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ABSTRACT

T lymphocyte activation relies on the cognate recognition by the TCR of the MHC-associated peptide ligand (pMHC) presented at the surface of an antigen-presenting cell (APC). This leads to the dynamic formation of a cognate contact between the T lymphocyte and the APC: the immune synapse (IS). Engagement of the TCR by the pMHC in the synaptic zone induces a cascade of signalling events leading to phosphorylation and dephosphorylation of proteins and lipids, which ultimately shapes the response of T lymphocytes. Although the engagement of the TCR takes place at the plasma membrane, the TCR/CD3 complexes and the signalling molecules involved in transduction of the TCR signal are also present in intracellular membrane pools. These pools, which are both endocytic and exocytic, have tentatively been characterized by several groups including ours. We will herein summarize what is known on the intracellular pools of TCR signalling components. We will discuss their origin and the mechanisms involved in their mobility at the IS. Finally, we will propose several hypotheses concerning the functional role(s) that these intracellular pools might play in T cell activation. We will also discuss the tools that could be used to test these hypotheses.

1. INTRODUCTION

Regulated T lymphocyte activation, which involves TCR stimulation, plays a key role in establishing tolerance or immune response. Signalling through the TCR is thus tightly regulated. The TCR is a multi-subunit receptor consisting of the antigen-binding α and β chains, and the signal transducing CD3 γ, ε δ, and ζ chains. TCR signalling is initiated by the recognition, by the TCR clonal αβ chains, of a peptide bound to pMHC, expressed at the surface of an APC (1). TCR-induced signalling involves a cascade of phosphorylation/dephosphorylation events of the TCR/CD3 complex itself, of proteins with enzymatic activity such as kinases, phosphatases, phospholipases and of adaptor proteins such as LAT which by assembling signalling complexes propagate the signal (2). Although pMHC recognition by the TCR takes place at the plasma membrane, some of the key actors of the signalling cascade, i. e. Lck, CD3ζ and LAT, are not uniquely present at the plasma membrane. They are sometimes more abundant in endosomes, which have characteristics of exocytic and/or endocytic compartments. Upon TCR binding to pMHC, these intracellular pools of signalling molecules are rapidly polarized towards the IS formed between the T lymphocyte and the APC.

Several groups including ours have shown that the tight regulation of their traffic is playing a role in T lymphocyte activation. Yet, how these endosomes containing signalling molecules regulate T cell activation is unknown. We will herein summarize

what is known on the nature of the endosomes containing the TCR/CD3 complexes, the Lck kinase and the adaptor LAT. We will also propose potential roles for the regulated traffic of these intracellular pools and discuss the fact that endosomes can be "signalling competent" thus challenging the mainstream thought that all TCR signalling takes place at the plasma membrane. We will finally propose methods that can be used to address these questions.

2. INTRACELLULAR POOLS OF TCR AND ASSOCIATED SIGNALLING PROTEINS

2.1. TCR ENDOCYTOSIS

Various groups have studied TCR endocytosis using most of the times as a read-out an antibody, recognizing just one of the chains of the TCR complex. Yet, in most of the cases, conclusions are drawn on the endocytosis of the TCR or the TCR/CD3 complex and one has to look carefully in the methods to find out which chain was followed in each case. Since there is clear evidence that at least the CD3ε and CD3ζ chains follow different endocytic routes and kinetics, we suggest that it would facilitate further studies if the authors clearly stated which chain is studied. Hence, in this chapter we will review TCR endocytosis findings stating the chain concerned wherever that is possible.

2.1.1 TCR constitutive versus activation-triggered endocytosis

Similar to other cell surface receptors, the TCR undergoes both ligand-dependent (activation induced) and ligand-independent (constitutive) endocytosis, followed by

either receptor recycling to the plasma membrane or receptor degradation in lysosomes (3-5).

The constitutive and activation-induced TCR endocytosis involve distinct mechanisms.

Constitutive internalization of TCRα, TCRβ, CD3γ, CD3δ and CD3ε involves clathrin-dependent endocytosis (CDE) and the di-Leucine motif of the CD3γ chain, which associates with the AP-2 clathrin adaptor. This clathrin dependent internalization is most probably associated with receptor recycling (6, 7). The constitutive endocytosis and recycling of CD3ε are important for T cell function, since the disruption of the CD3γ di-Leucine motif leads to low CD3ε levels at the plasma membrane and prevents its accumulation at the IS. This suggests that constitutively internalized CD3ε maintains a pool of receptors, susceptible to a polarized recycling, and that contributes to IS formation (8). At the plasma membrane, in fully assembled TCRs, the di-Leucine motif of CD3γ is masked by the CD3ζ chain, which limits constitutive endocytosis of the TCR complex, as detected by the F101.01 conformational antibody (9) and by anti-TCRβ and anti-CD3ε antibodies (9-11)

TCR ligand-induced internalization occurs via both CDE and clathrin-independent (CIE) mechanisms. While CIE mechanisms are involved mainly in the downregulation of CD3ε and CD3ζ, CDE is involved in the internalization of non-engaged, bystander CD3ε and CD3ζ. Bystander CD3ε was detected by anti-CD3ε,

while bystander CD3ζ was detected using CD3ζ reporters, such as CD8-CD3ζ fusion proteins (12, 13). CIE of the CD3ζ chain after TCR triggering by pMHC involves the activity of two Ras family GTPases: TC21 and RhoG (14). CD3ζ is internalized together with pMHC from the APC, in a phagocytosis-related process known as trogocytosis (14). The antigen-triggered TCRα, TCRβ, CD3ε and CD3ζ might be either recycled, especially at early time points (15, 16), or, at least for CD3ε, directed to lysosomes and degraded (9).

2.1.2 Role of Lck in TCR endocytosis

Both, antigen-triggered and bystander TCR endocytosis, require Lck activity (17). The involvement of Lck activity in constitutive TCR endocytosis is not fully understood, yet it does not seem to regulate TCR complex constitutive recycling, as detected by the F101.01 conformational antibody (9). However, Lck by inducing the phosphorylation of the clathrin heavy chain, has been shown to control endocytosis of CD3ε triggered by anti-CD3ε (18). In addition, Lck activity also affects TCR expression because it regulates the CD3ζ chain stability. Indeed, the total level of CD3ζ is reduced in T cells expressing a constitutively active form of Lck (Y505F mutation). Consequently, TCR cell surface expression, which depends on CD3ζ, is decreased and can be restored by chemical inhibition of Lck (17). Inversely, a partial knock-down of Lck leads to an increased expression of CD3ε at the cell surface (19), that is most probably due to less CD3ζ degradation (20). This regulation of the CD3ζ

chain degradation by Lck is at least partly due to activation of the ubiquitin ligase c-Cbl, which ubiquitinates the CD3 chains and leads to the degradation of antigen-triggered TCR (20, 21).

2.1.3 The role of the cSMAC and TCR microclusters in TCR endocytosis

Endocytosis of antigen-triggered TCR takes place in the central part of the IS, in the cSMAC. Indeed, the decreased tyrosine phosphorylation observed in the cSMAC suggested that this zone was related to extinction of TCR signalling (22, 23). The CD3ζ chains of TCR activated by pMHC bilayers are ubiquitinated by c-Cbl in the cSMAC. Ubiquitinated CD3ζ chains are recognized by the TSG101 component of the endosomal sorting complexes and transported to lysosomes (24). Other evidence for a role of the cSMAC in signal extinction comes from the discovery that the concentration of CD3ε, pCD3ζ, pZAP70 and pSLP76 in the cSMAC depends on dynein, the main protein complex involved in retrograde transport along microtubules. Dynein-depleted T cells show defective cSMAC formation, but enhanced phosphorylation of LAT and ERK (25), which suggest that T cell activation is negatively regulated by dynein-mediated transport of the TCR to the cSMAC.

