Membrane rafts in immunoreceptor signaling: new doubts, new proofs?

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Immunoreceptors are believed to initiate their signaling by association with membrane rafts rich in src-family kinases and other signaling molecules. Although a recent paper casts doubt over this concept, by exposing drawbacks of a commonly used procedure to disturb rafts by cholesterol extraction, several other recent papers give further support to the concept.

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T cells are stimulated to proliferate, terminally differentiate into effector cells and execute effector functions by appropriate ligation of their T-cell receptors (TCRs). In recent years, it has become widely (although not universally, see [1]) accepted that signaling through TCRs, as well as through other immunoreceptors, such as B-cell receptors and several Fc-receptors, is dependent on interactions of the crosslinked receptors with membrane rafts.

Membrane rafts are microdomains that are enriched in specific (glyco) lipids and proteins (Box 1). Rafts selectively accumulate essential cytoplasmic signaling molecules, such as src-family kinases, which attach to the raft membrane through covalently bound fatty-acid residues. Lipid rafts are relatively resistant to solubilization by some detergents (e.g. Triton X-100) and, owing to their high lipid content, they can be conveniently purified after bulk membrane solubilization by ultracentrifugal flotation in density gradients. According to the current model, the aggregated ligated immunoreceptors merge with membrane rafts and tyrosine motifs present in cytoplasmic tails of their signaling chains (CD3, CD79, ζ-family proteins) and become exposed to the src-family kinases present in the rafts. Importantly, several other signaling components {e.g. transmembrane adapter proteins LAT (linker for activation of T cells) and PAG [phosphoprotein associated with glycosphingolipid-enriched microdomains, also known as Cbp (Csk-binding protein)], phosphatidylinositol 4,5-bisphosphate} also reside constitutively in membrane rafts and these structures, therefore, become focal points of immunoreceptor signaling [2].

Methods used to demonstrate raft involvement: difficulties of interpretation

Because raft integrity is largely dependent on the presence of cholesterol, a widely used approach to demonstrate the involvement of lipid rafts in cellular functions has been based on depletion of cholesterol using methyl-β-cyclodextrin (MβCD). This agent does not bind or insert into the membrane but extracts cholesterol from the membrane. Detergent (Triton X-100) solubilization of cells after such treatment does not produce the typical insoluble low-density complexes of glycolipids, glycolipid-anchored proteins and cytoplasmic signaling molecules. Thus, if some cellular function is affected by the MβCD treatment, it is taken as evidence for raft involvement [3,4]. In principle, the cells depleted of cholesterol by the MβCD treatment can be partially replenished but this control is usually not performed.

One disturbing but largely neglected fact has been that MβCD is evidently not specific for cholesterol present in rafts but also extracts this membrane lipid from the bulk non-raft membrane. Furthermore, MβCD treatment is complicated by the fact that cells markedly change their shape and often die following treatment. A previous study demonstrated that MβCD treatment actually extracts cholesterol from outside rafts rather than from within rafts, partly solubilizes some glycosylphosphatidylinositol (GPI)-anchored and transmembrane proteins, and releases a fraction of rafts from the cells in a vesicular form [5]. Thus, it might be difficult to determine which effects unrelated to rafts are actually caused by MβCD treatment.

It is, therefore, important that a recent paper by Pizzo *et al*. [6] sheds light on these difficulties. The authors first confirm an earlier observation that MβCD treatment does not necessarily inhibit TCR-induced tyrosine phosphorylation in T cells [4], as claimed by others [3]. More importantly, they show convincingly that the previously reported MβCD-induced inhibition of TCR-induced cytoplasmic Ca^{2+} elevation is mainly a result of a nonspecific depletion of intercellular Ca^{2+} stores, as well as owing to plasma membrane depolarization inhibiting capacitative calcium channel function. When these nonspecific effects were properly accounted for, no specific effects of MβCD on the TCR-mediated elevation of cytoplasmic Ca^{2+} concentration could be demonstrated. By contrast, the elevation of cytoplasmic Ca^{2+} level elicited by the crosslinking of the GPI-anchored protein CD59 (an abundant protein component of T-cell membrane rafts) was completely inhibited in a specific manner. Interestingly, another recent study concluded that MβCD treatment effectively inhibited the activation of rat mast cells through another GPI-anchored glycoprotein, Thy-1, however, activation through FcεRI was not affected [7]. Pizzo *et al.* conclude that, although signaling through GPI-anchored proteins requires raft integrity, the TCR-induced signaling, or at least its initiation, occurs independently of the TCR–raft interaction. This is certainly a radical claim that would markedly change the prevailing view of the basic principles underlying immunoreceptor signaling.

