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Commentary Open Access

Membrane Lipids: The Final Frontier in Cellular Signaling

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Commentary

The cell membrane is a physical barrier that separates intracellular life processes from non-living outside environment, and controls the trafficking of substances and signals at this interface. This functional boundary is mainly made of lipids and proteins. The lipid components form bilayer structural scaffold where proteins are integrated mainly *via* their hydrophobic interactions. Although ligand-receptor or protein-based cell activation has been well established, there is ever-increasing evidence indicating the existence of receptor-independent cell activations. This commentary summarizes our work on membrane lipids-mediated phagocytosis, thus highlighting the importance of membrane lipids in cell activation and signaling in general.

Phagocytosis is a cellular process through which phagocytes, such as macrophages, dendritic cells (DCs), and neutrophils engulf and digest foreign particles, external pathogens, and cellular debris [1]. These professional phagocytes reply on this process to initiate innate immune response *in vivo* to defend against infections through events of antigen presentation. Phagocytosis is commonly believed to be a receptor-mediated process. Indeed, many receptor families, e.g. Fc receptors, opsonic receptors, and receptors sensing membrane change associated with apoptosis have been identified and their signaling pathways are well delineated [1]. Such specific receptor-ligand recognition-based sensing mechanisms originated from the coevolution of host/pathogen interactions and have been well studied. However, some solid particles including those synthesized or made available after the industrial revolutions can still trigger phagocytosis and/or strong immune responses. These observations cannot be explained by conventional receptor-ligand recognition mechanism since there are no biological ligand molecules on the surface of these solid particles [2]. They also fail to meet the basic premises of the coevolution theory due to huge time gaps between the emergence of receptors and these particles. Thus, there might exist an alternative sensing mechanism that does not depend on specific receptors and is perhaps more ancient than receptor-mediated cell signaling.

Our lab first faced this question while studying the activation mechanism of monosodium urate (MSU) crystals [3]. MSU is a strong endogenous stress signal that can activate phagocytosis and induces strong inflammation. However, its activation mechanism was not known at that time mainly because its presumed cell surface cognate receptor had not been identified. Instead of searching for specific receptor for MSU, we decided to focus on membrane lipids on the cell surface. Logically, when MSU interacts with the cell membrane, membrane lipids could physically engage with the crystal as well. This may initiate cell activation through an unidentified mechanism. This hypothesis emphasizes that the initial response is mediated by lipids instead of proteins or receptors yet the downstream cytosolic events are still conventional, i.e. in the form of typical Fc receptor mediated engulfment. To test this, we probed the binding interactions between MSU and DC using atomic force microscopy (AFM)-based single-cell force spectroscopy (SCFS) [4]. We found that MSU interacted with DC rather strongly. The adhesion forces are on the order of a few Nano newtons, much larger than pure receptor-ligand interactions, which are on the order of Pico newton. Moreover, when we removed cell-surface proteins with pronase, the binding force still remained. This clearly indicates that the strong MUS-DC interaction is independent of any specific surface receptors. In addition, we observed that the binding forces between MSU and DCs increased with time, which was reminiscent of phagocytic activity. The latter involves Syk, a key kinase for immune cells activation, and PI3K. Thus, we blocked Syk or PI3K in DCs, respectively, to see if such inhibitions could eliminate the strong binding interactions. Indeed, the binding forces dropped to the basal level upon those treatments. Parallel biochemical assays also confirmed the involvement of Syk and Src kinases, the latter phosphorates ITAM (Immunoreceptor Tyrosine-based Activation Motif) motifs in plasma membrane associated signaling proteins, and ITAM then recruits Syk [5]. We also found that cytoskeleton molecule actin was involved in this binding event. Collectively, the observed binding interactions between MSU and DCs pointed to a phagocytic event. However, unlike the classic receptors, the initial phagocytic event triggered by MSU is not receptor-dependent but lipid-based. Subsequent force and imaging measurements showed that it was membrane cholesterol on DCs that specifically interacted with MSU crystal and was sorted at the contact site. Therefore, MSU could directly engage cellular membrane, particularly cholesterol, a key component of lipid rafts [6]. This lipid alteration activates Syk kinase-dependent signaling in DCs, thus initiating phagocytosis and resulting in the strong binding interaction between MSU and DCs.

Later, we used the same force-based approach to investigate the adjuvantic mechanism of alum, an approved adjuvant for use in humans [7]. Similar to MSU, alum directly engages membrane lipids (sphingomyelin and cholesterol) of DCs, resulting in lipid sorting that involves the aggregation of ITAM-containing proteins and subsequent Syk and PI3K-mediated phagocytic responses. However, alum does not enter the cell; instead it facilitates the delivery of the admixed soluble antigen across the plasma membrane. Such activated DCs, without further association with alum, present high affinity and stable binding with $CD4^+$ T cells *via* surface adhesion molecules ICAM-1 and LFA-1. Thus, we provide not only the working mechanism of alum adjuvanticity, but another piece of evidence for membrane lipid-mediated cell activation.

