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**Complex beauty complements membrane attack**

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The membrane attack complex (MAC), the final product of complement activation, is a fundamental component of immune defense that drills holes in target cell membranes to cause cytolysis, cell activation and pathogen killing. MAC lesions were first visualized by electron microscopy on complement-lysed erythrocyte membranes in 1964 yet, more than half a century later, critical details of its structure and assembly mechanism remain undiscovered. The demonstration in the 1980s that C9 spontaneously polymerized to form rings that resembled the MAC led to the established dogma that the MAC pore comprised a homogeneous, closed ring of C9 molecules with precursor proteins C5b-8 displaced peripherally. Here we use electron cryo-microscopy to visualize at sub-nanometer resolution the MAC pore assembled from purified complement proteins in lipid bilayers. We determined the protein composition of the MAC pore and identified interaction interfaces between individual components that hold the assembly together. The barrel of the pore comprised 22 subunits, 18 of the barrel “staves” provided by C9 and one each by C8 $\alpha$ , C8 $\beta$ , C7 and C6. Whereas C9 “staves” completely penetrated the membrane, precursor protein “staves” were shorter and penetrated incompletely, leading to pore asymmetry and likely increased membrane perturbation. Unexpectedly, the MAC was not a closed ring but instead displayed a “split-washer” conformation, unique among related pore-forming proteins. The images also provide clues to how this unique pore assembles as they capture three distinct structural transitions along the path to MAC formation that demonstrate how the pore  $\beta$ -barrel closes. The MAC asymmetric pore and “split-washer” shape suggest a killing mechanism that involves not only membrane rupture, but also distortion. Asymmetry and incomplete membrane penetration may also explain lipid selectivity and mechanisms of MAC signaling and elimination.

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**C1 assembly is revealed by mass spectrometry and electron microscopy**

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The complement system is necessary for the immune system's functioning, particularly for the destruction of infectious agents. Therefore, it is crucial to understand the molecular details of the

initial steps of complement activation. The classical pathway of complement system is initiated by a large macromolecular complex called C1. C1 is a 790-kDa complex, which consists of 6 recognition proteins C1q and a hetero-tetramer of serine proteases, C1r<sub>2</sub>C1s<sub>2</sub>. In serum, free C1r<sub>2</sub>C1s<sub>2</sub> hetero-tetramer has an elongated structure and changes its conformation to a more compact shape once it binds to C1q. The proteolytic cascade of complement is then triggered when C1 binds to antibody-antigen complexes on target surfaces.

Here we address the following questions. How do C1r<sub>2</sub>C1s<sub>2</sub> and C1q assemble into C1? What determines the (auto-)activation of C1? What is the structure of C1 bound to antibodies? We have expressed a variety of inactive and active C1r and C1s constructs; and, studied assembly, activity and structure of complete C1 bound to hexameric antibodies. We present EM and MS data, which provide new insights into the dynamic assembly and activation of the C1 complex and structural details of C1 bound to activating antibodies.

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**Imaging complement activation step-by-step on liposomes by phase-plate cryo-electron tomography**

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Very recent advances in cryo-electron microscopy facilitate imaging of proteins and protein complexes in complex biological samples at nm-scale resolution. We applied the latest technology, i.e. a Volta phase plate mounted on a Titan Krios with Falcon II detector that markedly increases the contrast in the tomographic images, to study the cascade of molecular events in antibody-mediated complement activation on lipid bilayers. To visualize the process step-by-step, we mixed DNP-carrying liposomes with polyclonal anti-DNP antibodies and sera depleted of single complement components for 20 min prior to cryo-fixation. For each step a series of tilted projection images were acquired and reconstructed into 3D tomograms. We will highlight the advantages and limitations of this new method and present 3D rendered movies to represent the comprehensive structural data on the molecular cascade of complement activation on a lipid bilayer surface.

The 3D tomograms revealed antibody layers bound to the vesicles, C1 bound to antibody platforms with a marked improvement in contrast and resolution compared to previous work by Diebolder et al. Science 2014, 343, 1261–1263. The majority of cases had C1r<sub>2</sub>s<sub>2</sub> bound to C1q and only ~1% showed ‘empty’ C1q. Densities connected to the C1r<sub>2</sub>s<sub>2</sub> N-terminal platforms protruding from the complex likely indicate the connected serine protease domains. In C2-depleted sera, these protruding densities were observed occasionally to contact densities, larger than antibodies, on the membrane layer, possibly representing transient interactions between C1 and C4b molecules. In the next steps, deposition of C4b and C3b was apparent, but individual interpretation of molecules was limited. Finally, formation of MAC pores was unambiguous in the tomograms. Formation of single and multimeric pores were observed, similar to observations made by Sharp et al. Cell Reports 2016, 15, 1–8 using purified C5b6, C7, C8 and C9

components. Moreover, the data contained instances where large densities (projected into the soluble phase) were associated with membrane-embedded MAC pores, strongly suggesting the growth and dissociation of sMAC. These observations imply that multiple C5b8-9 complexes may arise locally, yielding sMAC and multimeric MAC pores. These observations exemplify the potential of this new imaging method to study the complex process of complement on a target surface.

