Lipid rafts and regulation of the cytoskeleton during T cell activation

Karina F. Meiri*

Department of Anatomy and Cellular Biology, Tufts University School of Medicine,
136 Harrison Avenue, Boston, MA 02111, USA

The ability of polarized cells to initiate and sustain directional responses to extracellular signals is critically dependent on direct communication between spatially organized signalling modules in the membrane and the underlying cytoskeleton. Pioneering work in T cells has shown that the assembly of signalling modules critically depends on the functional compartmentalization of membrane lipids into ordered microdomains or lipid rafts. The significance of rafts in T cell activation lies not only in their ability to recruit the signalling partners that eventually assemble into a mature immunological synapse but also in their ability to regulate actin dynamics and recruit cytoskeletal associated proteins, thereby achieving the structural polarization underlying stability of the synapse—a critical prerequisite for activation to be sustained. Lipid rafts vary quite considerably in size and visualizing the smallest of them *in vivo* has been challenging. Nonetheless it is now been shown quite convincingly that a surprisingly large proportion—in the order of 50%—of external membrane lipids (chiefly cholesterol and glycosphingolipids) can be dynamically localized in these liquid ordered rafts. Complementary inner leaflet rafts are less well characterized, but contain phosphoinositides as an important functional component that is crucial for regulating the behaviour of the actin cytoskeleton. This paper provides an overview of the interdependency between signalling and cytoskeletal polarization, and in particular considers how regulation of the cytoskeleton plays a crucial role in the consolidation of rafts and their stabilization into the immunological synapse.

Keywords: T-lymphocytes; lipid rafts; actin; PIP2; linker for activation of T cells; Wiskott–Aldrich syndrome

1. INTRODUCTION

Lipid rafts are an ancient strategy used to generate signal transduction modules in prokaryotic and eukaryotic cells, whether polarized or not (Smart *et al.* 1999; Simons & Toomre 2000; Yin & Janmey 2002). It has been more than 20 years since the discovery that clustering glycosylphosphoinositide (GPI)-linked components of the extracellular lymphocyte membrane such as Thy-1 can induce T cell proliferation and IL2 synthesis even though Thy-1 contains no intrinsic means to transduce signals intracellularly (Maino *et al.* 1981; Kroczeck *et al.* 1986). We now understand that GPI confers a molecular address that targets Thy-1 to microdomains or rafts within the outer leaflet generated by hydrophobic interactions between sphingomyelin and glycosphingolipids and stabilized by ceramide and cholesterol. We are also beginning to understand that the T cell responses to the clustering of Thy-1 are achieved via communication with complementary rafts in the inner leaflet, which have a distinct lipid composition. This communication is brokered by the transmembrane ‘linker for activation of T cells’ (LAT) protein (Zhang *et al.* 1998). LAT is targeted to inner leaflet rafts by its dual palmitoylation, and so is a

component of both outer and inner rafts. Hence clustering of Thy-1 and, concomitantly, LAT, in turn initiates activation of inner leaflet associated Src kinases, leading to signalling events that partially mimic the T cell activation induced by antigen presentation. However, Thy-1 is a component of only a subset of rafts and its clustering can only recapitulate certain early events in T cell activation—later events leading to cytotoxicity require coordination between a number of different raft subtypes and the actin-based cytoskeleton that are dependent on regulation of inner leaflet phosphoinositides, such as PIP2 that Thy-1 alone cannot influence. Nonetheless, it is now quite clear that lipid rafts are crucial to T cell function, playing a critical role in coordinating the membrane–cytoskeletal communication that enables the full response of T cells to antigen.

2. RAFT-MEDIATED REGULATION OF MEMBRANE–CYTOSKELETAL COMMUNICATION DURING T CELL ACTIVATION

The notion that rafts form membrane platforms or modules that mediate cytoskeletal responses that are key to execution of T cell signalling depends on information that supports the following simple model: upon cross-linking by ligand, T cell receptors and membrane rafts consolidate so that the tyrosine kinase substrate motifs in the receptor tails become exposed to raft-associated kinases. Then, associated cytoskeletal

* (karina.meiri@tufts.edu).

One contribution of 16 to a Theme Issue ‘Immunoregulation: harnessing T cell biology for therapeutic benefit’.

changes result in the generation of a stable immune synapse that is able to act as a platform to enable activation of sustained T cell responses. There are many excellent reviews of different aspects of this process (for example [Alonso & Millan 2001](#); [Magee *et al.* 2002](#); [Horejsi 2003](#); [Pizzo & Viola 2004](#); [Thomas *et al.* 2004](#)).

The interplay between concentration of T cell receptors at the extracellular membrane and intracellular reorganization of the cytoskeleton occurs from the very beginning of the activation process: when a T cell encounters an antigen presenting cell (APC), movement of the T cell receptor (TCR) to the T cell/APC interface can only be induced if the major T cell costimulatory receptor CD28 is also engaged and activated, and if GPI-modified CD4 is crosslinked. This crosslinking causes formation of a juxtamembrane 'actin cap' that is intracellularly coincident with the extracellular activation site ([Wulfing *et al.* 1999](#)). The next event is formation of a macromolecular activation complex opposite the APC that has variously been called the supramolecular activation cluster or immunological synapse (IS) that couples the T cell receptor with signal transduction machinery. The IS integrates signalling cascades originating from the simultaneous activation of a wide variety of receptors and is essential for both the amplification and maintenance of T cell activation. It has become clear that the IS represents an extremely complex assemblage of rafts, each playing a specific role in the goal of initiating proliferation of the T cells.

As is apparent from the controversy surrounding the membrane distribution of the TCR complex (also referred to more generally as the multichain immune receptor complex, or MIRR, which includes both T and B cell receptors) both qualitative and quantitative differences in rafts appear to regulate T cell behaviour ([He *et al.* 2005](#)). On one hand the T cell receptor complex can be solubilized by the ionic detergents conventionally used to prepare rafts as detergent-insoluble moieties, but on the other, either extracting the membranes with less hydrophobic detergents (such as the non-ionic polyoxyethylene ether Brij series) or using colocalization immunocytochemistry in the absence of detergent clearly demonstrates that the TCR can codistribute with raft components. Moreover the rapid ζ chain phosphorylation that is the initial response of the T cell to binding to the APC is not raft dependent ([Pizzo *et al.* 2002](#)). A consensus has emerged that the T cell receptor only 'weakly' associated with rafts, meaning that whether the lipid association is via a raft with lipid content that is distinct from the fully assembled IS, or is via a shell consisting of fewer than 100 molecules of raft-like lipids that cocoon the MIRR, neither is sufficient to enable signal propagation ([Montixi *et al.* 1998](#); [Janes *et al.* 1999](#); [Kosugi *et al.* 1999](#); [Dykstra *et al.* 2003](#)). One attractive hypothesis is that a population of 'outer leaflet rafts' that are preassociated with the TCR have to associate with 'inner leaflet' rafts containing the kinases needed for the initiation of signalling before costimulation signal amplification can occur, and that this rearrangement requires cytoskeletal intervention ([Horejsi 2004](#)).

In this model initiation of TCR phosphorylation would cause the coalescence of the components of the IS such as the CD3 co-receptor, GPI linked CD4 and certain proteins that are involved in early signalling events such as the inner leaflet Src family kinases Lck and Fyn, all of which are associated with small yet distinct rafts ([Filipp & Julius 2004](#); [Tavano *et al.* 2004](#)). This model also proposes that the transmembrane raft protein LAT acts as a linker between outer leaflet rafts and their inner leaflet counterparts that is crucial to assembly of the IS ([Horejsi 2004](#)). Indeed LAT mobility in membranes does decrease as signalling is activated ([Tanimura *et al.* 2003](#); but see [Zhu *et al.* 2005](#)).

