Review

Lipid Rafts & Co.: An integrated model of membrane organization in T cell activation

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Abstract

The model of membrane compartmentalization by self-organizing functional lipid microdomains, named lipid rafts, has been a fruitful concept resulting in great progress in understanding T cell signal transduction. However, due to recent results it has become clear that lipid rafts describe only one out of several membrane organizing principles crucial for T cell activation besides fences and pickets and protein–protein interactions that take part in the formation of the immunological synapse as a highly organized structure at the T cell contact site to the antigen-presenting cell. This review describes the concepts of lipid rafts and other membrane organizing principles to evolve a novel integrated model on the functional role of microdomains in immunological synapse formation and T cell activation. Further research has to elucidate the relative contribution and interrelation of different modes of membrane organization in productive T cell activation.

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Keywords: Membrane microdomains; Immunological synapse; Single molecule tracking; T cell signaling

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Abbreviations: APC, antigen-presenting cell; ERM, ezrin/radixin/moesin; F-actin, filamentous actin; FRAP, fluorescence recovery after photobleaching; FRET, fluorescence resonance energy transfer; GFP, green fluorescence protein; GPI, glycosylinositolphosphatidyl; ICAM, intercellular adhesion molecule; l_d, liquid-disordered; l_o, liquid-ordered; LAT, linker for activation of T cells; LFA-1, leukokocyte functional antigen-1; MHC, major histocompatibility complex; PAG, phosphoprotein associated with glycosphingolipid-enriched microdomains; PIP_2, phosphatidylinositol 4,5-biphosphate; PUFA, polyunsaturated fatty acid; (c-/p-)SMAC, (central/peripheral) supramolecular activation cluster; TCR, T cell receptor; Tm, melting temperature.

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1. Introduction

T cell recognition of antigen that is presented in context of a major histocompatability complex (MHC) is the central theme of the adaptive immune response. Understanding the transduction of signals provided by ligation of the T cell receptor (TCR) and subsequent T cell activation is of high priority for immunologists and cell biologists. It is understood that spatial organization and compartmentalization of the membrane and its signaling machinery is a prerequisite for signal transduction. The lipid raft model has evolved to describe a compartmentalization of the cell membrane that is based on phase separation of membrane lipids due to their physical properties and the specific targeting of proteins into the resulting lipid microdomains. A plethora of publications deals with the functional role of lipid rafts in several cellular functions including signaling by TCR, B cell receptors, IgE receptors, neurotrophic factors, growth factors, chemokines, interleukins, and insulin, as well as lipid and protein trafficking [1,2]. Thus, it may be surprising that despite considerable efforts of many researches no consensus on the nature and even the existence of lipid rafts has been found by now, mainly due to limitations in assessing the nature of lipid fluid phases in living cells. Consequently, lipid rafts have been named “slippery platforms” and “unidentified floating objects” to satirically highlight the serious question whether rafts are just “illusive” [3,4].

In this review, the lipid raft model, relevant open questions, and problems with this concept are discussed in the context of T cell activation. A modified understanding of lipid rafts and up-to-date knowledge of membrane organization in general are integrated into a current model of membrane compartmentalization that is crucial for initiation of T cell signaling. In addition, the formation of an immunological synapse between antigen-presenting cells and T cells has emerged to be of vital importance for T cell activation. In this review we evolve an integrated up-to-date model for the membrane-associated mechanisms of T cell activation.

2. The lipid raft model

2.1. Phase separation in biological membranes

Cellular membranes contain glycerophospholipids, consisting of a glycerol backbone to which two fatty acids are linked via ester bonds. In addition, eukaryotic membranes, particularly the plasma membrane, contain sphingolipids (sphingomyelin and glycosphingolipids) and sterols, in vertebrates mainly cholesterol. The structures of these lipids determine the physical properties of the lipid bilayer. Whereas glycerophospholipids usually contain one unsaturated acyl residue at the sn2 position, sphingolipids comprise a long-chain (16–26 carbons) sphingoid base with an amide-linked usually saturated acyl chain. The ceramide backbone facilitates hydrogen bonds preferably with cholesterol [5]. Sterols are rigid structures comprising four carbon rings.

The relative abundance of these lipids within the plasma membrane varies between different cell types. Whereas glycerophospholipids usually predominate the plasma membrane lipid composition, sphingomyelin and cholesterol are present at 10–20 mol% and 30–40 mol%, respectively. Glycosphingolipids usually represent a low proportion of membrane lipids with some exceptions such as cells in barrier epithelia [6,7].
Sphingolipids are generally located exclusively in the outer (exoplasmic) leaflet of the plasma membrane bilayer. In contrast, distinct glycerophospholipids such as phosphatidylinositol, phosphatidylserine and also phosphatidylethanolamine largely prefer the inner (cytoplasmic) leaflet [8], whereas cholesterol is probably abundant in both leaflets.

The majority of information on the physical properties of membranes comes from model bilayers. Above their melting temperature (Tm), phospholipid bilayers exist in a fluid (liquid) state, below Tm a solid (gel) state exists that is of no physiological relevance in mammals. The cis double bonds present in the unsaturated acyl chains of phospholipids reduce their Tm by untightening the organization of the acyl chains and enable a Tm at subphysiological temperatures. Conversely, high concentrations of cholesterol mediate a compact and thickened bilayer structure [9] forming a liquid-ordered (lo) state. Of note, the higher order of the lo state mediated by cholesterol compared to the liquid-disordered (ld) state has little effect on the rate of lateral diffusion of proteins and lipids in the plane of the bilayer [10,11]. Also sphingolipids promote formation of the lo state due to the higher saturation of their acyl residues and their capacity to form hydrogen bonds [12]. As a starting point for the development of the lipid raft model, experiments indicated a coexistence of both lipid fluid states within one bilayer [13,14].