Despite this prevalent view of the cSMAC as a negative regulator of TCR signalling, there are several data indicating that the strength of TCR activation dictates the termination of TCR signalling in this zone. Thus, weak TCR stimulation does not lead to TCRβ targeting to lysosomes (24). Moreover, using lipid bilayers loaded with

serial dilutions of an antigenic peptide, Cemerski et al., demonstrated that at high pMHC concentration, phosphorylated tyrosines are predominant in the periphery of IS, while at low pMHC concentration, phosphorylated signalling molecules are enriched in the cSMAC. Chemical inhibition of endocytosis by chlorpromazine allowed the visualization of phosphorylated proteins in the cSMAC even when the cells were stimulated with high concentration of antigenic peptides (26, 27). This suggests that the recruitment of active signalling proteins at the cSMAC occurs in the presence of high ligand concentration, but is not usually detected because of TCR internalization at the cSMAC. In conclusion, both strong and weak TCR ligands recruit signalling molecules not only to peripheral TCR clusters but also to the cSMAC, while only strong TCR ligands induce TCR internalization from the cSMAC. This is consistent with the ability of both strong and weak TCR ligands to activate T cells, but raises questions about how signalling triggered by weak TCR agonists is terminated.

One possible explanation, which might apply also outside the cSMAC, is the internalization and degradation of essential players of the TCR signalling pathway. This might occur independently of TCR endocytosis, as shown for the LAT and SLP76 adaptors, which are rapidly recruited to TCR microclusters upon T cell activation and internalized independently of the TCR (28, 29).

As stated above, expression of the TCR/CD3 complexes at the surface is strongly dependent on the presence of the CD3ζ chain. Indeed, the TCR/CD3 complexes are

either retained in the Golgi stacks in the absence of ζ chain (30), or rapidly internalized from the plasma membrane (9-11). In contrast CD3ζ can be expressed in the absence of the other chains of the TCR/CD3 (31). We will now see how traffic of this chain is regulated.

2.2 The intracellular pools of CD3ζ

CD3ζ is mainly localized in vesicular compartments at steady state (75% is intracellular). It shows partial colocalization with the Rab4b recycling compartment, but is more abundant in Rab3d and Rab8b exocytic compartments (32). CD3ζ is released from the vesicular compartment towards the plasma membrane upon cytosolic calcium increase induced by thapsigargin or ionomycin. Yet, this release is incomplete, consistent with the presence of CD3ζ in the Rab4b recycling compartment. CD3ζ colocalizes at about 40% with VAMP7, a v-SNARE that interacts with a calcium sensor, synaptotagmin-7 (SYT7). Once SYT7 is silenced in CD4+ T cells, the calcium induced CD3ζ release towards the plasma membrane is impaired, though its cellular distribution remains unaltered. In addition, inhibition of Lck traffic which is calcium-independent also results in inhibition of CD3ζ vesicle release to the IS, showing that Lck is involved (32). The recruitment of intracellular pools of CD3ζ to the IS was also shown to depend on Rab35 and its GAP, EPI64C (33). These Rab35 endosomes contain a vesicular SNARE, VAMP3 (34), which was also shown to control delivery of recycling pools of TCR at the IS (8). Thus, it is

possible that at least two distinct exocytic pathways contribute independently, or in a coordinated manner, to the recruitment of the CD3ζ pool to the IS.

TC21 (Rras2) is a GTPase, which has been shown to bind and recruit p110δ, the catalytic-subunit of the phosphatidylinositol 3-kinase (PI3K), resulting in PI3K activation. TC21 was found to bind to all CD3 subunits, including CD3ζ, through their ITAMs. Interestingly, ITAM motifs preferentially interacted with the GDP-bound form of TC21 or with a TC21 inactive mutant. Moreover, CD3ζ levels at the IS reached their peak at the same time and site as TC21 (35). Using time-lapse confocal videomicroscopy and fluorescent-tagged fusion proteins, CD3ζ was shown to colocalize with TC21 in internalized vesicles after synapse formation. Interestingly, part of the internalized CD3ζ could translocate towards a second IS in less than 20 minutes. This suggests that the CD3ζ internalized by the TC21 pathway can be recycled, even if endocytosis of the TCR/CD3 complexes in the cSMAC is generally considered to lead to TCR degradation. This rapid TC21-dependent recycling of CD3ζ might be relevant *in vivo*, where sequential contacts between T cells and APC are often required for full activation of T cells (36, 37).

The CD3ζ vesicles do not contain clathrin heavy-chain indicating that they are formed through CIE. Moreover, clathrin depletion resulted in partial inhibition of TCR downregulation but did not affect the pool of CD3ζ-TC21 coming from the IS. CD3ζ internalized from the IS colocalized extensively with another GTPase: RhoG

that is involved in phagocytosis. Of note, internalization of a transgenic TCR induced by a superantigen from the IS was also affected in T cells from both TC21 and RhoG KO mice (14). Yet, these T cells had a minor defect in internalization of the TCRβ chain. This suggests that TCR internalization from the IS, which requires TC21 and RhoG, represents only about 20% of the TCR internalized upon T cell activation. Therefore, after TCR ligation to the pMHC, the majority of internalized TCR does not come from the IS.

Interestingly, TC21 or RhoG KO T cells proliferated significantly less than wt cells while displaying increased CD69 expression, IL-2 secretion and total levels of pCD3ζ and pERK (14). This could indicate that distinct modules of TCR signalling differentially regulate CD69 expression, IL-2 secretion and T cell proliferation. It is possible that TCR internalization from the IS, which is compromised in the TC21 KO cells, is required for a distal TCR signalling pathway that triggers and supports cell proliferation, such as the activation of mTORC complexes.

In a more recent study performed in resting Jurkat T cells, using a photoactivation approach, CD3ζ was shown to undergo constitutive internalization, that became much faster in activated T cells. The activation-induced internalization was shown to depend on Lck and ZAP70, while it was independent of LAT (16). In the same study, CD3ζ endocytosis is shown to be clathrin-independent and even enhanced after clathrin chemical inhibition, though a silencing approach needs to be used to confirm

this. In agreement with this result, CD3ζ vesicles do not colocalize with clathrin but with flotillin-2, which is known to participate in CIE. Yet, flotillin depletion in T cells did not inhibit CD3ζ endocytosis but rather induced an accumulation of CD3ζ vesicles, which do not recycle normally to the IS (16). In an interesting study, Yudushkin and Vale performed FRET analysis (see BOX 1) to monitor CD3ζ phosphorylation (38). Characterizing the CD3ζ endosomal compartments, they showed that CD3ζ colocalized with early (Rab5, EEA1) endosomes, recycling (Rab11) endosomes, the *trans*-Golgi compartment (TGN46) and lysosomes (LAMP1). At steady-state, only a small percentage of intracellular CD3ζ colocalized with lysosomes. Activation of the cells resulted in its depletion from the cell membrane with simultaneous increase in the intracellular pool. This intracellular CD3ζ colocalized poorly with lysosomes and the phosphorylated form of CD3ζ was clearly in non-lysosomal compartments (38). These results suggest that the internalized pool of CD3ζ is present in recycling endosomes rather than in degradative compartments. These endocytic pools of CD3ζ can either be already phosphorylated or available for phosphorylation by Lck, probably in order to sustain long-term signalling (38).

Hence, the role of the endocytic pools of CD3ζ is still unclear and should benefit of further analysis.

