the binding partners.

CR2 binds multiple ligands, including the C3d fragment and the Epstein-Barr virus (EBV). CR2 binding is essential for EBV entry into B cells. The binding moiety in EBV is the gp350 protein and this interacts with a site in the N-terminal two SCRs of CR2. Hannan et al. undertook structure-guided mutagenesis through the putative binding areas identified in previous studies and/or part of the epitope recognised by a blocking monoclonal antibody against gp350. They identified a large binding surface in contiguous areas of SCR1-2 and created a molecular docking model for the interaction.

Lambris and colleagues presented an overview of progress in the development of the complement inhibitor compstatin, including a new structure of compstatin complexed with C3c. The structure demonstrates that compstatin undergoes conformational rearrangement to bind a unique site in C3. Inhibition of C3 cleavage then occurs by steric hindrance.

The major complement regulatory activities of factor H reside in the first four SCRs. Hocking et al. have examined the structure of this region by expressing pairs of SCRs, analysing these by NMR and rebuilding to obtain the complete structure. They have then examined the structural effects disease-associated variations in this region of factor H. The V62I polymorphism protective in multiple AMD cohorts caused only minor, local structural perturbations. The R53H mutation, linked with renal disease, also caused only minor local perturbations but did reduce the thermal stability of the expressed domains.

The AMD associated factor H polymorphism is in SCR7. Prosser et al. set out to generate crystals of factor H SCR6-8 containing either Y or H at position 402. In the presence of a sugar ligand, crystals of the H402 variant were successfully produced, but no crystals of the Y402 variant formed. The data suggest that the H402 residue creates a sugar-binding site that stabilises the crystal structure. Whether this differential sugar binding is involved in the well-documented AMD association remains to be determined.

C1 inhibitor is the sole plasma regulator of C1 enzymatic activity; deficiency causes the disease hereditary angioedema. C1 inhibitor is a member of the SERPIN family of protease inhibitors; structures for many SERPINs have been reported. Beirnhor and colleagues have now succeeded in crystallising C1 inhibitor. The structure held some surprises. The reactive centre was much more constrained compared to other SERPINs and it had a unique latent structure. There was marked polarisation of charged residues in the molecule that might explain the mechanism of heparin activation of C1 inhibitor.
Garlatti et al. crystallised the fibrinogen domain of M-ficolin in the presence of sugar ligands. Crystallisation was pH sensitive, shown to be due to a pH dependence of ligand binding. Binding of sialic acid was possible because of a large binding pocket into which it fitted. The disruption of the binding site with changes in pH might enable the molecule to release ligand and recycle after internalisation into lysosomes.

Like MBL, L-ficolin and H-ficolin bind the MASP proteins. Thielens et al. performed mutagenesis studies to identify the MASP binding sites in the ficolins. In each, a key Lys residue is essential for binding MASPs, a situation analogous to that found in MBL.

Session 8: Novel roles of complement.

It is astonishing that new roles of complement proteins, regulators and receptors continue to be discovered on a regular basis! Bulla et al. showed that C1q is abundant on the invading edge of the trophoblast. The trophoblast cells make C1q which then binds locally to extracellular matrix. The bound C1q appears to aid trophoblast cell adhesion and migration.

There are five factor H related proteins encoded in the factor H gene locus. Factor H-related protein 4 is present in plasma but its functional significance is unknown. Mihlan et al. showed that both isoforms of the protein bind C3b and also binds native pentameric C-reactive protein (CRP). A role in recruiting CRP to the surface of necrotic cells to aid clearance is suggested.

Multiple receptors for C3a and C5a have been described. The receptor C5L2 binds both C3a and C5a and their des-Arg metabolites and were first described as non-signalling decoy receptors. However, contradictory data has been published suggesting that C5L2 does signal. Monk et al. reported that C5L2 did not signal in response to C5a. In common with other decoy receptors, C5L2 is abundant in intracellular stores in expressing cells and undergoes constitutive recycling. This controversy will likely continue!

A new role for complement in causing the renal tubular injury that causes proteinuria was proposed by Tang et al. Tubuloendothelial cells in culture activated complement spontaneously and this induces an endothelial-mesothelial transition – a pro-fibrotic change in the cells. The effect is mediated through C3a and C5a and can be blocked using specific antagonists.

Paul Morgan
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