The lipid raft model is based on the assumption that, similar to model membranes, cholesterol and sphingolipids are not distributed evenly in the plasma membrane as suggested by the classical fluid mosaic model [15], but rather assemble to microdomains ("rafts") in an lo state that float within the rest of the membrane that is in the ld state [16,17]. In contrast to model lipid bilayers, biological membranes contain a great variety of lipids and many proteins that may as well organize their nearest lipid environment. Thus, results obtained from model membranes are to be related to cell membranes with cautions.

The formation of lipid domains within the cell membrane facilitates a spatial sequestration of membrane proteins, depending on their affinity for the lo phase. The most common biochemical method to analyse lipid rafts is based on the partial insolubility of membranes in non-ionic detergents such as Triton X-100 at 4 °C. As a consequence, when such membrane or cell lysates are subjected to density gradient ultracentrifugation, the detergent-insoluble membranes float to low-density fractions and can be separated from soluble and non-membrane fractions. Lipids from detergent-insoluble fraction are enriched in cholesterol and sphingolipids [18] and liposomes containing a lipid composition in lo behave the same way [19] suggesting that detergent-insoluble fractions represent membrane domains in a lo phase according to the lipid raft model. As discussed below, this does not necessarily mean that detergent resistant membrane fractions represent lipid rafts. Moreover, the size of rafts in biological membranes is very difficult to determine. Together with lipid components, membrane proteins are also separated into detergent soluble and insoluble fractions whereby proteins found in detergent-insoluble fractions fulfill specific roles in cellular processes, particularly in cell signaling, suggesting a functional role of rafts in these processes [20].

2.2. Compartmentalization of membrane proteins

Several mechanisms target proteins to detergent insoluble membrane domains. Signals targeting proteins to rafts can be divided into lipid modifications and protein-based signals. Glycosylinositolphosphatidyl (GPI)-anchored proteins are generally targeted to lipid rafts and have frequently been used as lipid raft markers [21]. GPI anchors consist of a phosphatidylinositol typically containing two long-chain acyl moieties [22] that insert into the exoplasmic leaflet of the membrane and a head group linked via an amide bond to the C-terminal residue of the protein, which usually has no other direct connection to the membrane. The second type of lipid modification that targets proteins to lipid rafts is acylation such as myristoylation and palmitoylation [23,24]. In general, two acyl moieties target proteins to lipid rafts, independent of whether a protein spans the membrane or is linked to the membrane merely by the lipid anchor [25–29]. Prenylated proteins generally do not associate with rafts. Ras proteins may be prenylated and additionally palmitoylated allowing differential targeting to raft and non-raft fractions due to variations of lipid modifications [27,30,31]. Of note, acylated proteins are attached to the inner leaflet of the cell membrane, in contrast to GPI-anchored proteins, which are located at the cell surface. Also protein-based interactions with membrane lipids or other raft proteins may target proteins to rafts as discovered for the transmembrane protein influenza virus haemagglutinin [32], and for cytosolic proteins containing domains that associate with raft residing flotillin [33]. The fact that
the $l_o$ phase of a lipid bilayer is thicker than the $l_d$ phase forms the basis for the possibility of raft targeting of transmembrane proteins based on the length of their transmembrane domain [34].

Hence there are several mechanisms that target proteins to rafts enabling a spatial organization of membrane proteins that preferentially reside in, or are excluded from rafts. In consequence, confined zones with specialized functions due to particular protein composition are created. Moreover, a fine-tuning of protein affinity for distinct lipid phases may be achieved by interactions between lipid- and protein-based targeting, as it is suggested by experiments with acylated green fluorescence protein molecules [35] and has recently been shown for the linker for activation of T cells (LAT) [36], an important T cell signaling molecule (see below). Also variations within one type of raft-targeting mechanisms are possible. Such variations include different acyl moieties of GPI-anchors [37] or different types and numbers of acyl moieties attached to proteins. Protein targeting to specific lipid environments may be a basis for lipid raft heterogeneity. Raft heterogeneity is indicated by experimental data, underlies recent models for lipid raft functions in cell signaling (see below), and could not only include the existence of different subsets of rafts, but also zones within one raft [35].

Strikingly, many cytosolic proteins that are linked to the membrane by lipid anchors are crucial signaling mediators. Accordingly, Src family kinases such as Lck and Fyn [38] and GTPases such as H-Ras [39] are enriched in rafts. In contrast, most transmembrane proteins, e.g. the phosphatase CD45, are generally excluded from rafts unless they are acylated such as the TCR coreceptors CD4 and CD8b [40] and adaptor proteins LAT [25], phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG, also named Cbp) [41,42], and Lck-interacting molecule (LIME) [43], all of which are also important molecules of the T cell signaling machinery.

Because GPI-anchored proteins and glycolipids are typical raft constituents, the previously puzzling finding that cross linking of such molecules, which have no direct contact to the inner side of the cell membrane, induces cytoplasmic signaling and cellular activation events [44,45] was interpreted as a strong indication for a functional role of rafts in T cell signal transduction. As an explanation for this phenomenon it was assumed that cross-linking of raft constituents at the outer membrane leaflet would concentrate the components of the signaling machinery at the inner leaflet and thus juxtapose enzymes and its substrate sufficient for initiation of the observed signaling [46]. This common opinion has to be reconsidered taking into account recent results showing that cross-linking of artificial lipids that clearly do not reside in rafts induce similar signaling effects [47]. However, the lipid raft model would provide a putative mechanism for the costimulatory signal of GPI-anchored proteins and initiated studies that focused on the role of lipid rafts in TCR mediated signals.