2.3 The intracellular pools of Lck

The Lck kinase is a key player of the TCR-induced signalling cascade. This kinase,

from the Src family, phosphorylates tyrosine residues present in the immunoreceptor-based activation motifs (ITAM) of the TCR/CD3 complexes. Phosphorylation of these ITAM creates binding sites for the tandem SH2 domains of the ZAP70 kinase, which once activated propagates signalling events from the TCR (reviewed in (2)). This propagation is essentially due to the phosphorylation by ZAP70 of LAT, a scaffolding protein that, when phosphorylated, assembles signalling proteins (see below for more information on LAT).

In T lymphocytes, Lck is associated to the plasma membrane and is also present in pericentrosomal endosomes containing mannose 6-phosphate receptor (39, 40). Lck does not have any transmembrane domain and its targeting to the plasma membrane requires both myristoylation and palmitoylation (41, 42). The exact nature of the pericentrosomal pool of Lck is not entirely clear. Yet, Lck is associated with vesicles that bear the Rab11 GTPase (32, 43), the uncoordinated 119 protein (UNC119) (43) and the tetraspanning transmembrane protein MAL (44, 45). Upon activation, the endocytic pool of Lck is recruited to the IS (40).

Rab11 are small GTPase proteins that have been implicated in a variety of cellular traffic pathways. They localize to the TGN and post-Golgi vesicles of the secretory pathway (46). They are involved in maintaining the apical/basolateral polarity of epithelial cells (47). Rab11 proteins are also present in pericentriolar recycling endosomes (48, 49) and have been shown to regulate recycling of various receptors

(50). Rab11 proteins thus function in exocytic processes at the TGN, and in recycling processes via pericentriolar recycling endosomes. Rab11 proteins interact with different adaptor proteins and hence can form complexes with distinct motor proteins, which enable the transport of Rab11 vesicles along microtubules or actin filaments (reviewed in (51)). In T lymphocytes, one of these adaptor proteins, the Rab11-family interacting protein 3 (Rab11-FIP3), has been shown to co-localize with Lck in pericentriolar endosomes (52). Rab11-FIP3 silencing or overexpression induces both a relocalization of Lck at the steady state and a defective delivery of Lck at the IS upon activation. This defect in Lck trafficking is accompanied by a defect in TCR early signalling and IL-2 production (52).

UNC119 colocalizes with Lck both at the plasma membrane and in endosomes (53). It is involved in the intracellular distribution of Lck at steady state and in its trafficking to the IS (43). In UNC119 KO cells, the plasma membrane pool of Lck is reduced whereas the Rab11 positive pool is increased (43). Moreover, the recruitment of Lck to the IS is also inhibited in the absence of UNC119. This might be due to the fact that UNC119 decreases Rab11 GTPase activity, thereby maintaining it in its active form, which binds effectors such as Rab11-FIP3, thus facilitating vesicular transport. UNC119 is also involved in trafficking of cargos in the primary cilium (54). This might be linked to its capacity to regulate the trafficking of ciliary vesicles between the Golgi and the ciliary membrane. Alternatively, it might be due to its

ability to bind and solubilize myristoylated cargos in the cell body (55) and to traffic these cargos to the ciliary membrane. This trafficking model has recently been proposed to regulate Lck transport to the IS. Indeed, UNC119A has been shown to extract and solubilize Lck from membranes of T cells. Lck is then conveyed and delivered to the IS by a mechanism involving the small GTPase ARL3 (53). This non-vesicular trafficking model is compatible with the fact that cytosolic non vesicular Lck has been found in the vicinity of the plasma membrane and shown to incorporate rapidly to the membrane (56). Although, the mechanism underlying this incorporation is still unknown, palmitoylation and depalmitoylation might play a key role. The vesicular and non-vesicular trafficking of Lck may both be involved in the maintenance of distinct intracellular pool of Lck and in the delivery of Lck at the IS.

The MAL protein has also been involved in Lck sorting to the IS. MAL is a highly hydrophobic integral protein expressed by T lymphocytes and polarized epithelial cells, which, like Lck, partitions into detergent-resistant membrane fractions (57, 58). At steady state, MAL co-localizes with the Rab11 positive pool of Lck and upon IS formation MAL travels together with Lck toward the plasma membrane (44, 45). In epithelial cells, MAL silencing impairs the transport of proteins to the apical surface and their missorting at the basolateral surface (58, 59). In T lymphocytes, MAL silencing induces a missorting of Lck, which is not partitioned anymore in the detergent resistant membranes, is not distributed at the cell surface and is retained in

endosomes (32, 44). MAL interacts with the inverted formin-2 (INF2), a protein of the formin family, which promotes actin filament assembly (60) and also binds and bundles microtubules (61). In INF2-deficient T lymphocytes, the formation of vesicles containing MAL and Lck is impaired as well as the levels of Lck at the plasma membrane, leading to an abnormal IS formation of the IS (32, 62). Hence, the current model is that INF2 collaborates with MAL in the formation of the transport vesicles containing Lck and thus to their correct localization in resting and activated T lymphocytes (reviewed in (63)). This might be in part linked to the role of INF2 in the reorientation of the microtubule organizing center towards the IS (64).

Lck activity is finely tuned by phosphorylation and dephosphorylation events. In resting unstimulated cells, up to 50% of Lck is active (65, 66). The presence of this active pool raises the question of the mechanisms that prevent it from inducing T lymphocyte activation. Compartmentalization of the activated molecules in specific endosomes might contribute to the regulation. Although, much is known about the distribution of Lck, its recruitment to the IS and the machinery controling these aspects, the specialization of the different pools of Lck, their activity status and how they contribute to T lymphocyte activation still remain to be found.

2.4 Intracellular pools of LAT

The Linker for activation of T cells (LAT) is a transmembrane protein that plays a key

role in T lymphocyte signalling and function (67-70). Upon T lymphocyte activation, LAT is phosphorylated on multiple tyrosines by the ZAP70 kinase (67). These phosphorylated tyrosines create docking sites for different adaptor proteins and enzymes inducing the formation of multimolecular signalling complexes, also called LAT signalosomes (71). These complexes control downstream signalling and T lymphocyte development (67) and activation (72). LAT has peculiar features: it has no signal peptide thus uniquely relying on its transmembrane domain to be incorporated to membranes; its extracellular domain is very short (4 amino acids); and it is palmitoylated. This palmitoylation is necessary for LAT localization in detergent-resistant membranes (73), as well as its localization at the plasma membrane. Indeed, a mutated form of LAT, which cannot be palmitoylated, is absent from the plasma membrane and localizes in the Golgi (74-76).

LAT is present both at the plasma membrane and in a pericentrosomal intracellular pool (77). At steady state, up to 75% of LAT is intracellular (32). Upon activation, LAT-containing endosomes are rapidly recruited to the IS (Figure 1). The origin of these intracellular pools is multiple.