A major undeniable point of the Pizzo *et al.* paper is that we should be much more careful when making conclusions from experiments based on MβCD or similar treatments because these reagents have serious side-effects unrelated to raft disruption. However, it should be noted that disruption of rafts by MβCD can currently be demonstrated only by loss of their detergent resistance; and owing to their small size, rafts cannot be directly observed on the intact cell surface by currently available microscopic techniques. Therefore, it can be speculated that the MβCD treatment disturbs the native structure of rafts only partially, in such a way that they lose GPI-anchored molecules, such as CD59 or Thy-1 and become more susceptible to Triton X-100; however, the 'disturbed rafts' might still be

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structurally and functionally intact enough to support TCR signaling. It will be important to check whether the buoyant rafts are preserved when the MβCD-treated cells are solubilized by milder detergents, such as Brij-98 [8].

Other raft-modifying treatments

Other potentially raft-modifying treatments do exist. One is based on cell treatment with cholesterol oxidase, which disrupts rafts by chemical modification of cholesterol; this treatment does affect TCR signaling [8]. Another relies on biosynthetic replacement of a fraction of saturated fatty acids in membrane lipids by polyunsaturated fatty acids. Such a treatment of T cells led to a markedly diminished Ca^{2+} response to stimulation by either TCR (CD3) or GPI-anchored CD59, mainly as a result of displacement of the crucial adapter protein LAT from the rafts [9]. However, Pizzo *et al.* [6] argue (based on previously published evidence) that these treatments might also produce nonspecific effects unrelated to the disturbing of rafts, presumably because of unavoidable targeting of lipids in the non-raft bulk membrane as well as in the membrane rafts themselves. It should probably be admitted that no reliable methods exist for disrupting membrane rafts in a highly specific manner.

Raft involvement in TCR signaling: conflicting results

On the basis of their other recent studies [10], Pizzo *et al.* [6] further suggest that membrane rafts might be more important as amplifiers of later phases of TCR-mediated stimulation. Interestingly, these 'costimulatory rafts' containing the GM_1 ganglioside appear to originate mainly from poorly defined cytoplasmic vesicles recruited to the patches of aggregated TCRs [10]. However, this concept remains controversial and requires further analysis. Nevertheless, it should be emphasized that the current model implicating membrane rafts in early phases of immunoreceptor signaling is not solely based on the experiments involving cholesterol depletion by MβCD or by other treatments. It has been clearly demonstrated that palmitoylation of crucial signaling proteins (e.g. Lck, LAT), which clearly targets them to the rafts, is indispensable for their normal function [11,12]; moreover, artificial targeting of a protein tyrosine phosphatase, SH2-domain-containing phosphotyrosine phosphatase 1 (SHP-1), into T-cell rafts blocks early signaling events elicited by crosslinking of CD3 [13]. In addition, the previously demonstrated effects of MβCD treatment are not limited to the presently questioned Ca^{2+} response; several previous studies demonstrated inhibitory effects on tyrosine phosphorylation [3] or production of inositol trisphosphate and diacylglycerol [4,14] (i.e. reactions placed upstream of the Ca^{2+} release). The discrepancies might be explained by differences in the MβCD concentrations used, the duration of the treatment or other experimental details. Furthermore, stimulation by anti-CD3 antibody might in some respects be significantly different from the physiological stimulation of normal T cells because it does not involve the CD4 and/or CD8 coreceptors present constitutively in the rafts.

Box 1. Basic features of membrane rafts and their interactions with immunoreceptors

Membrane rafts, also known as glycosphingolipid-enriched microdomains (GEMs), are submicroscopic membrane areas enriched, as compared with the rest of the membrane, in sphingolipids (sphingomyelin and glycosphingolipids) and cholesterol. These structures are held together mainly by hydrophobic interactions between long, saturated fatty-acid residues present preferentially in the sphingolipids. These, in the presence of optimal amounts of cholesterol, form a specific 'ordered liquid phase' distinguished from the 'less ordered' rest of the membrane composed mainly of lipids possessing polyunsaturated fatty acids [a]. Owing to the tight lipid packing, the lipid rafts are, especially at low temperatures, relatively resistant to solubilization by some detergents commonly used for membrane solubilization, such as Triton X-100; by contrast, the lipid rafts are readily solubilized in other detergents, such as octylglucoside or sodium dodecyl sulfate (SDS). Most transmembrane proteins are excluded from the rafts, exceptions being mostly palmitoylated molecules, such as the coreceptors CD4 and CD8, adhesion receptor CD44, several members of the tumour necrosis factor (TNF) receptor family, as well as transmembrane adaptor proteins LAT (linker for activation of T cells) and PAG [phosphoprotein associated with GEMs, also known as Cbp (Csk-binding protein)]. By contrast, membrane rafts accumulate extracellularly orientated proteins anchored in the membrane through a glycolipid moiety (glycosylphosphatidylinositol; GPI), such as Thy-1, Ly-6, CD14, CD55 and CD59. In addition, several lipid-modified cytoplasmic molecules, such as src-family kinases, heterotrimeric and small G-proteins, are characteristic raft components. Owing to their high lipid content, these insoluble complexes can be easily purified from a detergent-solubilized membrane by density gradient ultracentrifugation [b]. Several types of membrane rafts probably exist in a single cell, differing in their lipid and protein composition.