2.3. The role of lipid rafts in the initiation of T cell signaling

Like most transmembrane proteins, components of the TCR, i.e. a clonally specific heterodimer non-covalently associated with the invariant CD3 adapter complex, are found outside of lipid rafts and thus spatially separated from raft resident molecules that mediate the cytosolic signaling processes. The most proximal TCR-induced signaling involves phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) tyrosine residues by Src family tyrosine kinases, mainly Lck. The phosphorylated ITAMs mediate recruitment of Syk family kinase ZAP-70 and its activation by Lck-dependent tyrosine phosphorylation, which in turn leads to docking and activation of other Src homology two domain (SH2)-containing molecules involved in the hydrolysis of inositol-containing phospholipids, Ca$^{2+}$ mobilization and activation of the Ras/MAPK cascade [48]. A substrate of activated ZAP-70 is LAT [25], which upon tyrosine phosphorylation is involved in the recruitment of SH2-containing molecules and thus assembly of multiple signaling molecules within a so-called “signalosome” [49].

The decisive clue for the common model how ligand binding to TCR mediates intracellular signaling was the observation that depending on the Src family kinase activity phosphorylated CD3$\zeta$ chains can be found within lipid rafts upon TCR stimulation together with phosphorylation-activated ZAP-70 kinases [50]. Hence, it appeared plausible that upon ligand binding the TCR/CD3 complex is recruited to membrane rafts and thus moves from an environment containing inhibitory phosphatases such as CD45 to confined zones with enhanced signaling activity. However, the CD45 phosphatase has recently been found to predominantly activate Lck activity by inducing conformation changes, and small amounts of CD45 are targeted to rafts as
assessed by Triton X-100 extraction. A recent interpretation of the role of CD45 is to keep Lck activation balanced by counteracting the Lck-deactivating kinase Csk [51,52].

The notion of lipid rafts as functional signaling microdomains was corroborated by experimental disintegration of lipid rafts by cholesterol depletion with methyl-β-cyclodextrin and polyene antifungal agents filipin and nystatin, which impair TCR-mediated signaling [53]. Because only phosphorylated CD3ζ is recruited to lipid rafts and, due to the raft localization of Lck, CD3 phosphorylation is supposed to occur only inside rafts, it remained unclear which mechanisms could mediate the induced translocation of TCR components to rafts. A possible solution for this problem may be the finding that TCR components can be recovered from isolated rafts constitutively and independent of Src-family kinase activity when raft were isolated at physiological temperature using Brij-98 as detergent, characterized by its relatively bulky polyoxyethylene headgroup and mono-unsaturated ether moieties [54]. Tyrosine phosphorylation has been suggested to occur upon ligand-induced conformational changes of the TCR complex [54].

Important contributions to the elucidation of functional properties of lipid rafts have come from molecules designed to localize or not in rafts. The first molecule investigated in T cells this way was Lck, the function of which was found to depend on dual acylation of its N-terminus [55]. Importantly, the localization of Lck at the membrane is not sufficient for its function, as shown with a transmembrane Lck chimera, but targeting of Lck to rafts reconstitutes Lck-induced signaling in Lck-deficient T cells [55,56]. Analogously, experiments using mutated palmitoylation sites of another crucial T cell signaling molecule, namely LAT, revealed LAT functions to be associated with partitioning of this molecules into lipid rafts [25]. Using another construct, LAT targeting to detergent insoluble membrane fractions was shown to be sufficient to maintain full early T cell signaling even when rafts are partially disintegrated, confirming the central role of LAT and its raft targeting for T cell activation [57].

The most common method for investigations on lipid rafts in living cells is disruption of lipid rafts by extraction of cholesterol with methyl-β-cyclodextrin, which in many studies leads to inhibition of several signaling events (reviewed in Ref. [1]). Another, relatively mild alteration of lipid rafts is caused by treatment of cells with polyunsaturated fatty acids (PUFAs). PUFAs are incorporated into the membrane and affect the acyl moiety composition of membrane lipids from both the inner and the outer leaflet of lipid rafts, as shown in in vitro and in vivo experiments [58–60]. Such raft alterations, probably supported by partially altered protein acylation [61], lead to displacement of proteins linked to the inner leaflet including Lck and LAT from detergent insoluble fractions, while the outer leaflet remains unaffected with respect to GPI-anchored protein and ganglioside targeting to rafts [58]. The displacement of signaling molecules from rafts directly correlates with impaired signaling [58]. Moreover, using a “PUFA-resistant” construct comprising the transmembrane domain of PAG and the functional cytoplasmic regions of LAT that remains in lipid rafts also when other molecules such as Lck are displaced by PUFA, it could be proven that particularly LAT displacement from rafts is the cause for inhibition of early TCR/CD3 mediated signals [57].

2.4. Signaling platforms and involvement of the cytoskeleton

Beyond the role of lipid rafts in the initiation of the most proximal signaling events, another function of rafts is the stabilization and amplification of signals enabled by aggregation of rafts to larger complexes accompanied by further recruitment of important signaling mediators such as LAT and associated molecules [56] as well as TCR components [62]. These raft complexes were given the sounding term “signaling platforms” [63]. Importantly, rafts and filamentous actin (F-actin) were found to be co-localizing [64] and the actin cytoskeleton drives the aggregation of lipid rafts [62,65,66]. Moreover, the cytoskeleton-driven movement of membrane molecules leads to an increase of the overall amplitude and duration of T cell signaling [67]. Such molecular movements induce an accumulation of molecules at the interface of the T cell and the antigen-presenting cell (APC) [67], underlying the concept of the immunological synapse (see below). Conversely to the function of the cytoskeleton in driving lipid raft aggregation, rafts are necessary for activation of the actin skeleton and GPI-anchored proteins provide costimulatory signals leading to reorganization of the cytoskeleton [68].