LAT is present in exocytic pools that colocalize with Rab27a and Rab37 (32), which have both been involved in the transport of exocytic vesicles (78, 79). We were the first to show that the vesicular soluble N-ethylmaleimide-sensitive factor attachment protein receptor (v-SNARE) VAMP7 co-localized with LAT vesicles (80). Two other

groups confirmed this finding (32, 81). VAMP7, like other v-SNAREs, controls the fusion of vesicles with target membranes by interacting with its complementary t-SNARE on the target membranes. VAMP7 associates with the syntaxin 4 t-SNARE (82) as well as with the synaptosome-associated protein of 23 kDa (SNAP23) (83), which together with the VAMP/syntaxin complexes regulates vesicular transport and fusion (84). In many cell types, VAMP7 is present mainly in the Golgi and in late endosomes (85, 86), whereas in T lymphocytes VAMP7 is distributed mostly in the Golgi (Zucchetti A., unpublished data). This v-SNARE protein might have vesicular transport functions that are independent of the fusogenic activity. This is compatible with the biochemical properties of VAMP7, which contains an autoinhibitory Longin domain (85) and has a low fusion efficiency. Indeed, the vesicles decorated with VAMP7 have been shown to leave the cell center and travel on microtubules to the periphery thanks to the interaction of VAMP7 with a protein complex containing Varp, the GolginA4, Rab21, a kinesin and the microtubule regulator MACF1 (87). Our results, obtained in VAMP7 silenced human T cells and in CD4+ T lymphocytes from conditional VAMP7 KO mice, showed that VAMP7 controls the recruitment of LAT vesicles to the IS (80). They also suggest that the VAMP7 decorated LAT-containing vesicles that are recruited to the IS do not fuse with the plasma membrane (80). This is in contradiction with results, obtained by A. Alcover's group, showing that calcium increase induces the disappearance of the intracellular pool of LAT and

that this depends on the Ca^{2+}-sensitive SYT7 (32). The authors thus proposed that Ca^{2+} increase induced by TCR triggering regulates the fusion of VAMP7 positive LAT-containing vesicles with the plasma membrane in a SYT7-dependent way. Yet, to our knowledge, no formal experimental proof of the fusion of the VAMP7/LAT-containing vesicles with the plasma membrane was given. Using high resolution microscopy, L. Balagopalan in S. Samelson's group described kinetically the recruitment of LAT to the IS. They showed that a VAMP7-positive vesicular pool of LAT is recruited after few minutes of activation, that this pool is interacting with microclusters of LAT at the plasma membrane and that vesicles move from microcluster to microcluster (81). The authors observed, by TIRF and lattice light sheet microscopy, oscillations in the fluorescence of the microclusters and increase in LAT fluorescence when vesicles touched the synaptic plasma membrane (81). Although these "flares" could represent fusion events, they were not accompanied by a total collapse of the VAMP7 positive vesicles, as would be the case should the vesicle fuse with the synaptic membrane. However, these data may reflect "kiss and run" exocytosis (88), a much-debated unconventional fusion process that releases intravesicular content through a transient, nanometer-sized fusion pore (89). Alternatively, the VAMP7-positive vesicles may dock transiently to the plasma membrane via the VAMP/syntaxin/SNAP complexes. Such a docking would be sufficient to allow phosphorylation of LAT by ZAP70 and formation of LAT

signalosomes. A better characterization of the fusion/docking of LAT containing vesicles is thus required. In any case, the default of LAT recruitment to the IS, observed in the absence of VAMP7, was accompanied by a defect in formation of the LAT signalosome and of T lymphocyte activation (80), suggesting that the exocytic pool of LAT plays a key role in T cell activation.

LAT is also present in endocytic endosomes labeled with transferrin (77), suggesting a CDE, and endosomes labeled with cholera toxin, suggesting a CIE (90). Upon TCR activation, LAT and SLP76, another adaptor protein that binds phosphorylated LAT, undergo endocytosis (28, 29). The endocytic pool of LAT is at least in part ubiquitylated by c-Cbl, which has been shown to induce the degradation of LAT (91). Yet, internalized LAT can also recycle and be recruited to the IS (Figure 2).

We have recently shown that once internalized, at least part of the endocytic pool of LAT undergoes retrograde trafficking to the Golgi-trans-Golgi network (TGN) (92). This retrograde pathway allows the trafficking of proteins and lipids from endosomes to the TGN-Golgi complex (93, 94). It is conserved from yeast to mammals and is regulated by a complex cargo sorting machinery involving small GTPases, tethering factors, and SNARE proteins (93). This trafficking route was first shown to allow the delivery of some toxin proteins in the Golgi and the endoplasmic reticulum (ER) (95). Yet, this route is followed by several cargos such as the Glut4 transporter (96), the Wntless receptor (97-99), the Alzheimer-associated beta secretase 1 (BACE1) (100),

the cell polarity complex component Crumbs (101, 102) and the β1 integrin (103). In the case of LAT, we were able to demonstrate that the retrograde transport of LAT from endosomes to Golgi depended on the tSNARE Syntaxin-16 and the GTPase Rab6, which are both involved in retrograde transport of other cargos (104, 105).

The retrograde transport of cargos has important roles. It counterbalances the anterograde flow of proteins and lipids, thus maintaining resident proteins in their correct compartments. It also regulates lipid homeostasis (93, 94). Retrograde transport from endosomes to Golgi is also important to maintain cell polarity (101, 102), persistent polarized migration (103) and rapid local delivery of postsynaptic receptors to the shaft domain of neuronal dendrites (106). In T lymphocytes, it controls the polarized local delivery of LAT to the IS (92) and might control the delivery of other molecules, yet to be found. One striking result is that this retrograde transport of LAT, which happens at steady state, is increased by activation of T lymphocytes by APCs (92). A five-fold increase of the retrograde transport of the alpha 5-beta 1 integrin was also observed in RPE1 cells under polarizing conditions (103). Thus, the machinery involved in T cell polarity at the IS might be involved in retrograde transport of LAT.

The defect in LAT retrograde trafficking is associated to a defect in TCR-induced signalling and T cell activation (cytokine production), in the absence of Rab6 and syntaxin 16 (92), showing that it regulates T cell function.

It is worth noting that results obtained in our laboratory indicate that the Golgi has a pivotal role in LAT trafficking. Indeed, as discussed above, the endocytic pool of LAT undergoes a Rab6-dependent retrograde trafficking to the Golgi-TGN (92) and VAMP7, which is present at the Golgi, controls vesicular transport of LAT to the IS. On the same line, we have shown that the intraflagellar transport protein IFT20 controls LAT recruitment to the IS (107). IFT20, unlike other intraflagellar transport proteins, is not only localized to the primary cilium but is present in the Golgi complex in ciliated cells (108, 109) and in T lymphocytes (110). Understanding the links between the vesicular pools of LAT, their trafficking and the Golgi will require more investigation.

In conclusion, several intracellular pools of molecules involved in TCR signalling co-exist. Remarkably, different signalling molecules are present in different vesicles associated to a unique combination of regulators and effectors of intracellular traffic, such as Rab GTPase and v-SNARE proteins. This reflects the fact that these proteins follow different endocytic and exocytic pathways. This happens in the very crowded environment of the synapse and must be regulated. Characterization of this regulation is crucial for full understanding of TCR signalling.

3. THE POTENTIAL ROLE OF LAT RETROGRADE TRANSPORT

We have shown that LAT, once endocytosed, follows the retrograde route to the

Golgi (92). Although the role of this retrograde transport of LAT is still unknown, we will propose here two hypotheses that need to be addressed.

The first hypothesis is that the retrograde transport of LAT allows its association with proteins present in the Golgi and thereby, the formation of specific signalling platforms. Indeed, the Golgi complex is not only a sorting and biosynthetic center for glycoproteins and lipids, it is also a platform for signalling (111). In T lymphocytes several studies have shown that signalling in the Golgi may play a specific role in T cell activation in particular in the activation of the Ras/ERK/MAPK pathway. Ras proteins are small GTPases that regulate MAPK activation. TCR stimulation triggers activation of Ras from the inactive GDP-bound state to the GTP-bound form (112). This GDP-GTP exchange is induced by guanine nucleotide exchange factors such as RasGRP1 (113), which is localized in the Golgi (114, 115). Recruitment of Raf1, by the GTP-bound Ras was essentially thought to take place at the plasma membrane (116). However, it was also shown to take place at the Golgi (114, 115, 117). It was proposed that "the force" of TCR activation controls the localization of the activation of Ras: strong stimulation would lead to activation of Ras at the plasma membrane whereas weaker stimulation would lead to Ras activation at the Golgi (115). Subcellular localization of MAPK activation was also shown to regulate thymocyte differentiation. Negative selection led to activation of MAPK at the plasma membrane, whereas positive selection led to activation of MAPK at the Golgi membrane (118).