(a) *Dual role of the actin cytoskeleton during T cell activation*

After the TCR has been engaged and clusters opposite the APC, rafts coalesce into a much larger complex. This coalescence of rafts which in turn potentiates formation of the IS ([Janes *et al.* 1999](#); [Rao *et al.* 2004](#); [Thomas *et al.* 2004](#)) crucially requires engagement of the cytoskeleton ([Miceli *et al.* 2001](#); [Harder & Engelhardt 2004](#)); thus treatment of T cells with drugs that interfere with different aspects of actin treadmilling or prevent regulation of actin dynamics by the Rho family GTPases result in inhibition of both early and late events in the TCR signalling cascade ([Wulfing *et al.* 2003](#)).

(i) *How are T cell activation and cytoskeletal regulation coordinated?*

Current evidence suggests that there are two distinct phases, with rafts playing a decisive role in the latter phase. The first encounter with the APC ligates and activates both the TCR and the CD28 coreceptor in parallel. Activation of the TCR causes phosphorylation of the Src family kinase Lck ([Tavano *et al.* 2004](#)) and the tyrosine kinase ZAP-70 which in turn induces cortical actin to polymerize forming a cap beneath the APC. Concurrently interaction of the APC with CD28 also induces phosphorylation of the Rho family GTPase exchange protein Vav-1, which is constitutively associated with the TCR component CD3. This also affects actin dynamics through its guanine nucleotide exchange activity on Rac1 and CDC42 ([Salazar-Fontana *et al.* 2003](#)). Moreover, TCR signals are further boosted by the synergistic cooperation between Vav-1 and the linker protein SLP-76 that is also recruited to the T cell-APC interface upon activation of the TCR ([Michel & Acuto 2002](#)). This initial cytoskeletal reorganization does not appear to be regulated by rafts—for example it does not involve raft-mediated regulation of actin dynamics via phosphoinositide bis phosphate (PIP₂; [Salojin *et al.* 1999](#); [Viola *et al.* 1999](#)). Moreover Vav-1 is also located either outside rafts or is only weakly associated with them. Hence it seems likely that the first wave of Vav-1 phosphorylation that initiates F-actin reorganization in turn induces aggregation of small rafts circumferentially around the TCR, which helps to stabilize them and promote their further coalescence ([Villalba *et al.* 2001](#)).

The second stage of TCR activation, during which the T cell response is amplified, requires lipid raft reorganization to directly drive actin regulation (Gomez-Mouton *et al.* 2001; Valensin *et al.* 2002). This second stage is achieved by coalescence of smaller individual rafts that recruits raft-associated tyrosine kinases to the TCR complex and thereby sustains signalling. For example, cooperation between activated CD28 and CD4 recruits Src family kinases, particularly Lck and Fyn into the TCR complex. CD28 then induces Lck autophosphorylation thereby sustaining its activation (Harder & Engelhardt 2004; Horejsi 2004). This initiates a second wave of Vav phosphorylation, most likely by Fyn, to stimulate actin reorganization (Valensin *et al.* 2002). Meanwhile, coengagement of GPI linked CD48 with the TCR promotes TCR phosphorylation, enhancing F-actin binding directly to CD3, and thereby inducing cytoskeletal reorganization (Valensin *et al.* 2002). This actin reorganization can itself stimulate Vav-1 phosphorylation setting up a positive feedback loop that is independent of TCR receptor activation.

(ii) *LAT: linking early and late stages of T cell activation*

A major question about T cell activation is how the proximal and distal portions of the TCR signalling pathway are linked so that the initial stimulation mediates a sustained response. A likely candidate is the transmembrane linker protein LAT which, by virtue of dual palmitoylation in its cytoplasmic domain, acts to bridge external and internal leaflet rafts so that they can function in concert (Horejsi 2003). After TCR engagement LAT becomes rapidly tyrosine phosphorylated so that it directly binds the Src homology 2 (SH2) domains of other proteins, including Grb2, Gads, Grap, 3BP2, and Shb, and indirectly binds SOS, c-Cbl, Vav, SLP-76, and Itk. Tyrosine phosphatases such as CD45 are excluded from rafts, and this potentiates the amplification effect (Zhang *et al.* 2005). In the absence of LAT or mutation of its phosphorylatable tyrosines, TCR engagement does not lead to activation of distal signalling events, including actin reorganization, further supporting the notion that during the sustained portion of the response it is raft dynamics that influences actin reorganization (Wange & Samelson 1996; Sommers *et al.* 2001).

(iii) *Involvement of PIP2 in raft-mediated regulation of actin dynamics*

The relationship between raft clustering and actin polymerization becomes crucial for the downstream functional consequences of T cell activation; thus protein kinase C (PKC), which is required to activate AP-1 and NF κ B leading to the IL2 production that precedes proliferation, is located in the core of the IS in antigen stimulated cells and undergoes Vav/Rac dependent translocation to the membrane and cytoskeleton when T cells are activated (Gomez-Mouton *et al.* 2001; Villalba *et al.* 2001). Importantly also, LAT binds the PIP2 regulatory enzyme PLC γ 1 as well as PI3-K, thereby playing a major role in amplifying the signalling associated with the rafts (Tanimura *et al.* 2003; Horejsi 2004).

(b) *Control of actin polymerization at the IS*

It has been proposed that the physical and functional link between lipid rafts and the actin cytoskeleton is provided by the Wiskott–Aldrich syndrome (WASP) proteins that control *de novo* nucleation of actin branches on preexisting filaments through regulated activation of the seven-subunit Arp2/3 complex (Higgs & Pollard 2000). WASP integrates multiple signals to promote the formation actin networks in contact with membrane rafts, thus sustaining assembly of the IS (Barda-Saad *et al.* 2005). The crucial role of WASP in T cells was demonstrated from Wiskott–Aldrich syndrome (WAS) patients and WASP-deficient mice, both of whose T cells proliferate poorly after TCR/CD28 activation and whose capacity to cluster the lipid raft marker GM1 and to upregulate GM1 cell surface expression is impaired. In the latter case T cell proliferation and lipid raft clustering can be restored by viral transfer of the WASP gene (Klein *et al.* 2002; Charrier *et al.* 2004), therefore potentially identifying a mechanism underlying the T cell defect affecting WAS patients. Nonetheless WASP is neither a trans-membrane protein nor lipid-modified so its association with rafts presumably occurs via non-covalent interactions with lipids and/or proteins. Indeed WASP is not detected in the isolated rafts found in resting T cells, but only a few seconds after TCR/CD28 activation, it can be detected in rafts at the site of contact between the T cell and the APC together with its activators Vav-1 and PIP2 (Krause *et al.* 2000; Cannon *et al.* 2001), indicating that it has the potential to act very early during activation. Recruitment of WASP to the T cell–APC interface involves ZAP-70, which forms a complex at the TCR–APC interface with WASP and CrkL plus another interacting protein, WIP, that is inhibitory (Martinez-Quiles *et al.* 2001). First, autophosphorylation of ZAP-70 generates the phosphotyrosine(s) that are essential for CrkL docking. Then PKC-mediated phosphorylation of WIP allows WASP to dissociate, concurrently releasing it from inhibition and allowing it to be activated by membrane-bound Cdc42 and thereby initiate Arp2/3 complex-dependent actin polymerization (Sasahara *et al.* 2002). ZAP-70-deficient cells that also expressed dominant-negative CrkL, WIP $-/-$ and PKC $(-/-)$ T cells all failed to accumulate F-actin at the T cell–APC interface, or to increase total cellular F-actin content following TCR engagement (Anton *et al.* 2002). These data suggest that WASP plays a central role in promoting the organization of actin at the IS. WIP also binds and stabilizes actin filaments directly via PIP2, and therefore plays an additional independent role which helps stabilize the IS. Thus the failure of WIP $(-/-)$ T cells to increase F-actin following TCR ligation is due both to their inability to recruit WASP to ZAP-70 and their inability to bind F-actin directly.