Functional links between the cytoskeleton and lipid rafts promoted the characterization of physical links between lipid raft constituents and the cytoskeletal machinery. One such interaction was shown by the finding that a proportion of CD3ζ is associated with actin and this association is augmented by TCR stimulation.
[68–70]. Also CD2, which segregates to lipid rafts upon ligation [71] is linked to the actin cytoskeleton [72]. Another interesting link could be the binding of the raft protein PAG to the cytoplasmic adapter protein EBP50 (ezrin/radixin/moesin (ERM)-binding phosphoprotein of 50 kDa) [73,74]. ERM proteins had previously been known to link actin filaments to the membrane and to be colocalized with GPI-linked surface molecules [75]. An interesting aspect for the function of these proteins in linking lipid rafts to the cytoskeleton may be the action of phosphatidylinositol 4,5-biphosphate (PIP$_2$) within lipid rafts that provides a local environment to recruit and activate ERM proteins [76]. However, recent studies question the confinement of PIP$_2$ and PIP$_2$-dependent signaling within lipid rafts [77].

3. Novel aspects demanding modifications of the raft model

3.1. Unresolved questions concerning the nature of rafts

A prerequisite for the proposed function of lipid rafts is the association of the two membrane lipid leaflets that allows lipid raft residents of the outer leaflet such as GPI-anchored proteins to colocalize with lipid raft residents of the inner leaflet such as acylated cytosolic proteins without directly spanning into the other lipid leaflet. Of note, the major raft organizing lipids besides cholesterol, the sphingolipids, are exclusively located in the outer leaflet and in a model lipid bilayer resembling the inner leaflet lipid composition cholesterol does not induce l$_o$ domains [78]. It has been proposed that the outer leaflet l$_o$ phase is able to organize the lipids of the inner leaflet as a cholesterol-rich domain. In particular, the greater length of acyl chains of sphingolipids allows them to interdigitate with lipids from the inner leaflet [20,79]. However, this interdigitation is reduced in the presence of cholesterol [79]. Also transmembrane proteins could theoretically facilitate raft formation in biological membranes, but so far no such organizing protein has been found. Hence, there is no clear explanation for the existence of the inner lipid raft leaflet. Although a recent study confirms that depending on its cholesterol content the inner leaflet is physically coupled to the outer leaflet, it raises new questions by showing that the TCR-induced clustering of rafts is independent of cholesterol [80].

Another important question concerning lipid rafts is how accurately the lipid raft model describes the distribution of membrane proteins. Of note, only a small proportion of membrane proteins are found in the detergent-insoluble raft fraction [81]. A protein is often regarded as a raft protein even if only 10–30% of this protein is found in the detergent resistant fraction, particularly in case of cytosolic or transmembrane proteins. Depending on the membrane area covered by lipid rafts, a considerable proportion of a so-called raft protein could reside in soluble membrane fractions. The still unresolved question of the relative occurrence of lipid rafts within the cell membrane could hence question the idea that distinct proteins are highly concentrated within rafts. Thus, the rather simple static lipid raft model rather insufficiently describes the situation as it probably occurs in biological membranes irrespective of additional caveats arising from commonly used methods for analysing lipid rafts.

3.2. Limitations of common methods for analysing lipid rafts

Several problems of the lipid raft model arise from the nature of the standard biochemical method for lipid raft isolation as buoyant detergent-resistant membrane fractions. First, proteins could be resistant to detergent extraction due to general biochemical properties such as hydrophobicity rather than reflecting their distribution to membrane microdomains in the living cell. Moreover, addition of non-ionic detergents such as Triton X-100 could create l$_o$ domains in a homogeneous lipid bilayer resisting further solubilization by the detergent [82]. Besides this phenomenon, a number of non-physiological rearrangements of the bilayer could occur during detergent extraction. Most importantly, lipid phase behavior is highly temperature dependent and hence the usual extraction temperature of 4 °C has raised doubts from the very beginning of the raft theory. A recent study showed that cold temperatures are indeed sufficient for raft aggregation in living T cells resulting in induction of signaling processes [83]. On the other hand, membrane microdomains containing GPI-anchored proteins together with Src family kinases can be isolated also in the absence of detergents [84] and detergent insoluble membrane microdomains can be extracted from cells at physiological temperatures when using the appropriate detergent [54].
Also optical methods are insufficient for accurate analysis of membrane microdomains. Conventional immunofluorescence microscopy of raft constituents is accomplished on fixed cells at cold temperatures and often by using bivalent antibodies, thus it bears severe caveats of artifact generation as a result of temperature- or antibody-triggered redistribution and inadequate fixation [85]. Hence, conventional immunofluorescence is hardly suitable to prove the existence of rafts and definitely not to visualize their size, which is also a matter of debate since the emergence of the raft theory. Rafts would need to have a diameter in the scale ~300 nm to become visible, but rafts of resting cells probably do not reach this size (see below). Furthermore, detergent resistance and microscopic co-localization of proteins with each other do not necessarily correlate, as shown, e.g. for acylated green fluorescence protein (GFP)-tagged model molecules [35]. Such discrepancy, however, could be explained by raft heterogeneity. Although the use of more sophisticated fluorescence resonance energy transfer (FRET) microscopy on model membranes at 37 °C revealed the possibility of raft existing under physiological conditions [86], other studies using FRET microscopy and fluorescence recovery after photobleaching (FRAP) on living cells provided results inconsistent with significant clustering of raft markers in membrane domains unless they are very unstable and/or very small in the range of few nanometers [87–89]. These results are corroborated by methods analysing single molecule movements (see below).