More recently, the TRAF3-interacting protein 3 (TRAF3IP3) was involved in the maturation of thymocytes and shown to recruit MEK, which is downstream of Ras and upstream of MAPK, to the Golgi (119). Whether activation of Ras by the TCR takes place at the plasma membrane or at the Golgi membrane is still a matter of debate (120).

To test if LAT retrograde transport can transfer phosphorylated LAT to the Golgi, where it might assemble specific signalling complexes, one can use the capture assay we developed (92), which allows to retain and concentrate the pool of LAT following this pathway in the Golgi (Figure 3).

The second hypothesis is that LAT retrograde transport is required for its "re"-palmitoylation. LAT is a transmembrane protein with an extracellular region of only a few amino acids and a large cytoplasmic domain. LAT is palmitoylated on two Cys residues localized at the junction between the transmembrane and the cytoplasmic domain (73). Palmitoylation of LAT is essential for its scaffolding activity, since optimal recruitment and/or phosphorylation of several interacting proteins such as Grb2, PLC-γ1, Vav and SLP76 require LAT palmitoylation (121, 122). The involvement of palmitoylation in LAT function has been attributed to a role in targeting LAT to specific membrane microdomains enriched in sphingolipids and cholesterol, also called lipid raft (123, 124). Yet, a study challenged this hypothesis. A chimeric protein composed of the cytoplasmic domain of LAT and the

transmembrane and extracellular domains of LAX, another adaptor protein, was introduced in LAT KO T cells. This LAX/LAT fusion molecule, which was excluded from the "raft" microdomains, restored normal T cell activation and T cell development in LAT KO mice whereas a non palmitoylable LAT mutant did not (125). LAT palmitoylation may serve functions distinct from raft targeting. For instance, it has been demonstrated that non-palmitoylated LAT mislocalizes to the Golgi region and has a shorter half-life than the wild type protein, indicating that palmitoylation ensures proper plasma membrane localization and stability (74, 75). This role was also shown for other signalling proteins such as Ras and Src kinases (126). Palmitoylation is a reversible post-translational modification, it may be compared to other reversible post-translational modifications such as phosphorylation or ubiquitination. The majority of the enzymes responsible for palmitoylation reside in the ER and in the Golgi (127). In contrast, acyl-protein thioesterases (APTs) have been shown to localize at the plasma membrane due in part to their palmitoylation (128). Hence, retrograde transport of de-palmitoylated LAT from the plasma membrane proteins, back to the Golgi, where LAT will be re-palmitoylated, may ensure a correct sorting and secretion of endocytic LAT to the IS.

It will be interesting to look at the status of LAT palmitoylation in different compartments and to study if the palmitoylation and de-palmitoylation of proteins is regulated by TCR triggering. On this line, it is worth noting that in anergic T

lymphocytes palmitoylation of LAT is decreased, leading to defective LAT recruitment to the IS and defective LAT functions (129). These results suggest that LAT palmitoylation can be modified by specific signals.

4. NEW HYPOTHESES ABOUT THE FUNCTION OF VESICULAR POOLS OF TCR SIGNALLING COMPONENTS

4.1 Intracellular pools as signalling competent platforms

The presence of signalling platforms on the membranes of the endocytic system is a well-established feature of intracellular receptors, including some of the best studied in immunology: the endosomal Toll-Like Receptors (130). Nevertheless, the signalling platforms of cell surface receptors are most often depicted exclusively at the plasma membrane and receptor endocytosis after cell activation is considered to terminate signalling. This initial concept changed when the epidermal growth factor receptor (EGFR) was shown to signal from endocytic vesicles (131). Starting with this pioneer work, evidence accumulated that several tyrosine-kinase receptors (RTK) and G protein coupled receptor (GPCRs) signal not only from the plasma membrane, but also from endocytic compartments (132, 133). By analogy with RTK and GPCR receptors, one might postulate that the complete TCR receptor or some of the components of the TCR signalosome, assemble at least a part of their signalling platforms on endosomes. This scenario is suggested by several experimental observations, such as the presence of phosphorylated CD3ζ associated to ZAP70 in

the endosomal compartment (38) and the fact that, after their internalization, the SLP76 and LAT adaptors remain phosphorylated in the endocytic compartments, being probably signalling competent (28, 29).

In addition to the detection of signalling components of the TCR signalosome in the endocytic pathway, results obtained on dynamin 2-deficient T cells also suggest a role for endosomes in TCR signalling. Dynamin 2-deficient T cells failed to internalize their TCRβ chain after *in vitro* stimulation with anti-CD3ε antibodies, could not sustain TCR-induced mTORC1 activation *in vivo* and were unable to proliferate (134). At first glance, this defect in T cell activation seems in contradiction with previous reports in which deficiencies in TCR internalization or degradation were accompanied by exacerbated T cell responses. For example, in a transgenic mouse model expressing a dominant negative form of Rab5 that inhibits CDE of TCRβ, T cell activation was accompanied by increase in IL-2 secretion and Ca^{2+} response (135). However, it is conceivable that some endocytic pathways lead to TCR internalization for degradation and signalling termination, while others target the TCR and its signalling associated partners to intracellular compartments compatible with sustained signalling.

Such fine regulations of the balance between receptor signalling and receptor degradation were demonstrated for non-immune receptors such as the EGFR. Indeed, at low ligand concentrations, the EGFR is internalized via CDE, while at high ligand

concentrations it is internalized via both CDE and CIE. CDE of EGFR leads to increased signalling and receptor recycling, while CIE targets the EGFR to lysosomes, leading to receptor degradation (136). Interestingly, for LRP6 (low-density receptor-related protein 6) it is the opposite; CIE of LRP6 sustains signalling, while CDE induces LRP6 degradation (137). Therefore, it seems that the endocytosis pathways can finely tune receptor trafficking and signalling, probably in a receptor and cell type-specific manner. This might apply to the TCR. Identification of endocytic mechanisms that could finely tune the balance between receptor degradation and sustained TCR signalling need further studies. This will be facilitated by the development of new tools, that allow the visualization of TCR signalling (BOX 1) and can be used in combination with cells and animals genetically inactivated for specific endocytic pathways.

4.2 Polarized transport of vesicles may regulate the local lipidic composition in the IS

Several interesting reviews have been written on the role of lipids and lipid modifications in the formation of the IS and the activation of T lymphocytes (for example (138-141)). The goal of this paragraph is not to review the literature on this subject, but to highlight the role that vesicular trafficking might play in the dynamic regulation of the IS lipidic composition.

One striking characteristic of the IS is that the distribution of phospholipids and phosphoinositide is entirely remodeled. For example, diacylglycerol (DAG) accumulates at the inner leaflet of the membrane in the center of the IS (142, 143). PI(3, 4, 5)P3 accumulates in the peripheral zone of the IS (144). Recently, the group of G. Griffiths showed that the formation of the IS in cytotoxic T cells was accompanied by a re-distribution of phosphoinositides in a specialized domain of the plasma membrane (145). Phosphatidylserine is enriched at the IS (146, 147) and more generally, activation of T lymphocytes has been shown to increase the presence of unsaturated lipids at the plasma membrane (148). Hence, the lipidic landscape at the IS is totally remodeled.