3. UNANSWERED QUESTIONS IN T CELLS

A major challenge in immune system cell biology remains the identification and functional characterization of the heterogeneous raft populations that are dynamically regulated during the course of T cell activation.

(a) Biochemical isolation of structurally distinct raft fractions

The first lipid raft structures to be identified in the plasma membrane were flask shaped invaginations of the plasma membrane that thus became named caveolae (Anderson & Jacobson 2002). Their resistance to extraction with cold non-ionic detergents, particularly Triton-X100, suggested that their major lipids, cholesterol and sphingolipids, must be highly structured (liquid ordered). Antibodies to the major protein component, the cholesterol binding protein caveolin (Rothberg *et al.* 1992), immunoprecipitated caveolae in the absence of detergent thereby identifying other molecular constituents without the potential aggregation artefacts that confound detergent-induced extractions (Schnitzer *et al.* 1995). However, it has become clear that morphologically identifiable caveolae or rafts containing caveolin are absent from many cell types, including T cells. In these instances biochemical isolation has relied on detergent extraction and membrane microdomains having similar lipids to caveolae and/or caveolin itself have been variously called detergent-resistant membranes (DRMs), glycosphingolipid enriched membranes (GEMs), detergent-insoluble glycolipids (DIGs) or lipid rafts (Simons & Toomre 2000).

Rafts have been operationally defined on the basis of their insolubility in cold non-ionic detergents (chiefly TX-100 or TX-114) which solubilize non-raft membranes (Brown & Rose 1992; Fra *et al.* 1994; Friedrichson & Kurzchalia 1998). Centrifugation, utilising density gradients of either sucrose or Optiprep (a solution of iodixanol in water) causes rafts to float with a buoyant density of about 1.09–1.13 g ml⁻¹. (Membrane proteins normally have a buoyant density of around 1.3 g ml⁻¹ in the absence of lipid.) Any cytoskeleton that is also detergent-resistant will pellet under these conditions, as will rafts that are tightly associated with cytoskeleton (Timasheff 1995). Isolation of rafts following TX-114 extraction requires raising the temperature to 37 °C to induce phase separation, after which solubilized transmembrane proteins partition into the detergent phase whereas raft components are collected from the pellet. Because both methods are operationally defined they are beset with problems, the chief among them being the potential for artefactual aggregation between raft components during the extraction, by virtue of the mutual hydrophobicity of the lipid components. Similarly, if the detergent to protein ratio is less than 5–10:1, solubilization of non-raft transmembrane proteins may be incomplete and they may also become artefactually associated with raft lipids. The propensity of detergent extraction to induce artefactual aggregation cannot be overemphasized: for instance, *in vivo* spectroscopy of individual rafts shows clusters of 3500 sphingolipid molecules containing 20 protein components that are between 10 and 300 nm (average 50 nm) in diameter, well below the limit of resolution of light microscopy (Anderson & Jacobson 2002)—and yet fluorescence light microscopy is commonly employed to infer raft structure and function. While extraction of live cells on cover slips may somewhat limit the extent that aggregation can occur, it is of

limited value in uncovering the intricate signalling mechanisms that underlie raft-mediated regulation of complex cell behaviours such as T cell activation.

(b) Are structurally distinct classes of rafts also functionally heterogeneous?

The experimental challenge of how to separate structurally and potentially functionally heterogeneous populations of rafts from each other has been approached in two ways: the first involves paying careful attention to differences in buoyant density and solubility in conventional non-ionic detergents. Many published methods leave the impression that rafts have a single buoyant density, but this is only true when a high-density cushion is included in the gradient. In fact in polarized cells, several raft-containing bands can be recovered with a high degree of reproducibility after detergent extraction. For instance, we have found that neuronal growth cones (the motile tip of growing axons) contain five reproducible fractions all of which contain bonafide raft components such as GPI-linked and transmembrane, dually palmitoylated neural cell adhesion molecules (NCAM120 and NCAM 140, respectively). The fractions differ with respect to the activation status of cell-adhesion molecule-mediated signal transduction mechanisms and the association of cytoskeletal proteins (He & Meiri 2002). Only the lightest fraction contains the canonical raft marker caveolin. In this fraction NCAM signal transduction is activated via Fyn kinase. In contrast, in heavier raft fractions that do not contain caveolin and in which Fyn kinase is inactive, NCAM signal transduction mediated via PKC is upregulated. The heaviest raft fraction is associated with components of the actin cytoskeleton and both ultrastructurally and biochemically resembles the juxtamembrane ‘membrane skeleton’ described in erythrocytes and platelets as well as growth cones to be the primary target of extracellular signals that impact on the cytoskeleton. Similarly, when activated bovine neutrophils are extracted with TX-100 and fractionated with Optiprep, rafts with different functional characteristics are also generated. In those experiments Nebl *et al.* first collected a mixture of detergent-resistant rafts on a sucrose cushion and then subjected them to progressively shallower gradients that emphasized the small differences in buoyant density corresponding to structural differences in composition (Nebl *et al.* 2002). The lighter fraction (DRM-L) had a buoyant density between 1.09 and 1.13 g ml⁻¹ similar to rafts containing GPI anchored proteins, heterotrimeric G proteins and Src family kinases, whereas the slightly heavier fraction (DRM-H) fraction had a buoyant density of 1.15–1.18 g ml⁻¹. It too contained rafts associated with cytoskeletal material. It seems that the key to reproducibility in both instances was establishing and maintaining a constant detergent: protein ratio. Both approaches showed a direct association between rafts and the cytoskeleton: when the ultrastructure of the DRM/cytoskeleton fraction was examined by electron microscopic analysis fragments of membrane that had resisted detergent solubilization were seen to be associated with fibrils of actin cytoskeleton (probably the juxtamembrane actin-based membrane skeleton). Other attempts to identify

different raft subfractions have substituted other detergents for TX-100, particularly those that are less hydrophobic. For example, extracting T cells with the non-ionic detergent Brij 58 produced cholesterol-enriched fractions that differed with respect to activation of the Src family kinase Lck (Giurisato *et al.* 2003). Significantly active Lck fraction also contained considerably more of the TCR coreceptor CD3, whose association with rafts has been controversial. The results have been used to argue that different subpopulations of rafts may also exist in T cells, but an alternative explanation may be that proteins like CD3 are surrounded by a shell of only a few lipid molecules that allow them to associate with larger rafts under some circumstances (Anderson & Jacobson 2002). Whether any of these associations are artefactual cannot be determined from these experiments.

This confounding issue of aggregation has inspired attempts to isolate rafts in the absence of detergent. Several have been more or less successful although yields may be low and the necessary sonication step predictably increases significant interexperimental variation (Smart *et al.* 1995; Song *et al.* 1996). A very recent protocol was able to produce higher yields with more reproducibility and less cell type to cell type variation by substituting divalent cations for EDTA in the extraction buffer. However, the ensuing Optiprep gradient, while rapid, did not resolve raft fractions into the distinct bands obtainable with more time-consuming sucrose density centrifugation (Macdonald & Pike 2005). None of these methods have yet been employed in T cells.