Effects of the extraction of cholesterol with methyl-β-cyclodextrin in living cells are frequently regarded as a confirmation that the function of various signaling proteins depends on lipid rafts. Notably, non-specific depletion of intracellular Ca^{2+} stores and plasma membrane depolarization of T cells contributes to the reported inhibition of TCR-induced Ca^{2+} influx by methyl-β-cyclodextrin treatment [90]. Also with other methods of raft disruption such as cholesterol sequestrating antibiotics (filipin, nystatin) unspecific effects apart from lipid raft alterations can hardly be excluded. Recent studies further question the assumed link between cholesterol extraction and lipid raft function. First, FRAP experiments revealed that cholesterol depletion had similar effects on the diffusional mobility of raft and non-raft proteins [88]. Second, in contrast to cholesterol depletion from membranes other than rafts, selective extraction of cholesterol from lipid rafts did not affect CD3-induced protein tyrosine-phosphorylations and oxidation of raft cholesterol did not inhibit the activation of T cells [91]. Moreover, cholesterol depletion by blocking its synthesis with lovastatin inhibits calcium response induced by GPI-anchored proteins but not that induced by CD3 [92]. PUFA-mediated inhibition of membrane-proximal T cell signaling appears to be due to lipid raft alterations at least up to the level of calcium response [57,93] but the implication of rafts for PUFA action on selective downstream signaling events [93,94] has not been proven. Thus, PUFA effects on T cells cannot be causally linked to lipid raft alterations in all instances, since PUFAs inhibit T cell activation by a variety of mechanisms [95].

Altogether, although a large number of experiments on disruption/modification of lipid rafts in living cells point towards a functional role of rafts in T cell signaling often correlating with biochemical and/or microscopic evidence of raft alterations, these experiments cannot explicitly prove the lipid raft model by definitely elucidating the physiological nature of membrane compartmentalization. Also studies using molecular constructs do not support direct information about the nature of rafts but rather give evidence for the dependency of protein function on distinct lipid modifications or targeting sequences that lead to a specific distribution as detected by density gradient centrifugation or microscopy.

3.3. Membrane compartmentalization as determined by single molecule tracking

A different approach to analyse membrane properties is the analysis of single molecule movements. This is achieved using fluorescent probes (dyes and optimized GFP variants) or by colloidal gold. Although a colloidal gold particle appears to be rather large (~40 nm), gold-labeling diffusion of membrane lipids as assessed by colloidal gold does not differ from fluorescence labeling [96]. For details on the advantages and limitations of either labeling, we refer to the excellent recent review by Kusumi et al. [97], and summarize both methods as “single molecule tracking” here.

From early studies on membrane molecule dynamics using FRAP it was known that molecules in biological membranes diffuse puzzlingly slower than in model membranes [98,99]. For a long time it remained unexplained how the diffusion coefficients of molecules are reduced by a factor of 5-50 in cell membranes. A second paradigm arose from the finding that molecular diffusion is drastically reduced by oligomerization much more than predicted by the two-dimensional continuum fluid model for lipid bilayers [100]. These observations by FRAP
were confirmed by fluorescence correlation spectroscopy [101]. Only the amazingly high time resolution (25 μs) of novel single molecule tracking techniques could provide an explanation for these phenomena [96,102]. Following the track of single membrane lipids a characteristic behaviour of these molecules was observed. For a period in the range of milliseconds (between 1 and 17 ms, depending on the cell type) the molecules diffuse in a confined area of 30–230 nm in diameter and then “hop” into the next compartment, where they are trapped for some milliseconds before the next hopping [96,102]. Thus it turned out that the previously observed slow diffusion of molecules in biological membranes is a result of an averaging of this “hop diffusion” over time or by many molecules under observation. These data correlate with previous studies on single molecule tracking of transmembrane molecules [103,104]. While compartmentalization of transmembrane proteins may easily be explained with cytoskeletal “fences” at the cytoplasmic side of the cell membrane [105], the observation that also lipids of the outer leaflet undergo hop diffusion demands the postulation of posts for such fences made up by various transmembrane proteins anchored to the actin skeleton meshwork termed “pickets” [96].

According to results from single molecule tracking experiments, lipids and proteins are trapped in confined zones between these fences and pickets, where they diffuse freely. With a distinct statistical probability, molecules break through that barrier (“hop over fences”) into the neighbouring compartment, and become again confined for a distinct time period. This model also gives a plausible explanation for the oligomerization-induced decrease of diffusion of receptor molecules: Not diffusion itself, but the probability of complexes to break through the fences-pickets barriers and thus the average diffusion rate is drastically decreased by oligomerization. From further studies, for instance tracking of two single molecules [106], new general insights into molecular dynamics during receptor ligation can be expected.

The results of single molecule tracking discussed so far came from molecules that are not regarded to be lipid raft residents. Single molecule tracking of lipid probes with saturated acyl and unsaturated chains in living cells revealed confined and predominantly unconfined diffusion, respectively, indicated a raft-dependent behaviour of saturated membrane lipids [107]. In contrast, studies analysing movements of single GPI-anchored and transmembrane molecules revealed similar behaviour of raft and non-raft proteins and could not provide evidence for a specific confinement of raft proteins [108,109]. These results were essentially confirmed by FRAP experiments under steady-state and raft disintegrating conditions indicating that raft association is not the major determinant of membrane protein diffusion [88]. In conclusion, raft molecules are highly mobile, which is in some contrast to the classic picture about lipid rafts, although, as mentioned above, the Iₐ phase may not be understood to significantly alter molecule diffusion anyway [10,11].