Modifications in the lipid composition have important consequences; they drastically change locally the membrane charge (reviewed in (141)). By modifying the local interaction with the cortical cytoskeleton, they change the mechanical stability (145, 149) and the dynamic of proteins in the membrane (144, 150, 151). Modifications of the lipid composition can also change the curvature of the membrane and favor membrane fusion (152, 153).

At steady-state, the relative abundance of major lipid species differs between cellular organelles (154-156). For example, the ER is rich in monounsaturated lipids, whereas polyunsaturated lipids are extremely abundant in synaptic vesicles from neurons (154, 157). Moreover, the lipidic composition of a given membrane is heterogeneous. This

is the case for the plasma membrane of T lymphocytes. This organization of the plasma membrane in domains regulates the partitioning of transmembrane proteins in the different domains according to the length of their transmembrane domain but also to post-translational modifications such as palmitoylation (76, 158).

Little is known about the lipidic composition of the vesicles, yet subsynaptic vesicles that are recruited to the IS have been shown to be heterogeneous, some containing "ordered lipids", whereas others contain "disordered lipids" (159). Moreover, DAG enriched organelles have been shown to be recruited to the IS upon strong TCR activation (160). We herein propose that the polarized delivery of vesicles containing given lipids could efficiently, rapidly and locally induce a change in the lipidic content of the IS. The partitioning of lipids at the IS would thus not only be due to the local activation of enzymes modifying phospholipids (145, 161, 162) but also to the delivery of a "bolus" of vesicular lipids.

Hundreds of different lipid species can be found in the membrane of a single organelle, it is thus particularly relevant to develop tools that will allow the analysis of the lipidic composition of the different vesicles in T lymphocytes. This can be done both by lipid mass spectrometry analysis of purified vesicles (163) and by developing fluorescent probes that can specifically label different lipids without disturbing their structures. It would indeed be informative to characterize and to follow the lipids not only by the identification of their polar heads, i.e. phosphatidylcholine,

phosphatidylserine, phosphatidylethanolamine, but also by their acyl chain content. Indeed, the number of bonds (=unsaturation) in the acyl chains of phospholipids has a great impact on many physical and chemical properties of cellular membranes, such as deformation, fusion and fission (164) and fluidity (reviewed in (165)). Membrane order can be assessed by measuring the "packing order", as loosely packed membranes are more polar because water molecules can more easily penetrate into the lipid bilayer. This change in polarity can be employed to measure the degree of membrane condensation by utilizing fluorescent membrane probes whose emission spectra are solvent-polarity dependent and can report on membrane order. Some lipid dyes have already been used successfully to evaluate the "lipid order state" of membranes. Indeed, Laurdan, a fluorescent membrane probe, that detects change in membrane "packing" or "condensation" (166), was used to image the "condensation" of the plasma membrane at the IS (167). However, such probes have several technical limitations (168) and do not give indications on the exact nature of the lipids.

Development of alternative methods should allow a better analysis of the biophysical properties of the IS and of their lipid composition.

5. IMPACT OF ENDOSOMAL SIGNALLING ON T CELL FUNCTION: OPEN QUESTIONS AND FUTURE DIRECTIONS

Endosomal signalling has been experimentally proven for the GPCR and RTK receptors and it is highly probable that it contributes to TCR signalling. This is suggested, as discussed previously, by the detection of signalling-competent components of the TCR signalosome in endosomes and Golgi stacks (38, 90, 92, 114, 115, 169) and by impaired activation of T cells deficient in endocytic factors (14, 134).

For the TCR, like for all plasma membrane receptors, the initial signals are generated at the plasma membrane and should be accurately communicated to the nuclear transcriptional machinery. This is often realized by a cascade of protein and lipid phosphorylation, which extends the wave of signal over the cytosol and leads to the activation of nuclear transcription factors. The phosphorylated proteins that spread the signal either passively diffuse in the cytosol, or associate with organelles undergoing a retrograde transport. A mathematical model that considers the kinetics of phosphorylation and dephosphorylation reactions predicts that the signals generated at the plasma membrane can spread by diffusion over a distance of 200 nm. In contrast, propagation of the signal beyond this distance is predicted to be much more efficient by receptor association with the membrane of actively transported vesicles (170).

In addition to a wider spread of the signalling waves predicted by modelling, internalization of signalling competent TCR components in endosomes and their retrograde transport to Golgi can sustain TCR signalling in several ways.

First, if we consider the equilibrium between phosphorylation and dephosphorylation, the signalling proteins can be protected, at the endosomal level, from dephosphorylation by phosphatases present at the plasma membrane. The major plasma membrane associated phosphatase that dephosphorylates several components of the TCR is CD45. Although CD45 is required for Lck activation, high concentrations of CD45 inhibit TCR signalling and for this reason CD45 is excluded from signalling-active TCR microclusters. Yet, CD45 persists in the cSMAC (22) and in the synapse of memory T cells (171), situations in which TCR internalization might be beneficial for sustained signalling.

The other important phosphatase that negatively regulates TCR signalling is SHP1. Although SHP1 is a soluble protein, 20-30% of SHP1 is associated to lipid rafts in the plasma membrane through its SKHKED motif (172). In the absence of this motif, SHP1 does not associate with lipid rafts and is unable to exert an inhibitory function on TCR signalling. This suggests that the inhibitory function of the second major phosphatase involved in TCR signalling is exerted mainly at the plasma membrane (173).

In addition to isolating signalling molecules from phosphatases, a second advantage of endocytosis of signalling-competent TCR components could be to facilitate the interaction of the TCR with signalling partners that are not present at the plasma membrane. Indeed, as discussed before, depending on the activation conditions, ERK

and Ras activity were detected in Golgi stacks of T cells (114, 115). Other signalling proteins, such as AKT, mTORC1 and mTORC2 are also present in endocytic compartments (174, 175), although this has not yet been investigated in T cells.

Internalization of TCR signalling components could be particularly important for T cell stimulation by weak TCR ligands, not only for intracellular activation of Ras, but also to avoid negative cross-talk between signalling proteins. On this line, it has been shown that when the T cells are activated by a weak ligand, Lck phosphorylates SHP1. Phosphorylated SHP1 binds Lck and is thus rapidly recruited to the TCR complex, leading to TCR dephosphorylation and inactivation (176). In contrast, upon TCR activation by strong ligands, the kinase ERK modifies the SH2 domain of Lck and this inhibits SHP1 recruitment to the TCR signalosome. The cellular localization of this crosstalk between Lck, SHP1 and ERK, by which the TCR signalling might discriminate between weak and strong ligands, is still unknown. However, Lck and TCR internalization from the plasma membrane might limit the activation of SHP1 by Lck. The analysis of Lck-SHP1 interaction by cell imaging and cell fractionation methods (described in BOX1) could clarify these questions. Understanding how low affinity peptides succeed to activate T cells is important, since during the immune response, T cells with a low avidity for pMHC are the first to egress from the lymph nodes after immunization (177). Although they are short-lived in comparison with the

T cells activated by stronger ligands, they provide a first wave of competent effector T cells that efficiently control the viral load during infection (178).

Finally, the existence of separate pools of proteins involved in TCR signalling that could function as qualitatively distinct signalling modules might be essential not only to discriminate between different affinities of the ligand, but also to regulate the differentiation of T cells and their polarization in Th1, Th2 or Th17. Moreover, precise mechanisms of TCR endocytosis seem to be specific to TCR signalling in each T cell subset, as demonstrated by the deletion of DENND1B, which impairs TCR internalization exclusively in Th2 cells (179). Identification of T cell sub-set specific endocytic factors will be essential to understand the complexity of TCR signalling and to evaluate the function of intracellular pools of TCR signalling molecules in each population of T cells.