(c) *How outer and inner leaflet T cell rafts differ*

The liquid ordered array characteristic of lipid rafts is due to the tight association between the saturated fatty acid residues of their major components—sphingomyelin and glycosphingolipids—in T cells, chiefly the gangliosides GM3 and GM1. The interactions are further stabilized by the presence of intercalating cholesterol. Both the structural and functional integrity of rafts is compromised if cholesterol is depleted (Ilangumaran & Hoessli 1998). Likewise the sphingolipids also play a key role—if they are substituted by polyunsaturated fatty acids (PUFAs), T cell activation is downregulated (Stulnig *et al.* 1998) apparently because interactions with the cytoskeleton are inhibited (Geyeregger *et al.* 2005). Interestingly the presence of PUFAs also displaces the linker protein LAT from rafts, suggesting that its inability to play a key coordinating role may underlie the functional defect (Zeyda *et al.* 2002).

The characteristic protein components of outer leaflet rafts in T cells are extracellularly oriented GPI-linked proteins that include (but are by no means limited to) CD24, CD52, CD59, CD73, CD90 (Thy-1) and CD100 (semaphorin). In contrast, most transmembrane proteins are excluded with the notable exceptions of the coreceptors CD4 and CD8 $\alpha\beta$, the adhesion receptor CD44, the pre TCR, and of course LAT. Like LAT (Zhang *et al.* 1998), CD4 (Fragoso *et al.* 2003), CD8 (Arcaro *et al.* 2000) and CD44 (Guo *et al.* 1994) are dually palmitoylated at the point where

their cytoplasmic tails pass through the membrane, and their function crucially depends on their concomitant insertion into inner leaflet rafts. In the case of the pre-TCR, the combination of a cytoplasmic cysteine and multiple proline residues appear to act in concert for raft association (Aifantis *et al.* 2002). Most protein components of inner leaflet rafts are targeted via fatty acid acylation, either dual palmitoylation or myristoylation (Webb *et al.* 2000). Key among them are the Src family kinases crucial for T cell function, namely Fyn (van't Hof & Resh 1999) and Lck (Kosugi *et al.* 2001) as well as G-proteins (Moffett *et al.* 2000). The membrane lipids of inner leaflet rafts have in general been less studied than their outer leaflet counterparts, but clearly are not identical. Nonetheless there are functional similarities: for example, substitution of saturated fatty acids with PUFAs also inhibits the association of cytoplasmic proteins with rafts, revealing another route whereby PUFAs may cause immune suppression, in addition to disrupting LAT (Webb *et al.* 2000).

One class of inner leaflet raft lipids are of particular functional significance to raft-mediated regulation of the cytoskeleton: the phosphoinositides (PPIs), of which phosphoinositide 4,5-bisphosphate (PIP2) and phosphoinositide 3,4,5 trisphosphate (PIP3) are key members, play important roles in regulating cytoskeletal dynamics that impact on a number of fundamental cellular functions such as polarization, membrane trafficking, growth and apoptosis (Sechi & Wehland 2000; Martin 2001; Takenawa & Itoh 2001). Rafts provide a structural substrate to attract PIP2 accumulation via electrostatic interactions, and also provide the means to control its homeostasis via the enzymes phosphatidylinositol-4-phosphate 5-kinase (PIP5KI) and phospholipase C (PLC), key raft components that synthesize and hydrolyse PIP2, respectively (Rozelle *et al.* 2000). Indeed, agonist sensitive regulation of PIP2 homeostasis, a major means of cytoskeletal regulation, occurs specifically in rafts (Yin & Janmey 2002). In general, stimulation of PIP5KI to increase synthesis of PIP2 initiates actin assembly: for instance, overexpression of PIPKI induces cholesterol-dependent formation of actin comet tails that extend preferentially from Golgi-derived vesicles that are enriched in rafts. Conversely, the number of PIP5KI-induced actin comets is dramatically reduced when rafts are disrupted (Rozelle *et al.* 2000). In a similar vein, hydrolysis of PIP2 by PLC causes immediate downregulation of the PIP2 signal and, in general, depletion of PIP2 triggers actin depolymerization (Takenawa & Itoh 2001). Hence agonist-mediated regulation of PIP2 homeostasis may impact cytoskeletal behaviour directly because of the complement of PIP2-regulated actin regulatory proteins that are present in individual rafts, or indirectly via the metabolites generated upon hydrolysis (Ins (1,4,5) P3 and diacylglycerol) that themselves are second messengers for PKC-mediated signalling pathways. In sum rafts have now also emerged as platforms for the integration of PIP2 signalling and actin polymerization (Laux *et al.* 2000; McLaughlin *et al.* 2002). In T cells regulation of PIP2 and PIP3 in rafts may provide the mechanism that couples initial events in T cell

Figure 1. (a) Before activation outer and inner leaflet rafts are likely segregated in the membrane. Activation of the TCR causes phosphorylation of Lck and ZAP-70, which initiates formation of an actin cap at the APC interface. In addition interaction of the APC with CD28 causes activation of Rac-1 and CDC42 GTPases thereby stimulating actin polymerization via Vav-1 activity. This helps stabilize coalescence of rafts circumferentially around the TCR complex. At the same time LAT plays an important role in bringing outer and inner leaflet rafts into concert. (b) During the second stage of T cell activation, coordination between raft function to amplify signalling mechanisms and actin polymerization to stabilize the IS involves PIP2 production, which occurs following LAT-mediated translocation of PLC γ and Vav-1 mediated translocation of PKC. PIP2 in turn activates WASP and WIP, which further consolidate the actin cytoskeleton, allowing the IS to fully mature.

activation to maturation of the signalling process (Yin & Janmey 2002; see figure 1).

4. ASSEMBLING AN OVERVIEW OF PIP2 FUNCTION IN T CELL ACTIVATION

Raft-mediated recruitment of PIP2 synthetic and metabolic enzymes is a key means by which cytoskeletal regulation occurs in many polarized cell types; however, the story is still incomplete in T cells. PIP5KI is recruited to rafts by two families of GTPase raft components: both Rho and Rac bind PIP5KI so that it can be activated by Rho-kinase (Chong *et al.* 1994; Chatah & Abrams 2001; Weernink *et al.* 2004). Rac1 stimulation of PIP2 synthesis can then induce actin polymerization directly (Tolias & Carpenter 2000) and it is thought that transient Rac-1 mediated activation of PIP2 synthesis occurs at sites where dynamic membrane movement and cytoskeletal remodelling converge, such as in ruffling lamellae or in filopodia (Doughman *et al.* 2003). In T cells transient activation of Rac-1 is also associated with changes in morphology (Sanchez-Martin *et al.* 2004); however, the connection to PIP2 synthesis has not been elucidated. A second GTPase that can activate PIP5KI, the GTPase Arf-6, appears to be primarily involved in vesicle recycling (Brown *et al.* 2001) and phagocytosis. Whether it is also involved in T cell function is not yet clear. Rafts also mediate down-regulation of PIP2 via hydrolysis by PLC. This does drive the cytoskeletal rearrangements important in

T cells—for example, activation of T cells recruits PLC to rafts where it is in turn activated by the Src family kinase Lck. This causes PIP2 function to be down-regulated and causes a local release of PIP2-binding proteins that impact cytoskeletal organization underlying T cell activation (Veri *et al.* 2001).