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### C3a and C5a control central and peripheral circadian rhythms

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The complement system is an ancient part of innate immunity that regulates tissue homeostasis and protects the host from a hostile microbial environment. Biological clocks are an ancient system that control the circadian rhythm of organism and their cellular compartments. The suprachiasmatic nucleus (SCN) within the hypothalamus serves as a central master clock to coordinate all peripheral body clocks. Here, we demonstrate that complement and body clocks are intertwined. More specifically, we demonstrate that C3a and C5a regulate central and peripheral clock functions. We subjected C3aR<sup>-/-</sup>/C5aR1<sup>-/-</sup> (dKO) and wild type (wt) mice to a running wheel experiment. Mice were kept in 12 h/12 h light/dark (L/D), then 12 h/12 h LL and 12 h/12 h DD conditions over several days. Surprisingly, we observed in DD or LL conditions that combined C3aR and C5aR1 deficiency extended the circadian cycle by 2 h demonstrating a marked impact of the anaphylatoxins on the central clock.

To determine whether the C3aR/C5aR1 signaling also affects peripheral clocks, we isolated fibroblasts from skin of wt and C3aR, C5aR1 single and double mutant mice. We observed that only dKO cells suffered from a lengthened period when compared to wt cells ( $p < 0.001$ ). Further, we isolated lung cells from wt and C5aR1<sup>-/-</sup> mice every 6 h during a 24 h cycle. We determined the numbers of lung conventional dendritic cells (cDC) and macrophages by flow cytometry using CD11c, SiglecF, MHCII, CD11b, CD103 and CD64 cell surface markers. At steady state, the numbers of pulmonary macrophages and CD11b<sup>+</sup> cDCs oscillated in C5a-dependent manner. In wt mice, the macrophage numbers peaked at Circadian Time (CT) 15 (CT 0 defines the beginning of sleeping phase in mice during total dark conditions, where normally the light was turned on in a 12 h L/D cycle) and reached their minimum at CT9 ( $p < 0.05$ ). Intriguingly, such oscillations were absent in C5aR1<sup>-/-</sup> mice. Cell numbers of C5aR1<sup>-/-</sup> CD11b<sup>+</sup> cDCs peaked at CT9 and declined at CT15 ( $p < 0.01$ ), while wt mice had their maximum already at CT3 ( $p < 0.05$ ).

In summary, our findings strongly suggest that complement not only has a strong impact on the SCN-driven central clock, but also controls peripheral clocks at the cellular level. In the lung, the C5a/C5aR1 axis controls the circadian fluctuation of macrophages and CD11b<sup>+</sup> cDC levels. These data suggest that complement

may modulate inflammatory, metabolic and neurological disease through its impact on central and peripheral clocks.

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### The C3-like molecule CD109 controls Th1 versus Th17 induction in CD4<sup>+</sup> T cells



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The GPI-linked protein CD109 belongs structurally to the C3/C4/C5/α-2-Macroglobulin family. CD109 expression has been reported on keratinocytes, and on endothelial, stem and activated T cells. CD109 negatively regulates TGF-β responses, via mediating TGF-β receptor internalization and while increased expression of CD109 has been associated with several epithelial cell cancers, reduced CD109 levels may contribute to autoimmunity, particularly the IL-17-driven disease psoriasis. Because we noticed that CD109 is among the most strongly induced genes during TCR and CD46-driven Th1 induction, and a function for CD109 on T cells has not yet been described, we assessed the role of CD109 in human CD4<sup>+</sup> T cells.

In line with our initial gene array data, CD109 was absent from the surface of resting CD4<sup>+</sup> T cells but could be induced by CD3 + CD46 stimulation. Likewise, T cells from CD46-deficient patients failed to up-regulate CD109. CD109 is normally cleaved and released from activated T cells. When we disturbed temporal control of CD109 expression, either by addition of soluble CD109 to *in vitro* cultures or via cytotopic tethering of myristol-tailed-CD109 to T cells, we observed significantly decreased IL-10 production, a marker of Th1-cell contraction. Remarkably, IL-17, a cytokine co-produced by pathogenic Th1 cells driving autoimmunity, was also inhibited. Mechanistically, this was due to increased expression of Suppressor of Cytokine Signaling 3 (SOCS3), a negative regulator of STAT3 activity (STAT3 is critical to both IL-10 and IL-17 expression). Conversely, addition of peptides that block CD109 function led to reduced SOCS3 and uncontrolled IL-10 and IL-17 secretion. The *in vivo* importance of CD109 in regulating the Th1-IL-10/Th17 axis was further supported by *Cd109*<sup>-/-</sup> mice. T cells from these mice showed exaggerated Stat3 activation and spontaneous Stat3-dependent IL-17 and IL-10 secretion under non-skewing conditions. These animals developed spontaneous lethal inflammatory disease when removed from controlled, specific pathogen free, housing conditions.

Thus, the complement-related molecule CD109 serves as an important and novel molecular switch on CD4<sup>+</sup> T cells: initial CD109 expression on activated T cells blocks TGF-β signalling, which suppresses IL-17 generation and allows optimal Th1 lineage induction; later, cleavage and loss of CD109 permits TGF-β signal transduction required for IL-10 induction and Th1 contraction.