Detailed analysis of Lck single molecule movements revealed that after TCR stimulation at a defined spot on the cell surface Lck comes into close vicinity of the TCR complex independently of its kinase activity [110]. A TCR-induced rapid actin reorganization, as previously described [67,111], could explain this initial Lck movement. Formation of a dense mesh of fences and pickets around the TCR complex would facilitate trapping of Lck molecules. Though these cytoskeletal rearrangements could theoretically occur also in the absence of rafts, the molecular complexes within rafts are much more susceptible to minor changes in the density of the actin filaments than are monomeric molecules [110]. Hence, there may be a close functional interaction of fences and pickets and lipid rafts in mediating the vicinity of kinases and its substrates necessary for the induction of T cell signaling.

3.4. The function of rafts in initiation of TCR signaling – An integrated model

The hop diffusion model is not in discrepancy with the existence of rafts, but “reduces” them to a very small size and/or very short lifetime. Accordingly, early single molecule analyses suggested a raft size in resting cells between 50 and 200 nm [112,113]. Strikingly, it seems that the better one looks, the smaller lipid rafts appear. Thus, novel single molecule techniques and FRET-based experiments showed that clusters of GPI-anchored proteins consist of only few proteins (up to 4) and exist for a lifetime in the range of milliseconds [89,97]. It is plausible that the organization of molecules in such small clusters does not effectively interfere with the diffusion including hop diffusion within the membrane bilayer. Of note, these clusters are sensitive to cholesterol depletion [89] which may allow us to classify them as lipid rafts. A frequent exchange of molecules between different rafts as well as the non-raft fraction occurs, but nevertheless, raft targeting results in a higher probability for molecules to reside in a lipid raft and to interact with each other.
Even though this integrating view on membrane organization does not involve stable spatial confinements it supports highly dynamic spatial confinements resulting in a high probability of raft-targeted molecule species to come into vicinity with each other but with rapidly exchanging individual molecules. This integrated model emphasizes a function of lipid rafts in cell signaling by providing an environment that facilitates selective vicinity of distinct (raft-targeted) signaling proteins. Importantly, we have to understand lipid rafts as dynamic structures with enhanced probability for distinct molecules to get into contact with each other.

The integrated model of recent insights into membrane organization questions several assumptions but explains previously unresolved issues. First, the estimated number of raft-targetted proteins (approx. 250 in HeLa cells [81]) in consideration of the small size of rafts (e.g. 4 GPI-anchored molecules at a time) implicates the existence of lipid raft heterogeneity. Raft heterogeneity was found by different sensitivity of Lck and LAT to cholesterol extraction [114] and recently further supported by the discrimination of sphingomyelin-rich and ganglioside GM1-rich membrane domains [115]. Raft heterogeneity may explain why, after stimulation, TCR-enriched plasma membrane immunosolutes are enriched in various signaling molecules including LAT, but not other raft-associated molecules such as Lck showing that the TCR signaling machinery assembles without generally concentrating raft-associated membrane proteins [116]. Of note, LAT tyrosine phosphorylation sites are required for assembly of signaling complexes such as the TCR/LAT signalosome [117]. Hence, raft targeting alone appears not to be sufficient to concentrate selected raft proteins since they may be located in different types of rafts and protein-protein interactions are necessary to build up signaling complexes.

A predominance of protein–protein interaction in signalosome formation is supported by the finding, that, similar to non-raft protein CD45, raft molecules such as Lck and LAT are highly mobile in the cell membrane until TCR stimulation leads to a substantial immobilization that depends on distinct activation sites within these molecules and, interestingly, also on F-actin [118]. Stimulation-induced immobilization is due to formation of a complex that includes CD2, a protein known to be associated with the cytoskeleton [72] and previously shown to provide a costimulatory signal by lowering T cell activation threshold [119,120]. Nucleation of lipid raft-based TCR signaling complexes was suggested as a mechanism for the CD2-mediated signal [71]. Interestingly, the signal-induced Lck and LAT association with CD2 and the resulting immobilization of these proteins is generally transient, indicating that not only lipid raft-dependent but also protein–protein interactions are highly dynamic processes [118].

Of note, the protein–protein interaction-based mechanisms agree with a raft function for induction of signaling events, since rafts may facilitate distinct protein–protein interactions by increasing their local concentration and hence the probability for contact of signaling molecules. Moreover, results from single molecule tracking indicate that receptor ligation induces raft stabilization as shown for GPI-linked CD59 [121,122]. Raft stabilization leads to larger and more stable but still dynamic rafts [121,122]. Receptor-induced raft stabilization could be explained by small energy changes provoked by receptor clustering causing raft lipids and cholesterol to be trapped into initially small-sized rafts [123]. Although the exact impact of raft stabilization on induction and enhancement of TCR-induced signaling remain to be elucidated, it could be speculated that stabilized rafts provide a basis for raft aggregation by cytoskeleton-driven events to form signaling platforms as described above.

In summary, the presented integrated model on membrane organization, depicted in Fig. 1, point towards three different mechanisms that act in parallel to compartmentalize membrane molecules thereby facilitating T cell activation: (i) lipid rafts, (ii) fences and pickets that result in hop diffusion, and (iii) protein–protein interactions that include not only association of signaling proteins but also links to the actin cytoskeleton that in turn controls hop diffusion and raft aggregation. Future studies are warranted to elucidate the relative extent by which each of these organizing principles contributes to the initiation of T cell signaling.