(a) PIP2 regulation of actin nucleation and dendritic branching is important in T cell function

Of particular significance to T cell function is PIP2-mediated control of cytoskeletal behaviour at the membrane. This is achieved via the Arp2/3 complex that regulates nucleation at preexisting actin filaments. Components of the Arp2/3 complex, normally cytoplasmic, become enriched in the lamellae of stimulated fibroblasts (Machesky 1997) where they can intercalate into an existing filament, nucleating branching growth in concert with profilin and capping protein. The complex also caps the (+) end, inhibiting treadmilling (Mullins *et al.* 1998). The activity of the Arp2/3 complex is quite low, but it is stimulated by the Wiskott–Aldrich syndrome (WASP) family proteins, WASP itself, neuronal WASP (N-WASP) and Scar/WAVE proteins. This stimulation of Arp2/3 by WASP is of particular importance in T cells: basal autoinhibition of WASP family proteins is relieved upon T cell activation by Rho-GTPase signalling via Cdc42 which in turn activates PIP2. These activators bind to WASP and synergistically increase its activation by more than

300 fold *in vitro* (Prehoda *et al.* 2000). Binding of activated Cdc42 and PIP2 to WASP also induces tyrosine phosphorylation via Fyn which in turn creates the SH3 domains that enable WASP to interact with Hck, Nck and importantly Grb2, in addition to both the Arp2/3 complex and actin nuclei themselves. This capacity to bind with multiple partners underlies WASP function as a premier adaptor protein at the immune synapse. Moreover the persistence of WASP phosphorylation beyond the short-term activation of GTPase and kinase signalling allows the actin remodelling to persistent and permits the transition between short-term and long-term responses to T cell activation. WASP null T cells have been instrumental in determining that WASP-dependent T cell responses include proliferation, apoptosis, antigen receptor endocytosis and cytokine secretion (Badour *et al.* 2004). While this responsiveness of WASP proteins makes them very effective in generating an extended range of responses to specific signals, the redundancy between them also somewhat mitigates effects of absence or defects in individual family members (Snapper *et al.* 2001).

(b) Is raft-mediated regulation of interactions between the actin cytoskeleton and the membrane also PIP2 dependent?

In contrast to WASP proteins, which interact with rafts indirectly via linker proteins, ezrin/radixin/moesin (ERM) proteins are important bifunctional binders that in T cells directly link of actin filaments to transmembrane proteins in rafts (Hirao *et al.* 1996). For instance ezrin binds to ICAM (Heiska *et al.* 1998), and moesin binds to CD44 (Neame *et al.* 1995; Tilghman & Hoover 2002). ERM proteins also bind F-actin *in vitro* (Bretscher *et al.* 2000). ERM and WASP behaviour show some commonalities: first, like WASP proteins, ERM proteins exist in both 'dormant' and active states. ERM 'dormancy' is caused by self-association inactivating the actin- and membrane binding sites, but stimulation of PIP2 production mediated by Rho signalling causes PIP2 binding to ERM which reveals the membrane binding sites. Like WASP too, Rho-dependent signal transduction also influences their phosphorylation state and activation. However, ERM proteins play dual roles in Rho protein signalling by acting both upstream and downstream of them: the N terminus of ERM proteins binds to RhoGDI, causing it to dissociate from Rho, thus activating it. Also in contrast with WASP proteins, ERM proteins appear to be involved in the early stages of activation. Thus, they are rapidly inactivated after antigen recognition via the Vav1–Rac1 pathway. The resulting disanchoring of the cortical actin cytoskeleton from the plasma membrane decreases cellular rigidity, leading to more efficient T cell-antigen-presenting cell conjugate formation (Faure *et al.* 2004). Interestingly, ERM proteins are also linked with assembly of the so-called 'distal pole complex' that is opposite the antigen presenting complex or APC on T cells and appears to be involved in cytokine secretion (Cullinan *et al.* 2002).

A final class of membrane/actin binding proteins that are known to be important in the cytoskeletal dynamics that underlie T cell activation are the

Ena/VASP/EVL proteins; however, their mechanism of action is much less clear. It seems likely that their interaction with rafts is indirect via Src-homology-3 (SH3) and proline-binding WW domains, and, as far as is currently known, they are not regulatable by PIP2. Nonetheless, disrupting EVL interactions with its adaptor protein SLAP mirrors the effect of disrupting WASP association with Arp2/3 (Badour *et al.* 2004).

(c) Does PIP2 regulation of actin treadmilling also contribute to T cell function?

PIP2 in rafts affects actin dynamics directly by regulating the behaviour of proteins that control actin treadmilling (net polymerization at the (+) end and net depolymerization at the (–) end) and nucleation. Most of this activity centres on gelsolin. Stimulation by agonist activates gelsolin in a Ca^{2+} -dependent fashion, increasing actin polymerization by severing actin filaments to generate new (+) end nuclei. In response, depolymerization from the (–) ends also increases and as a consequence of the increased activity, cell membranes ruffle (Barkalow *et al.* 1996). Gelsolin-null cells in which treadmilling is inhibited provide several examples of the interrelationship between treadmilling and membrane behaviour: in these cells decreased actin polymerization leads to a reduction of ruffling in response to growth factors and the concurrent reduction in actin depolymerization causes generation of excess actin stress fibres that in turn means cells migrate more slowly (Witke *et al.* 1995). Too much PIP2 itself can also inhibit treadmilling: cells overexpressing PIP5KI (and therefore PIP2) also cannot ruffle their membranes in a gelsolin-dependent response to growth factor stimulation, and also have more stress fibres (Yamamoto *et al.* 1998). However, interactions between PIP2 and gelsolin are not confined to regulating membrane dynamics of motile cells. In many cell types (including T cells) the principal role of PIP2 in gelsolin function appears to be to inhibit its cleavage by caspase, leading to inhibition of caspase-mediated apoptosis (Meerschaert *et al.* 1998; but see also Posey *et al.* 2000), but its contribution to the T responses to APC has not been extensively studied.

5. CONCLUSION

Lipid rafts are a structural substrate with a key role in amplifying and sustaining T cell responses to antigen. Central to that role is their ability to regulate cytoskeletal behaviour both directly and indirectly. In this paper we have merely touched on the different ways in which lipid rafts can influence the actin cytoskeleton, but there is mounting evidence that rafts also regulate the microtubule cytoskeleton via the microtubule organizing centre. How responses between the two cytoskeletal systems are transduced has not even begun to be explored in detail. The notion that T cell rafts are both structurally and functionally heterogeneous is also appealing as a basis for the variability in T cell dynamics that is observed upon antigen presentation, and the molecular basis for this variability is now beginning to be explored. However, investigations of raft function are always bedevilled by the experimental

constraints that may induce significant artefacts. Until there are significant technological breakthroughs in either biochemical raft isolation or in raft visualization in living cells, significant uncertainties are likely to remain, even in the T cell whose experimental tractability is especially appealing.

Since this is such a brief review, in many cases references have focused on relevant reviews rather than primary sources, and a number of omissions are therefore inevitable, but nonetheless regretted. This work was supported by NIH NS33118 (K.F.M.).

