cytoskeleton apposed to the plasmalemma. Here, we investigated the role of the cortical actin cytoskeleton and plasma membrane nanodomains in the control of CD36 activation in endothelial cells. Stimulation with multivalent ligands (TSP-1 and anti-CD36 IgM) resulted in the downstream phosphorylation of the Src Family kinase, Fyn. Disruption of the actin cytoskeleton or removal of cholesterol blocked this activation. To gain molecular details on the rearrangement of the receptors during TSP-1 binding, we conducted superresolution approaches (based on PhotoActivated Localization Microscopy or PALM) and quantitative spatial distribution analysis. Endothelial cell lines stably expressing CD36-PAmCherry were generated for that purpose. At steady state, CD36 receptors pre-exist in small clusters (average diameter of 100 nm), in which Fyn was also present. Upon TSP-1 binding, CD36 clusters increased in size (average diameter of 140 nm) and also became denser. The average distance between CD36 molecules in these clusters was in the range of 8 nm compare to 11 nm in the control condition. F-actin depolymerization or cholesterol depletion reduced the capacity of the ligand to induce formation of larger clusters resulting in a decreased recruitment and activation of Fyn. Our data demonstrate cooperation between cholesterol-dependent domains and the cortical actin cytoskeleton in the organization of CD36 receptors and Fyn before and during TSP-1 stimulation.

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Super-Resolution Imaging of IgE-FcεRI Stimulated with Structurally Defined Ligands

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IgE-mediated signaling via its receptor FcεRI in mast cells is of interest due to its role in allergic responses, and as a model system for immune receptor signaling. To investigate IgE-FcεRI redistribution and dynamics in response to antigen stimulation, we utilize stochastic optical reconstruction microscopy (STORM), a super-resolution imaging technique. STORM permits visualization of IgE at the membrane of live or chemically fixed cells at a resolution of about 30 nm, which allows us to examine the initial events of IgE receptor signaling at a new level of molecular detail.

Physiologically, cross-linking of IgE-FcεRI by multivalent antigen initiates the transmembrane signal, and we ask how this signaling capacity relates to the cross-linked structure of the clustered IgE-FcεRI. To investigate this question, we utilize STORM imaging in conjunction with structurally defined ligands, which correspondingly cross-link IgE with a high level of definition. We use trivalent ligands based on rigid Y-shaped scaffolds of double-stranded DNA, which have one 2,4-dinitrophenyl (DNP) hapten conjugated to each arm. The distance between DNP groups can be tuned by changing the lengths of the arms of the scaffolds. We show through STORM that changes in hapten spacing effectively change the structure of the cross-linked clusters, in terms of their average area and receptor density. To investigate more limited IgE aggregation, we also use other structurally defined ligands, including bivalent DNA-based ligands and monoclonal antibodies specific for IgE or FcεRI.

Our ongoing work focuses on how these structurally defined IgE-FcεRI clusters mediate subsequent molecular events at the plasma membrane. Using two-color super-resolution microscopy, we will characterize the recruitment of early signaling partners, including Lyn tyrosine kinase. We are also investigating whether changes to membrane lipid composition alter clustering of cross-linked IgE-FcεRI or consequent interactions with signaling partners.

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Sending Innate Immune Signals Across the Membrane: A Multiscale Simulation Approach to Toll-Like Receptor Assembly

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Toll-like receptors (TLRs) are single transmembrane-spanning proteins that sense pathogenic molecular patterns within the innate immune system. Upon activation, TLRs form homodimers or heterodimers and initiate immune response pathways. The ability of TLRs to dimerize is therefore critical to their function in responding to invading pathogens. Crystallographic structures have been solved for the ectodomains of various TLR homodimers, but high-resolution structural information is not available for the full-length proteins, or for the transmembrane (TM) regions. We performed ab-initio modelling of the TM regions of all ten human TLRs, based on secondary structural predictions and spectroscopic data. Subsequently, coarse grained (CG) molecular dynamics (MD) simulations were performed to follow assembly and homo/hetero-dimerization within a phospholipid membrane environment. Our results have been used to evaluate the stability of TLR dimers, and to identify key sequence motifs that stabilize TM interactions, helping to rationalize in vitro data. Using acceptor photobleaching FRET on live cells, it has been demonstrated that TM domains including TLR2-TLR1 and TLR2-TLR6 interact within the plasma membrane. Additionally, multiscale models of entire TLR receptors have been built to determine the link between ligand recognition, assembly and cross-membrane/downstream signaling. In particular, we have focused on TLR4, which recognizes lipopolysaccharide (LPS) from the outer membranes of Gram-negative bacteria, for which a variety of structural/biophysical and MD data are available. Modelling of mutant constructs containing variable linkers revealed the structural basis for experimentally demonstrated tight coupling between extra- and intra-cellular domains and the TM region, based on receptor stability and dimerization efficiency. These data improve our understanding of the assembly and signalling mechanisms in TLRs, and may facilitate design of ligands with specific immunomodulatory properties, paving the way for new therapeutic treatments of inflammatory diseases.

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A DNA Origami Platform for Protein Interaction Analysis

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Quantitative analysis of the interaction networks of membrane proteins is of crucial interest in cell biology and has a direct impact on our understanding of cell function. Such interaction networks are qualitatively comprehensive but lack. The formation of protein complexes in the immunological synapse, in the contact area of a T cell and an antigen-presenting cell, plays a key role in the initiation of the immune response. Despite extensive studies, the quantitative molecular details of complex formation, as well as the kinetics are still poorly understood.

Here, we use DNA origami structures as templates to immobilize proteins in specific arrays. Hereby, the origami structures serve as sequence specific binding sites for a variable number of single stranded DNA strands to which antibodies against proteins important in T-cell activation can be attached.The origami structures are presented to the cell in different ways to single out individual protein complexes in their native membrane environment, for quantifying their content, and for elucidating the mechanisms of their formation: first, the DNA origami can be immobilized on nanometre-sized carbon spots created by e-beam lithography. Second, they can be placed on a lipid bilayer surface and thereby imitate the antigen presenting cell (APC) more closely.

The development of the new platform will be exploited to address and answer central questions in T-cell immunology: the unknown composition of protein complexes in the T-cell plasma membrane, and the mechanisms of their cohesion.

Exocytosis and Endocytosis

501-Pos Board B281

Closure of Pre-Existing Ω Profiles Mediates Compensatory Endocytosis, Endocytosis Overshoot and Bulk Endocytosis

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In neurons and endocrine cells, depolarization induces exocytosis, whereas endocytosis retrieves exocytosed vesicles to maintain secretion. It is generally thought that after depolarization, endocytosis must involve three steps, the Ω-profile formation, vesicular membrane protein recruitment, and Ω-profile closure. Here we report a novel form of endocytosis that involves only one step after depolarization, the pore closure of pre-existing Ω-profiles, in neuroendocrine chromaffin cells. Pre-existing Ω-profiles were fusion-generated Ω-profiles from previous rounds of exocytosis that did not collapse, but retained vesicular membrane proteins. Their pore closure was triggered by calcium influx during subsequent depolarization and was mediated by dynamin, which generated vesicles within seconds without the need for recruiting vesicular proteins. Such closures substantially mediated compensatory endocytosis, endocytosis overshoot and bulk endocytosis as widely observed in neurons and endocrine cells. We conclude that pre-existing Ω-profile closure is a major mode of endocytosis enabling efficient vesicle retrieval in secretory cells.