4. Membrane organization during T cell activation by antigen-presenting cells

4.1. The immunological synapse

So far this review has focused on the initiation of TCR-mediated signals. T cell activation, however, depends on a complex interaction of T cells with antigen-presenting cells (APC). Interaction of a T cell with an APC occurs in three stages, namely contact, recognition, and stabilization [124]. Due to some similarities to
the neural synapse the junction between T cell and APC is named “immunological synapse” [125]. Using confocal microscopy to analyse protein distribution of T cells when they encounter an APC, it was discovered that T cell cytoskeletal, adhesion, and signaling proteins aggregate at the contact site to the APC, building “supramolecular activation clusters” (SMACs) [126] which are spatially and temporally organized structures crucial for controlling and balancing signals, the strength and nature of which depend on the abundance of antigen as well as on the type of APC [127,128]. Here, we mainly focus on the relationship between lipid rafts and the immunological synapse, for more details of the current view on the immunological synapse, particularly different stages, types, and functions of synapses see Ref. [129]. Moreover, also the active contribution of APCs to synapse formation as shown for dendritic cells [130,131] is not addressed here.

Since the molecules that relocalize towards the immunological synapse include lipid raft molecules such as Src family kinases and the ganglioside GM1 [67,126,132], it appears that the immunological synapse could be described as a large merger of lipid rafts. However, not only a recent study using FRET microscopy contradicts this idea [133], already early findings emphasized mechanisms beyond raft aggregation at the contact site that drive synapse formation. First, during synapse maturation, i.e. the complex relocalization of cell surface molecules that takes about 15 min, some proteins including the TCR complex accumulate at the center of the immunological synapse, named c-SMAC, whereas others such as the integrin leukocyte functional antigen (LFA)-1 become located at its periphery (p-SMAC) [126,134]. Since the LFA/intercellular adhesion molecule (ICAM) binding pair requires a larger intercellular distance than a TCR/MHC pair, the intercellular junction between T cell and APC displaces the larger binding pairs to the periphery and concentrates the shorter ones in the center. Second, the non-raft protein CD45 was found to segregate to the c-SMACs at early stages of synapse formation and to be displaced to outer regions after synapse maturation later on [135]. Also non-raft CD28 accumulates at the immunological synapse [136,137]. Although results from theoretical studies have hypothesized that the immunological synapse could form in the absence of active, energy-expending processes
[138], accumulation of molecules at the immunological synapse is an active process, driven by the cytoskeleton and depending on costimulatory signals such as those provided by B7/CD28 and ICAM-1/LFA-1 [66,67,132,136,137,139].

e-SMAC formation has been considered the basis for efficient TCR signaling. However, TCR-induced activation of ZAP-70 by Lck reaches its maximum even before the typical c-SMAC has been formed [140]. A speculation to resolve this apparent contradiction is that ZAP-70 activation could be rather inefficient unless downstream molecules are concentrated in the synapse. Whereas signals occurring before synapse maturation are sufficient to activate cell adhesion, productive T cell responses require orchestrated signaling in SMACs [135]. In line with these considerations, the APA1/1 epitope of the CD3e molecule that indicates productive TCR activation independent of CD3 tyrosine phosphorylation is strictly confined in c-SMACs [141]. Accordingly, disintegration of the immunological synapse results in diminished T cell activation [128] and interference with synapse formation appears to be a potential mode of action of immunosuppressive and antiinflammatory chemokines [142], orally-induced systemic immune hyporesponsiveness [143], as well as a promising target for immunosuppressive and antirheumatic drugs [144–146].

4.2. T cell activation by APC in three stages

To accurately describe the dynamics of membrane reorganization during T cell activation, we have to integrate the three membrane organizing principles (fences and pickets, lipid rafts, and protein–protein interactions) into the context of the immunological synapse and unravel their functional roles in synapse induction and maturation.

A molecule that may be a central linker for the membrane organizing principles involved in T cell activation is LAT. First, LAT is crucial for early T cell activation and its function depends on raft targeting [25,145]. Second, following TCR stimulation, LAT is very rapidly recruited from the cell membrane and intracellular storage vesicles to the c-SMAC [147–149]. Third, LAT is nucleating the TCR/LAT signalosome [49,117]. Of note, the LAT signalosome includes the GTP exchange factor Vav, which is a crucial molecule in controlling activation of the cytoskeleton [66,150]. Accordingly, T cell spreading, a process resembling synapse formation, depends on protein binding domains of LAT including the Grb2/Gads binding site that is necessary for the association of Vav with the LAT signalosome [151]. Another key for the mechanism of LAT function could be its association with CD2, the detailed nature of which is still to be elucidated [118]. Both LAT and CD2 are located at the c-SMAC upon T cell stimulation [72]. Moreover, ligation of CD2 activates LAT phosphorylation [152], a phenomenon also shown for other costimulatory receptors [153] and suggested also for GPI-anchored proteins, since their cross-linking induces calcium response [58].

Importantly, TCR-independent membrane reorganizations depending on ligand binding of ICAM-3 are crucial for cell–cell adhesion before antigen recognition and lead to an “immature immunological synapse” [134]. ICAM-3-mediated signals also induce LAT phosphorylation [134]. Hence, membrane reorganization depending on LAT activation that are induced by ICAM-3 and/or other costimulatory receptors putatively including CD2 could occur before ligation of the TCR, which subsequently mediates the association of the TCR/LAT signalosome. Besides protein–protein interactions, TCR/LAT signalosome formation can be explained by the fusion of distinct TCR and LAT rafts [154]. The TCR-independent activation of LAT and initiation of synapse formation may be the basis for the drastic lowering of the threshold of the number of TCR molecules that is needed to induce signals via CD2 and LFA-1 [120]. Additionally, also the early ligation of few TCR molecules may provide first signals sufficient to induce actin-mediated membrane reorganizations as suggested by temporal analysis of multiple proteins in the immunological synapse [135].