REFERENCES

- Aifantis, I., Borowski, C., Gounari, F., Lacorazza, H. D., Nikolich-Zugich, J. & von Boehmer, H. 2002 A critical role for the cytoplasmic tail of pTalpha in T lymphocyte development. *Nat. Immunol.* **3**, 483–488.
- Alonso, M. A. & Millan, J. 2001 The role of lipid rafts in signalling and membrane trafficking in T lymphocytes. *J. Cell Sci.* **114**, 3957–3965.
- Anderson, R. G. & Jacobson, K. 2002 A role for lipid shells in targeting proteins to caveolae, rafts, and other lipid domains. *Science* **296**, 1821–1825.
- Anton, I. M., de la Fuente, M. A., Sims, T. N., Freeman, S., Ramesh, N., Hartwig, J. H., Dustin, M. L. & Geha, R. S. 2002 WIP deficiency reveals a differential role for WIP and the actin cytoskeleton in T and B cell activation. *Immunity* **16**, 193–204.
- Arcaro, A., Gregoire, C., Boucheron, N., Stotz, S., Palmer, E., Malissen, B. & Luescher, I. F. 2000 Essential role of CD8 palmitoylation in CD8 coreceptor function. *J. Immunol.* **165**, 2068–2076.
- Badour, K., Zhang, J. & Siminovitch, K. A. 2004 Involvement of the Wiskott–Aldrich syndrome protein and other actin regulatory adaptors in T cell activation. *Semin. Immunol.* **16**, 395–407.
- Barda-Saad, M., Braiman, A., Titerence, R., Bunnell, S. C., Barr, V. A. & Samelson, L. E. 2005 Dynamic molecular interactions linking the T cell antigen receptor to the actin cytoskeleton. *Nat. Immunol.* **6**, 80–89.
- Barkalow, K., Witke, W., Kwiatkowski, D. J. & Hartwig, J. H. 1996 Coordinated regulation of platelet actin filament barbed ends by gelsolin and capping protein. *J. Cell Biol.* **134**, 389–399.
- Bretscher, A., Chambers, D., Nguyen, R. & Reczek, D. 2000 ERM–Merlin and EBP50 protein families in plasma membrane organization and function. *Annu. Rev. Cell Dev. Biol.* **16**, 113–143.
- Brown, D. A. & Rose, J. K. 1992 Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* **68**, 533–544.
- Brown, F. D., Rozelle, A. L., Yin, H. L., Balla, T. & Donaldson, J. G. 2001 Phosphatidylinositol 4,5-bisphosphate and Arf6-regulated membrane traffic. *J. Cell Biol.* **154**, 1007–1017.
- Cannon, J. L., Labno, C. M., Bosco, G., Seth, A., McGavin, M. H., Siminovitch, K. A., Rosen, M. K. & Burkhardt, J. K. 2001 Wasp recruitment to the T cell: APC contact site occurs independently of Cdc42 activation. *Immunity* **15**, 249–259.
- Charrier, S. *et al.* 2004 A lentiviral vector encoding the human Wiskott–Aldrich syndrome protein corrects immune and cytoskeletal defects in WASP knockout mice. *Gene Ther.* **12**, 597–606.
- Chatah, N. E. & Abrams, C. S. 2001 G-protein-coupled receptor activation induces the membrane translocation and activation of phosphatidylinositol-4-phosphate 5-kinase I alpha by a Rac- and Rho-dependent pathway. *J. Biol. Chem.* **276**, 34 059–34 065.
- Chong, L. D., Traynor-Kaplan, A., Bokoch, G. M. & Schwartz, M. A. 1994 The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. *Cell* **79**, 507–513.
- Cullinan, P., Sperling, A. I. & Burkhardt, J. K. 2002 The distal pole complex: a novel membrane domain distal to the immunological synapse. *Immunol. Rev.* **189**, 111–122.
- Doughman, R. L., Firestone, A. J., Wojtasiak, M. L., Bunce, M. W. & Anderson, R. A. 2003 Membrane ruffling requires coordination between type Ialpha phosphatidylinositol phosphate kinase and Rac signaling. *J. Biol. Chem.* **278**, 23 036–23 045.
- Dykstra, M., Cherukuri, A., Sohn, H. W., Tzeng, S. J. & Pierce, S. K. 2003 Location is everything: lipid rafts and immune cell signaling. *Annu. Rev. Immunol.* **21**, 457–481.
- Faure, S., Salazar-Fontana, L. I., Semichon, M., Tybulewicz, V. L., Bismuth, G., Trautmann, A., Germain, R. N. & Delon, J. 2004 ERM proteins regulate cytoskeleton relaxation promoting T cell–APC conjugation. *Nat. Immunol.* **5**, 272–279.
- Filipp, D. & Julius, M. 2004 Lipid rafts: resolution of the “fyn problem”? *Mol. Immunol.* **41**, 645–656.
- Fra, A. M., Williamson, E., Simons, K. & Parton, R. G. 1994 Detergent-insoluble glycolipid microdomains in lymphocytes in the absence of caveolae. *J. Biol. Chem.* **269**, 30 745–30 748.
- Fragoso, R., Ren, D., Zhang, X., Su, M. W., Burakoff, S. J. & Jin, Y. J. 2003 Lipid raft distribution of CD4 depends on its palmitoylation and association with Lck, and evidence for CD4-induced lipid raft aggregation as an additional mechanism to enhance CD3 signaling. *J. Immunol.* **170**, 913–921.
- Friedrichson, T. & Kurzchalia, T. V. 1998 Microdomains of GPI-anchored proteins in living cells revealed by cross-linking. *Nature* **394**, 802–805.
- Geyeregger, R., Zeyda, M., Zlabinger, G. J., Waldhausl, W. & Stulnig, T. M. 2005 Polyunsaturated fatty acids interfere with formation of the immunological synapse. *J. Leukoc. Biol.* **77**, 680–688.
- Giurisato, E., McIntosh, D. P., Tassi, M., Gamberucci, A. & Benedetti, A. 2003 T cell receptor can be recruited to a subset of plasma membrane rafts, independently of cell signalling and attendant to raft clustering. *J. Biol. Chem.* **278**, 6771–6778.
- Gomez-Mouton, C. *et al.* 2001 Segregation of leading-edge and uropod components into specific lipid rafts during T cell polarization. *Proc. Natl Acad. Sci. USA* **98**, 9642–9647.
- Guo, Y. J., Lin, S. C., Wang, J. H., Bigby, M. & Sy, M. S. 1994 Palmitoylation of CD44 interferes with CD3-mediated signaling in human T lymphocytes. *Int. Immunol.* **6**, 213–221.
- Harder, T. & Engelhardt, K. R. 2004 Membrane domains in lymphocytes—from lipid rafts to protein scaffolds. *Traffic* **5**, 265–275.
- He, Q. & Meiri, K. F. 2002 Isolation and characterization of detergent-resistant microdomains responsive to NCAM-mediated signaling from growth cones. *Mol. Cell Neurosci.* **19**, 18–31.
- He, H. T., Lellouch, A. & Marguet, D. 2005 Lipid rafts and the initiation of T cell receptor signaling. *Semin. Immunol.* **17**, 23–33.
- Heiska, L., Alfthan, K., Gronholm, M., Vilja, P., Vaheri, A. & Carpen, O. 1998 Association of ezrin with intercellular adhesion molecule-1 and -2 (ICAM-1 and ICAM-2). Regulation by phosphatidylinositol 4, 5-bisphosphate. *J. Biol. Chem.* **273**, 21 893–21 900.