The work on MSU and alum demonstrates that engagement of specific lipid-species with particles leads to rearrangement of those lipids on the plasma membrane. Such lipid sorting itself is a signal that can be transduced into the cytosol. For professional phagocytes, membrane lipid sorting triggers Syk-mediated phagocytosis, a program shared with FcR-mediated phagocytosis as well and much of ITAM-based immune signaling; thus, membrane lipids, to some

extent, can function as a protein-based receptor sensing extracellular environment. There are still two pieces of information missing in this proposed scheme. First, what is the identity of the ITAM-containing protein that relay such signal to Syk? Second, how does the signal transduce across the cell membrane?

To answer these questions, we recently began to search for the cryptic ITAM-containing protein *via* a bioinformatics approach [8]. We used a loosely defined ITAM motif sequence as the probe to search the mouse genome databases. We further narrowed down the hits by taking into account that the candidate protein should be expressed relatively high in phagocytes and be able to associate with the plasma membrane. Finally, we ended up with 7 probable genes for functional analysis in phagocytosis of polystyrene (latex) beads. Among them, only Msn or Moesin, an evolutionarily conserved structural linker protein, was shown to be tightly connected with beads uptake. The Moesin knockdown cells also show a reduced phagocytic binding force probed by AFM-based SCFS. Therefore, Moesin is the ITAM-containing protein that signals in receptor-independent phagocytosis. As a structural protein, Moesin is known to bind to PIP2 in the inner leaflet of the cell membrane *via* its N-terminal FERM domain and to interact with actin through its C-terminal actin-binding domain, thus bringing actin-based cytoskeleton network underneath the plasma membrane [9]. Therefore, it is likely that the upstream signal of Moesin is PIP2. This was confirmed by the observations that PIP2 and Moesin showed high colocalization at the interface of beads/membrane interaction, and most importantly, PIP2 accumulation at the contact site was an autonomous event that was independent of any cellular enzymatic activities as shown in the assays with giant plasma membrane vesicles (GPMVs). Thus, PIP2 sorting is indeed upstream of Moesin signaling.

At this stage, the key players involved in membrane lipid-mediated cell activation for phagocytosis have been gradually identified. Upon engaging solid particles, the membrane PIP2 molecules are autonomously sorted in the inner leaflet of the cell membrane at the contact site. The resulting accumulation of PIP2 works as a docking platform for Moesin. Once Moesin binds to PIP2, it adopts an extended (active) conformation, thus exposing its ITAM-motif for phosphorylation. This further recruits Syk for downstream activations. There is still one question remained: how is the signal transduced from the outer to the inner leaflet? We have initiated a systematic search with this regard and the final answer may not be upon us for several years.

The identified PIP2-Moesin-Sky axis and classic FcR signaling share similar features for initial signaling. Both pathways need sufficient membrane ligation with particles and subsequent ITAM-motif accumulation at the cell membrane for successful cell activation. Too confirm that these two approaches are similar in nature, we converted none-phagocytes to phagocytosing cells by manually introducing a ligation pair between non-phagocytes expressing a specific chimeric receptor and solid particles coated with its interacting molecules. For example, Cos-1 cells transfected with a chimeric CD4 receptor containing intracellular Moesin ITAM domain could phagocytose the anti-CD4 antibody coated latex beads very efficiently, comparable to FcRIIA-transfected cells in phagocytosing antibody-opsonized beads. This system even worked with the chimeric CD4 receptor without Moesin ITAM domain. In this case, endogenous Moesin provided the necessary ITAM motifs since Moesin-Knock down (KD) Cos-1 cells failed to phagocytose the beads. Therefore, to some extent, PIP2-Moesin together work as a "pseudo" receptor, functionally equivalent to FcR receptor; Considering that Moesin/Syk-based signaling is evolutionarily conserved, we believe that this ancient (about 0.8 billion years ago) activation mechanism is the origin of modern (about 90 million years ago) ITAM/Syk-based FcR activation. This ancient membrane-based sensing mechanism not only coexists with modern receptor-mediated signaling but still fulfils its duty when the latter is not operational, such as in the case of non-opsonized solid particles.

Our work clearly demonstrates that lipid-sorting itself is a triggering signal that can initiate downstream protein-based signaling events on eukaryotic cell membrane. Once again the unique features of eukaryotic membrane should be emphasized. First, cholesterol and sphingomyelin are two lipid species that only exist in eukaryotic cell membrane. Second, because of these two unique lipids, eukaryotic cell membrane is heterogeneous in nature. The cell membrane domains (liquid ordered domain or lipid raft) enriched with cholesterol and sphingomyelin are more rigid and thicker than their surrounding domains (liquid disorder domain or non-lipid raft). These physical constrains alone can be a driving force for protein sorting on the cell membrane as a result of hydrophobic matching between the length of hydrophobic domain of proteins and the thickness of the membrane. In addition, chemical constrains such as the existence of specific lipid-recognition motif in the transmembrane proteins can influence its distribution as well. Thus, lipid composition and membrane protein function are tightly entangled and changes in either of them will affect the other. Understanding how lipids domain regulates receptor activation or the other way around is a key step towards a full picture of cell activation at the cell membrane.

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