Lipid rafts are too small to be directly observed by light microscopy, although some advanced techniques are able to reconstruct their size and shapes on the cell surface [c,d]. It is not clear to what extent the preparations obtained from detergent-solubilized cells correspond to the native rafts. It seems probable that 'elementary rafts' are quite small (diameter <10 nm) and contain very few (perhaps even single and some none at all) protein molecules surrounded by a 'shell' of ~100 of the specific lipid molecules [e]. These units might easily coalesce into larger patches, especially after membrane exposure to certain types of detergents or after crosslinking of their protein or glycolipid components by antibodies or natural multivalent ligands.

Owing to the specific lipid environment and presence of important signaling molecules, membrane rafts have been recently implicated in: (1) signaling through a wide range of receptors, including immunoreceptors; (2) antigen presentation; (3) cell interactions with pathogens and bacterial toxins; (4) budding of viruses from the host-cell membrane; (5) the pathogenesis of prion and other neurodegenerative diseases; (6) specific forms of endocytosis and vesicle trafficking; and (7) establishing cell polarity [b]. Recently, it has appeared as if almost all important membrane processes involve lipid rafts.

Although the concept of membrane rafts neatly explains several biological phenomena, many important basic issues concerning their native composition, dynamics, heterogeneity and functional importance still remain unsolved.

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Although the results of Pizzo *et al.* [6] might cast doubt over the concept of raft involvement in the early phases of TCR signaling, other recent papers provide remarkable additional evidence indicating that the model is basically correct. TCR (CD3) mutants that either lack CD3δ or in which the αβTCR fails to associate with CD3δ owing to a mutation in the TCR α chain do not properly associate with membrane rafts following TCR ligation, and have severe defects in downstream signaling events [15,16]. Recently it was demonstrated that this effect of CD3δ is a result of its surprisingly robust interaction with the CD4 and CD8 coreceptors constitutively present in membrane rafts [17]. This interaction, therefore, couples TCRs to the rafts. Constitutive association of a fraction of TCRs with membrane rafts was demonstrated in another recent study, in which a mild detergent (Brij-98) preserved this association, even at 37°C [8]. This finding is important because it largely removes the objection that the detergent-resistant rafts obtained by solubilization at low temperatures and their associations with immunoreceptors could arise artificially as a result of lipid phase transitions induced by the low temperature.

Concluding remarks

The present controversy reminds us that there is an urgent need for more information on the nature and properties of membrane rafts and their physiological roles (Box 1). Among the unclear issues are not only the size, composition and dynamics of native rafts in different cell types but also their heterogeneity: it is becoming more and more clear that several, or perhaps many, types of rafts exist in a single cell differing in their lipid and protein composition [18,19]. The standard methods of raft isolation based on density gradient ultracentrifugation of detergent-solubilized membranes obviously produce a mixture of such rafts that are difficult to separate further. It is even possible that 'heavy rafts' resistant to certain detergents exist, with a much higher protein:lipid ratio; these would go largely undetected by the currently used methods because they would be lost in the bottom fractions of the gradient containing the large excess of fully solubilized membrane proteins. The field would enormously benefit from the development of new microscopic techniques for direct visualization of rafts. In addition, the development of mutant lymphoid cell lines devoid of any rafts would be very helpful. Indeed, T cells of the recently described knockout mice lacking acidic sphingomyelinase were devoid of conventionally defined (i.e. Triton X-100-resistant) membrane rafts and have serious defects in TCR-mediated activation [20].

Rafting evidently continues to be an exciting activity in molecular immunology and in cell biology in general. It is healthy that some investigators have the courage to critically examine even the widely accepted approaches used in the field

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