- Higgs, H. N. & Pollard, T. D. 2000 Activation by Cdc42 and PIP(2) of Wiskott-Aldrich syndrome protein (WASp) stimulates actin nucleation by Arp2/3 complex. *J. Cell Biol.* **150**, 1311–1320.
- Hirao, M., Sato, N., Kondo, T., Yonemura, S., Monden, M., Sasaki, T., Takai, Y. & Tsukita, S. 1996 Regulation mechanism of ERM (ezrin/radixin/moesin) protein/plasma membrane association: possible involvement of phosphatidylinositol turnover and Rho-dependent signaling pathway. *J. Cell Biol.* **135**, 37–51.
- Horejsi, V. 2003 The roles of membrane microdomains (rafts) in T cell activation. *Immunol. Rev.* **191**, 148–164.
- Horejsi, V. 2004 Transmembrane adaptor proteins in membrane microdomains: important regulators of immunoreceptor signaling. *Immunol. Lett.* **92**, 43–49.
- Ilangumaran, S. & Hoessli, D. C. 1998 Effects of cholesterol depletion by cyclodextrin on the sphingolipid microdomains of the plasma membrane. *Biochem. J.* **335**, 433–440.
- Janes, P. W., Ley, S. C. & Magee, A. I. 1999 Aggregation of lipid rafts accompanies signaling via the T cell antigen receptor. *J. Cell Biol.* **147**, 447–461.
- Klein, C. *et al.* 2002 Gene therapy for Wiskott-Aldrich syndrome: rescue of T-cell signaling and amelioration of colitis upon transplantation of retrovirally transduced hematopoietic stem cells in mice. *Blood* **101**, 2159–2166.
- Kosugi, A., Saitoh, S., Noda, S., Yasuda, K., Hayashi, F., Ogata, M. & Hamaoka, T. 1999 Translocation of tyrosine-phosphorylated TCR ζ chain to glycolipid-enriched membrane domains upon T cell activation. *Int. Immunol.* **11**, 1395–1401.
- Kosugi, A., Hayashi, F., Liddicoat, D. R., Yasuda, K., Saitoh, S. & Hamaoka, T. 2001 A pivotal role of cysteine 3 of Lck tyrosine kinase for localization to glycolipid-enriched microdomains and T cell activation. *Immunol. Lett.* **76**, 133–138.
- Krause, M., Sechi, A. S., Konradt, M., Monner, D., Gertler, F. B. & Wehland, J. 2000 Fyn-binding protein (Fyb)/SLP-76-associated protein (SLAP), Ena/vasodilator-stimulated phosphoprotein (VASP) proteins and the Arp2/3 complex link T cell receptor (TCR) signaling to the actin cytoskeleton. *J. Cell Biol.* **149**, 181–194.
- Krocze, R. A., Gunter, K. C., Seligmann, B. & Shevach, E. M. 1986 Induction of T cell activation by monoclonal anti-Thy-1 antibodies. *J. Immunol.* **136**, 4379–4384.
- Laux, T., Fukami, K., Thelen, M., Golub, T., Frey, D. & Caroni, P. 2000 GAP43, MARCKS, and CAP23 modulate PI(4,5)P(2) at plasmalemmal rafts, and regulate cell cortex actin dynamics through a common mechanism. *J. Cell Biol.* **149**, 1455–1472.
- Macdonald, J. L. & Pike, L. J. 2005 A simplified method for the preparation of detergent-free lipid rafts. *J. Lipid Res.* **46**, 1061–1067.
- Machesky, L. M. 1997 Cell motility: complex dynamics at the leading edge. *Curr. Biol.* **7**, R164–R167.
- Magee, T., Pirinen, N., Adler, J., Pagakis, S. N. & Parmryd, I. 2002 Lipid rafts: cell surface platforms for T cell signaling. *Biol. Res.* **35**, 127–131.
- Maino, V. C., Norcross, M. A., Perkins, M. S. & Smith, R. T. 1981 Mechanism of Thy-1-mediated T cell activation: roles of Fc receptors, T200, Ia, and H-2 glycoproteins in accessory cell function. *J. Immunol.* **126**, 1829–1836.
- Martin, T. F. 2001 PI(4,5)P(2) regulation of surface membrane traffic. *Curr. Opin. Cell Biol.* **13**, 493–499.
- Martinez-Quiles, N. *et al.* 2001 WIP regulates N-WASP-mediated actin polymerization and filopodium formation. *Nat. Cell Biol.* **3**, 484–491.
- McLaughlin, S., Wang, J., Gambhir, A. & Murray, D. 2002 PIP(2) and proteins: interactions, organization, and information flow. *Annu. Rev. Biophys. Biomol. Struct.* **31**, 151–175.
- Meerschaert, K., De Corte, V., De Ville, Y., Vandekerckhove, J. & Gettemans, J. 1998 Gelsolin and functionally similar actin-binding proteins are regulated by lysophosphatidic acid. *EMBO J.* **17**, 5923–5932.
- Miceli, M. C., Moran, M., Chung, C. D., Patel, V. P., Low, T. & Zinnanti, W. 2001 Co-stimulation and counter-stimulation: lipid raft clustering controls TCR signaling and functional outcomes. *Semin. Immunol.* **13**, 115–128.
- Michel, F. & Acuto, O. 2002 CD28 costimulation: a source of Vav-1 for TCR signaling with the help of SLP-76? *Sci. STKE* **2002**, PE35.
- Moffett, S., Brown, D. A. & Linder, M. E. 2000 Lipid-dependent targeting of G proteins into rafts. *J. Biol. Chem.* **275**, 2191–2198.
- Montixi, C., Langlet, C., Bernard, A. M., Thimonier, J., Dubois, C., Wurbel, M. A., Chauvin, J. P., Pierres, M. & He, H. T. 1998 Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains. *EMBO J.* **17**, 5334–5348.
- Mullins, R. D., Heuser, J. A. & Pollard, T. D. 1998 The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments. *Proc. Natl Acad. Sci. USA* **95**, 6181–6186.
- Neame, S. J., Uff, C. R., Sheikh, H., Wheatley, S. C. & Isacke, C. M. 1995 CD44 exhibits a cell type dependent interaction with triton X-100 insoluble, lipid rich, plasma membrane domains. *J. Cell Sci.* **108**, 3127–3135.
- Nebi, T., Pestonjamas, K. N., Leszyk, J. D., Crowley, J. L., Oh, S. W. & Luna, E. J. 2002 Proteomic analysis of a detergent-resistant membrane skeleton from neutrophil plasma membranes. *J. Biol. Chem.* **277**, 43 399–43 409.
- Pizzo, P. & Viola, A. 2004 Lipid rafts in lymphocyte activation. *Microbes Infect.* **6**, 686–692.
- Pizzo, P., Giurisato, E., Tassi, M., Benedetti, A., Pozzan, T. & Viola, A. 2002 Lipid rafts and T cell receptor signaling: a critical re-evaluation. *Eur. J. Immunol.* **32**, 3082–3091.
- Posey, S. C., Martelli, M. P., Azuma, T., Kwiatkowski, D. J. & Bierer, B. E. 2000 Failure of gelsolin overexpression to regulate lymphocyte apoptosis. *Blood* **95**, 3483–3488.
- Prehoda, K. E., Scott, J. A., Mullins, R. D. & Lim, W. A. 2000 Integration of multiple signals through cooperative regulation of the N-WASP-Arp2/3 complex. *Science* **290**, 801–806.
- Rao, R., Logan, B., Forrest, K., Roszman, T. L. & Goebel, J. 2004 Lipid rafts in cytokine signaling. *Cytokine Growth Factor Rev.* **15**, 103–110.
- Rothberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y. S., Glenney, J. R. & Anderson, R. G. 1992 Caveolin, a protein component of caveolae membrane coats. *Cell* **68**, 673–682.
- Rozelle, A. L. *et al.* 2000 Phosphatidylinositol 4,5-bisphosphate induces actin-based movement of raft-enriched vesicles through WASP-Arp2/3. *Curr. Biol.* **10**, 311–320.
- Salazar-Fontana, L. I., Barr, V., Samelson, L. E. & Bierer, B. E. 2003 CD28 engagement promotes actin polymerization through the activation of the small Rho GTPase Cdc42 in human T cells. *J. Immunol.* **171**, 2225–2232.
- Salojin, K. V., Zhang, J. & Delovitch, T. L. 1999 TCR and CD28 are coupled via ZAP-70 to the activation of the Vav/Rac-1/PAK-1/p38 MAPK signaling pathway. *J. Immunol.* **163**, 844–853.
- Sanchez-Martin, L. *et al.* 2004 Signaling through the leukocyte integrin LFA-1 in T cells induces a transient activation of Rac-1 that is regulated by Vav and PI3K/Akt-1. *J. Biol. Chem.* **279**, 16 194–16 205.
- Sasahara, Y., Rachid, R., Byrne, M. J., de la Fuente, M. A., Abraham, R. T., Ramesh, N. & Geha, R. S. 2002 Mechanism of recruitment of WASP to the immunological synapse and of its activation following TCR ligation. *Mol. Cell* **10**, 1269–1281.