BOX 1. NEW METHODS TO STUDY INTRACELLULAR SIGNALLING COMPETENT ORGANELLES

Powerful cell imaging methods have been developed in the last two decades. These include super resolution imaging techniques, such as Photo-Activated Localization microscopy (PALM) and Stimulated Emission Depletion (STED) microscopy, which achieve 50 to 20 nm resolution in x-y dimensions. The acquisition of fluorescent signals near the plasma membrane was improved by using total internal reflection fluorescence (TIRF) microscopy, which allows the separation of the signal from the fluorescent background of the proteins located deeper in the cytosol. These techniques were largely employed to study the signalling near the plasma membrane, which will not be exhaustively developed here, since they have been already covered by excellent reviews (91, 180, 181). We will underline the methods that can be used and improved to investigate the signalling events at the level of intracellular vesicles.

Lattice light sheet microscopy (LLSM)

It is particularly important to visualize in 4D the trafficking of the intracellular pools of signalling molecules. This allows the dynamic characterization of the relationship between different pools of signalling molecules, present at the plasma membrane, intracellular vesicles, associated with organelles or the cytoskeleton. Conventional 4D imaging techniques such as spinning-disk microscopy induce photobleaching and are too slow to follow very dynamic events. In contrast, LLSM is well adapted to follow such events (182). LLSM combines light sheet microcopy with the super-resolution structured illumination microscopy (SIM). This method rather than relying on the exclusion of out of focus light, like confocal microscopy, uses a “sheet” of light to illuminate the cells and is thus much less phototoxic. Moreover, the scanning rate and the z resolution obtained with LLSM are much better than with confocal microscopy

and hence LLSM is well suited to follow rapid events in 4D. This technique has been successfully used to image remodeling of the actin at the IS (182, 183) and dynamic of the microvilli in T lymphocytes interacting with APC (184). Recently, L. Balagopalan and co-workers used this technique to follow dynamically the vesicular pools of LAT and their recruitment to the IS (81) and as discussed in this review, showed that clustering of LAT at the plasma membrane precedes recruitment of the vesicular pools (81). Developing new reporters and thoroughly analyzing formation of signalling complexes spatially and dynamically by LLSM will surely increase our understanding of signalling in T lymphocytes.

Fluorescence resonance energy transfer (FRET) and biosensors

The FRET mechanism involves a donor fluorophore in an excited state that can transfer its excitation energy to an acceptor fluorophore if the two molecules are closer than 10 nm, a distance that usually corresponds to direct protein interactions. Bimolecular FRET, i.e. labelling two distinct molecules with donor and acceptor fluorophores, was used successfully to demonstrate that CD3ζ directly interacts with ZAP70 and Lck (185) upon TCR activation. It was also used to show that LAT and SLP76 adaptors recruit the actin remodelling factors Nck and WASp (186) and that PLCγ1 recruitment to the LAT-nucleated signalling complex involves not only direct interactions with LAT, but also direct contacts with SLP76, Vav and c-Cbl (187). These FRET experiments combined with TIRF microscopy do not necessarily visualize TCR signalling players at the plasma membrane level, since the TIRF method visualizes proteins within the 200 nm beneath the plasma membrane.

FRET can also be performed with probes containing both the donor and acceptor fluorophores. In an elegant study from Yudushkin et al. (38), CD3ζ-GFP was fused to SH2 domains of ZAP70-mCherry. Upon T cell activation, ZAP70 domains associated

to phosphorylated CD3ζ, generating a FRET signal, with GFP as donor and mCherry as acceptor fluorophore. The FRET signal was quantified by FLIM (fluorescence lifetime imaging), which can detect the decrease in the lifetime of the donor (GFP) when FRET occurs. An advantage of FRET-FLIM measurements over FRET by acceptor photobleaching or sensitised emission is that they are independent of fluorophore concentration or excitation intensity. This robust FRET-derived method provided information on the dynamics and localization of pCD3ζ in Jurkat T cells, showing an important accumulation of pCD3ζ associated to ZAP70 on endosomal vesicles, distinct from lysosomes. The pCD3ζ reporter overlapped in the original study with Rab5. Using the same reporter, we observed an important overlap of CD3ζ not only with endosome markers, but also with Golgi vesicles (Figure 4).

FRET was also used to detect tyrosine kinase activity by fusing a known substrate sequence for the kinase with a phosphotyrosine binding domain, such as SH2, flanked by FRET compatible donor and acceptor fluorophores. Hence, the ROZA (Reporter Of ZAP-70 Activity) probe was produced to detect ZAP70 activity (188). The ROZA reporter consists of a fusion between a peptide from LAT, encompassing the tyrosines 132, 175 and 195, which are phosphorylated by ZAP70, and the SH2 domain of Grb2 adaptor, which binds to phosphorylated LAT peptide. The LAT peptide and the SH2 Grb2 domain of the ROZA reporter are flanked by CFP and YFP. This reporter revealed that ZAP70 is not only activated at the IS, but also at the opposite cell pole.

Similar biosensors have been developed to reveal intracellular Ras activation. They are based on the fusion of the Ras binding domain (RBD) of Ras effectors, such as Raf1, with a fluorescent protein. The GFP-fused RBD domain was used to analyze the localization of the Ras active form, but also to develop both intramolecular and intermolecular FRET. For intramolecular FRET, Ras and RBD are connected by a

linker in a single fusion protein and flanked by donor and acceptor fluorophores, while for intermolecular FRET, CFP-Ras is co-expressed with YFP-RBD. The GFP-RDB domain of Raf1 detected Ras activity both at the plasma membrane and Golgi stacks in T cells (114, 115, 189). In addition to the localization of Ras activity, the localization of ERK activity is now testable, thanks to the development of reporters of ERK activity (190). In addition, the team of Matsuda optimized intramolecular FRET biosensors not only for ERK activity, but also for other kinases that are relevant for T cell activation, suck as PKC, S6K and Akt (191).

Using these reporters in T cells activated not only by anti-CD3 and superantigens, but also by physiological ligands of different affinities, should improve our understanding of the localization and the propagation of the TCR signal.

Biochemical tools fractionation of resting and activated T lymphocytes

Although "old fashioned", we think that biochemical tools may be useful to characterize the pools of intracellular signalling molecules. Proteomic analysis of signalling complexes has been used successfully to characterize the formation of signalosomes in different conditions of T lymphocyte activation. In particular, mice in which a tag for affinity purification was knocked into several genes encoding signalling molecules have been used to perform quantitative mass spectrometry of activated primary CD4(+) T cells. These tools allowed the characterization of multiprotein complexes formed around tagged proteins such as ZAP70, LAT, SLP-76, c-Cbl and others (71, 192, 193). Their thorough analysis gave precious and new information in the kinetic of their formation and the different partners recruited. Moreover, bioinformatic analysis of the data with the Cytoscape public software revealed the organization of these signalling complexes as well as their potential functions (194). Yet, these analyses, because they were performed on solubilized