Integrating the data discussed above, the process of T cell stimulation by APC could be described, very concisely, as follows: in unstimulated T cells the membrane is compartmentalized in a temporally and spatially very dynamic manner by lipid-based molecule interactions, i.e. small and unstable lipid rafts, and hop-diffusion of molecules with constraints by fences and pickets. Immediately after contact with an APC, costimulatory receptors including adhesion molecules, GPI-anchored receptors, CD28, and CD2, possibly together with stimulation of few TCR molecules mediate reorganization of the cell membrane by receptor-mediated raft stabilization, induction of tyrosine phosphorylation, and nucleation of signalosomes leading to activation of the actin cytoskeleton (stage 1). Thereby, the signaling machinery located in distinct lipid rafts is translocated
Fig. 2. T cell membrane organization during activation by APC. In unstimulated T cells only small and unstable lipid rafts exist as well as fences and pickets (not depicted). Stage 1 of stimulation. Ligation of costimulatory receptors such as GPI-linked receptors, actin-associated CD2, as well as few TCRs induce receptor-mediated raft stabilization and nucleation of LAT- and TCR-signalsomes providing signals for activation of the actin cytoskeleton. Further costimulatory signals, particularly those involved in activation of the actin cytoskeleton originate from ligation of adhesion molecules and CD28 and lead to formation of an immature immunological synapse. Stage 2: Stabilized lipid rafts aggregate and MHC-ligated TCR complexes associate with LAT-nucleated signalsomes forming TCR/LAT signalsomes that induce enhanced signals to reorganize the T cell APC contact site. Adhesion molecules segregate to the peripheral contact zone. Stage 3: Concentration of TCR signalsomes in c-SMACs and clearing of CD45 from the center. Formation of a mature immunological synapse and full signaling to the nucleus for induction of the T cell activation program. AM, adhesion molecule (e.g. ICAM-1, -3, or integrins such as LFA-1); GPI: GPI-anchored protein (e.g. CD59); yellow unspecified signaling molecules are part of the TCR and LAT signalsomes including, e.g. ZAP-70 and Vav. “Cytoskeleton” subsumes cytoskeletal proteins such as F-actin and adapters, e.g. ERM proteins. Signals indicated by red arrows include activity of protein kinases, phospholipases, GTPases, induction of calcium response, etc. For further information see main text.
to the APC contact site and induce cell–cell adhesion providing an environment that facilitates T cell stimulation. Due to TCR stimulation, lipid raft aggregation occurs and LAT-nucleated signalosomes associate with TCR complexes, forming TCR/LAT signalosomes (stage 2) that induce further cytoskeletal reorganization and formation of a mature immunological synapse (stage 3). Finally, activation signals are transduced to the nucleus to induce gene transcription and promote T cell-mediated immune responses.

A sketch illustrating three-stage T cell activation is given in Fig. 2. Beyond the obligatory oversimplification due to the large number of molecules involved and the complexity of their interactions, several aspects of T cell activation by APC, which dramatically increase the complexity of the topic and are not or only very briefly discussed in this review, have not been integrated into the depicted model. First, T cell activation involves induction of a high-adhesive state of integrins due to affinity and avidity changes [155–157]. Second, activation events are generally in a balance with mechanisms of negative feedback. These include activation of inhibitory signaling pathways [158] as well as downregulation of surface receptors including the TCR [127]. Moreover, activation of the cytoskeleton does not only involve actin polymerization and translocation of associated proteins but also a rapid inactivation of ERM proteins leading to disanchoring of the actin cytoskeleton from the plasma membrane and resulting in a decrease of cellular rigidity that facilitates T cell/APC conjugate formation [159]. Third, coreceptors CD4 and CD8 are involved in TCR activation, probably by lipid raft-dependent mechanisms [160,161]. Fourth, there are different types of T cells and APCs and accordingly variants of immunological synapses [162]. When anergized T cells, i.e. T cells that have been exposed to a specific antigen under distinct conditions and fail to proliferate upon restimulation with the same antigen, encounter an APC a peculiar type of immature synapse evolves that results in only partial activation of gene transcription [143,163].

The depicted course of events is a framework to describe the four-dimensional processes of T cell activation by APC. At any stage, signals are enhanced by increasing the degree of organization. Notably, each step demands particular stimuli to proceed to the next. Thus several control mechanisms allow T cells to sense not only the presence of an antigen but also the quantity and the duration of the primary (i.e. antigen presented by MHC) and secondary (i.e. costimulatory receptors) signals provided by the APC. Hence, this model is in accordance with several others describing control of amplification of TCR-induced activation, e.g. by costimulatory receptors that set quantitative and/or temporal signaling thresholds [120,128,164].

5. Conclusions/outlook

Despite all doubts and weakpoints of the raft theory, our concept of T cell activation includes lipid rafts. Naturally, today’s picture of membrane organization is more complex and sophisticated than earlier models, integrating at least two organizing principles in addition to lipid rafts closely interacting with each other. Future data will show whether the term “lipid raft” is suitable for the description of a particular mode of lipid-dependent membrane compartmentalization, or whether it will be replaced. Anyway, the raft model is currently favored to understand many aspects of T cell activation and has hence proven to be one of the most fruitful ideas of research on membrane biology and signal transduction. We are eagerly looking forward whether the quickly increasing knowledge on membrane biology will shed light not only on the details of membrane compartmentalization but also on major unresolved questions concerning T cell activation, i.e. detailed mechanism that are responsible for the very first steps of TCR-mediated signal transduction as well as the molecular control of formation of the immunological synapse and its function in T cell activation.

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References