- Schnitzer, J. E., McIntosh, D. P., Dvorak, A. M., Liu, J. & Oh, P. 1995 Separation of caveolae from associated microdomains of GPI-anchored proteins. *Science* **269**, 1435–1439.
- Sechi, A. S. & Wehland, J. 2000 The actin cytoskeleton and plasma membrane connection: PtdIns(4,5)P(2) influences cytoskeletal protein activity at the plasma membrane. *J. Cell Sci.* **113**, 3685–3695.
- Simons, K. & Toomre, D. 2000 Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* **1**, 31–39.
- Smart, E. J., Ying, Y. S., Mineo, C. & Anderson, R. G. 1995 A detergent-free method for purifying caveolae membrane from tissue culture cells. *Proc. Natl Acad. Sci. USA* **92**, 10 104–10 108.
- Smart, E. J., Graf, G. A., McNiven, M. A., Sessa, W. C., Engelman, J. A., Scherer, P. E., Okamoto, T. & Lisanti, M. P. 1999 Caveolins, liquid-ordered domains, and signal transduction. *Mol. Cell Biol.* **19**, 7289–7304.
- Snapper, S. B. *et al.* 2001 N-WASP deficiency reveals distinct pathways for cell surface projections and microbial actin-based motility. *Nat. Cell Biol.* **3**, 897–904.
- Sommers, C. L., Menon, R. K., Grinberg, A., Zhang, W., Samelson, L. E. & Love, P. E. 2001 Knock-in mutation of the distal four tyrosines of linker for activation of T cells blocks murine T cell development. *J. Exp. Med.* **194**, 135–142.
- Song, K. S., Li, S., Okamoto, T., Quilliam, L. A., Sargiacomo, M. & Lisanti, M. P. 1996 Co-purification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains. Detergent-free purification of caveolae microdomains. *J. Biol. Chem.* **271**, 9690–9697.
- Stulnig, T. M., Berger, M., Sigmund, T., Raederstorff, D., Stockinger, H. & Waldhausl, W. 1998 Polyunsaturated fatty acids inhibit T cell signal transduction by modification of detergent-insoluble membrane domains. *J. Cell Biol.* **143**, 637–644.
- Takenawa, T. & Itoh, T. 2001 Phosphoinositides, key molecules for regulation of actin cytoskeletal organization and membrane traffic from the plasma membrane. *Biochim. Biophys. Acta* **1533**, 190–206.
- Tanimura, N. *et al.* 2003 Dynamic changes in the mobility of LAT in aggregated lipid rafts upon T cell activation. *J. Cell Biol.* **160**, 125–135.
- Tavano, R., Gri, G., Molon, B., Marinari, B., Rudd, C. E., Tuosto, L. & Viola, A. 2004 CD28 and lipid rafts coordinate recruitment of Lck to the immunological synapse of human T lymphocytes. *J. Immunol.* **173**, 5392–5397.
- Thomas, S., Preda-Pais, A., Casares, S. & Brumeanu, T. D. 2004 Analysis of lipid rafts in T cells. *Mol. Immunol.* **41**, 399–409.
- Tilghman, R. W. & Hoover, R. L. 2002 E-selectin and ICAM-1 are incorporated into detergent-insoluble membrane domains following clustering in endothelial cells. *FEBS Lett.* **525**, 83–87.
- Timasheff, S. N. 1995 Solvent stabilization of protein structure. *Methods Mol. Biol.* **40**, 253–269.
- Tolias, K. & Carpenter, C. L. 2000 *In vitro* interaction of phosphoinositide-4-phosphate 5-kinases with Rac. *Methods Enzymol.* **325**, 190–200.
- Valensin, S., Paccani, S. R., Ulivieri, C., Mercati, D., Pacini, S., Patrussi, L., Hirst, T., Lupetti, P. & Baldari, C. T. 2002 F-actin dynamics control segregation of the TCR signaling cascade to clustered lipid rafts. *Eur. J. Immunol.* **32**, 435–446.
- van't Hof, W. & Resh, M. D. 1999 Dual fatty acylation of p59(Fyn) is required for association with the T cell receptor zeta chain through phosphotyrosine-Src homology domain-2 interactions. *J. Cell Biol.* **145**, 377–389.
- Veri, M. C. *et al.* 2001 Membrane raft-dependent regulation of phospholipase Cgamma-1 activation in T lymphocytes. *Mol. Cell Biol.* **21**, 6939–6950.
- Villalba, M., Bi, K., Rodriguez, F., Tanaka, Y., Schoenberger, S. & Altman, A. 2001 Vav1/Rac-dependent actin cytoskeleton reorganization is required for lipid raft clustering in T cells. *J. Cell Biol.* **155**, 331–338.
- Viola, A., Schroeder, S., Sakakibara, Y. & Lanzavecchia, A. 1999 T lymphocyte costimulation mediated by reorganization of membrane microdomains. *Science* **283**, 680–682.
- Wange, R. L. & Samelson, L. E. 1996 Complex complexes: signaling at the TCR. *Immunity* **5**, 197–205.
- Webb, Y., Hermida-Matsumoto, L. & Resh, M. D. 2000 Inhibition of protein palmitoylation, raft localization, and T cell signaling by 2-bromopalmitate and polyunsaturated fatty acids. *J. Biol. Chem.* **275**, 261–270.
- Weernink, P. A., Meletiadis, K., Hommeltenberg, S., Hinz, M., Ishihara, H., Schmidt, M. & Jakobs, K. H. 2004 Activation of type I phosphatidylinositol 4-phosphate 5-kinase isoforms by the Rho GTPases, RhoA, Rac1, and Cdc42. *J. Biol. Chem.* **279**, 7840–7849.
- Witke, W., Sharpe, A. H., Hartwig, J. H., Azuma, T., Stossel, T. P. & Kwiatkowski, D. J. 1995 Hemostatic, inflammatory, and fibroblast responses are blunted in mice lacking gelsolin. *Cell* **81**, 41–51.
- Wulfig, C., Chien, Y. H. & Davis, M. M. 1999 Visualizing lymphocyte recognition. *Immunol. Cell Biol.* **77**, 186–187.
- Wulfig, C., Purtic, B., Klem, J. & Schatzle, J. D. 2003 Stepwise cytoskeletal polarization as a series of checkpoints in innate but not adaptive cytolytic killing. *Proc. Natl Acad. Sci. USA* **100**, 7767–7772.
- Yamamoto, M., Toya, Y., Schwencke, C., Lisanti, M. P., Myers Jr, M. G. & Ishikawa, Y. 1998 Caveolin is an activator of insulin receptor signaling. *J. Biol. Chem.* **273**, 26 962–26 968.
- Yin, H. L. & Janmey, P. A. 2002 Phosphoinositide regulation of the actin cytoskeleton. *Annu. Rev. Physiol.* **65**, 761–789.
- Zeyda, M., Staffler, G., Horejsi, V., Waldhausl, W. & Stulnig, T. M. 2002 LAT displacement from lipid rafts as a molecular mechanism for the inhibition of T cell signaling by polyunsaturated fatty acids. *J. Biol. Chem.* **277**, 28 418–28 423.
- Zhang, W., Sloan-Lancaster, J., Kitchen, J., Tribble, R. P. & Samelson, L. E. 1998 LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell* **92**, 83–92.
- Zhang, M., Moran, M., Round, J., Low, T. A., Patel, V. P., Tomassian, T., Hernandez, J. D. & Miceli, M. C. 2005 CD45 signals outside of lipid rafts to promote ERK activation, synaptic raft clustering, and IL-2 production. *J. Immunol.* **174**, 1479–1490.
- Zhu, M., Shen, S., Liu, Y., Granillo, O. & Zhang, W. 2005 Cutting edge: localization of linker for activation of T cells to lipid rafts is not essential in T cell activation and development. *J. Immunol.* **174**, 31–35.