proteins did not give information on the distribution of the different signalosomes in specific intracellular compartments. We described a protocol of purification of membrane compartments containing the adaptor molecule LAT (163). It is based on the preparation of membranes from T lymphocytes expressing a strep-tagged protein (LAT in our case), by mechanically breaking the cells in the absence of detergent. Membranes are then submitted to flotation on a gradient of iodixanol, which allows the separation of different intracellular organelles in different fractions. The enrichment of membranes from different origins can then be characterized by Western blot analysis using markers of the organelles (markers of the Golgi, of the recycling endosomes, of the ER…see figure 5A) and purifications of the membranes containing the strep-tagged transmembrane protein of choice can be realized with Strep-Tactin Sepharose resin. This strategy revealed new trafficking pathways for LAT. Indeed, mass spectrometry analysis of the membranes containing the Strep-tagged LAT revealed the presence of a complex of proteins involved in the retrograde transport of LAT to the Golgi (92). This protocol can thus be useful to characterize the proteomic and lipidomic content of different intracellular pools of signalling proteins in resting and activating conditions. Kinetic analysis of the presence of phosphoproteins in the fractions can also reveal a change in their distribution during time. In the example presented in Figure 5B, the ratio between phosphorylated CD3ζ and total CD3ζ is decreasing in some fractions but it stays stable in fraction 3 where markers of recycling endosomes are found. Such results suggest that upon activation different signalling complexes are present in different pools of membrane and thus different intracellular compartments. These analyses are challenging since they require a large amount of T lymphocytes. Moreover, as for the analysis of the signalosomes in solubilized cells, it would be better to perform these analyses in cells expressing

normal amount of signalling proteins rather than in cells overexpressing these proteins. Designing models adapted to these protocols would certainly bring new information on the vesicular pools of signalling molecules and their dynamic. Using the bioinformatic tools developed to analyze the signalosomes purified from solubilized T lymphocytes (194) and comparing the analysis performed on the different membrane compartments will help inferring their functions.

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FIGURE LEGENDS:

Figure 1: **Kinetic of recruitment of LAT to the IS.** (A) Schematic representation of the "mean cell" method used to analyse LAT recruitment to the IS. Jurkat T cells forming conjugates with Raji B cells (blue) in the presence of SEE were fixed and label with LAT Abs. One z plane confocal images is chosen for each conjugate, images are cropped to keep only the T cell and they are all oriented the same way regarding their synapse (here T cell on the right side). Images are resized to the smallest image (here different times of activation) and fluorescence intensities are normalized by the mean fluorescence intensity of all images. Stack of "normalized" images are finally projected giving "a mean cell" image for each experimental group (here LAT recruitment at the IS at different time of activation). (B) Mean images performed as explained in (A) showing the polarization of the intracellular pool of LAT at 5 min and the progressive enrichment of LAT to the IS. N represents the number of independent images, i.e. T/B synapses labelled for LAT, used to create the "mean cell".

Figure 2: **LAT is internalized in endosomes that are polarized toward the immune synapse upon activation.** (A) Jurkat cells expressing a chimeric LAT protein tagged in its extracellular domain with HA (HA-LAT) were labelled with a mouse anti-HA Ab, washed, and either left at 4°C or incubated for 4h at 37°C. Cells were then fixed and labelled with anti-mouse Ig coupled to Alexa-Fluor568. Confocal images showing the distribution of HA-LAT. (B) Jurkat cells incubated with anti-HA, washed and left at 37°C, to allow HA-LAT endocytosis, were seeded on glass slides together with Raji B cells for 30 min in the absence (-SEE) or presence of SEE (+SEE). Cells were fixed and labelled as in (A). Confocal images showing conjugates

between Raji and Jurkat cells, the localization of the endocytic pool of HA-LAT and its recruitment to the IS. Scale bars: 5μm

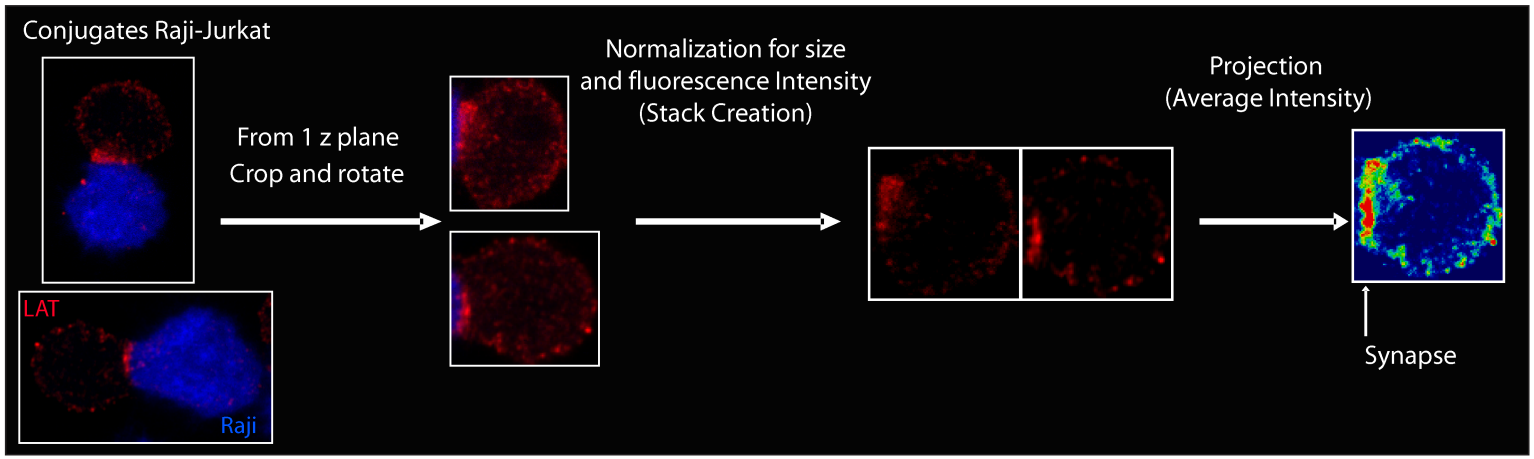
Figure 3: **Capture assay revealing LAT retrograde transport to the Golgi.** Schematic representation of the SNAP-tag capture assay. Jurkat cells expressing HA-LAT and the Golgi-resident GalT-GFP-SNAP chimera are incubated with anti-HA at 4°C. After washing, cells are incubated at 4°C with membrane-impermeable BG-PEG9-NHS to label surface proteins including anti-HA Ab. Cells are then incubated at 37°C, allowing Ab uptake. BG-modified anti-HA Abs that reach the Golgi compartment are covalently captured by the GalT-GFP-SNAP fusion protein. (B) Jurkat cells expressing both GalT-GFP-SNAP and HA-LAT were incubated at 4°C with a mouse anti-HA Ab, washed, and incubated at 4°C without (-BG) or with (+BG) BG-PEG9-NHS. After washing, cells were activated on slides for 30 min with Raji cells pulsed with SEE. After fixation and permeabilization, cells were labeled with anti-mouse Ig (Alexa Fluor 568) to reveal the anti-HA Ab and anti-GFP to reveal the GalT-GFP-SNAP. Representative confocal images representing the maximum intensity from projection of two to four z planes covering the Golgi are shown. A magnified image of the zone in the white dotted square is shown. The profile plots of RGB images from ImageJ are depicted on the right. White scale Bars, 5μm. Gray scale bar, 1μm.

Figure 4: **Intracellular pools of CD3ζ are interacting with ZAP70.** (A) The reporter molecule coding for CD3ζ-GFP-linker-mCherry-SH2 domains of ZAP70 was expressed in Jurkat cells via lentiviral transduction (Yudushkin IA and Vale R, PNAS, 2010). Upon cell activation by plate bound anti-CD3ε antibodies, the interaction of CD3ζ with ZAP70 was quantified by FRET-FLIM both in intracellular compartments and at plasma membrane. The control cells expressing Rab14-mCherry

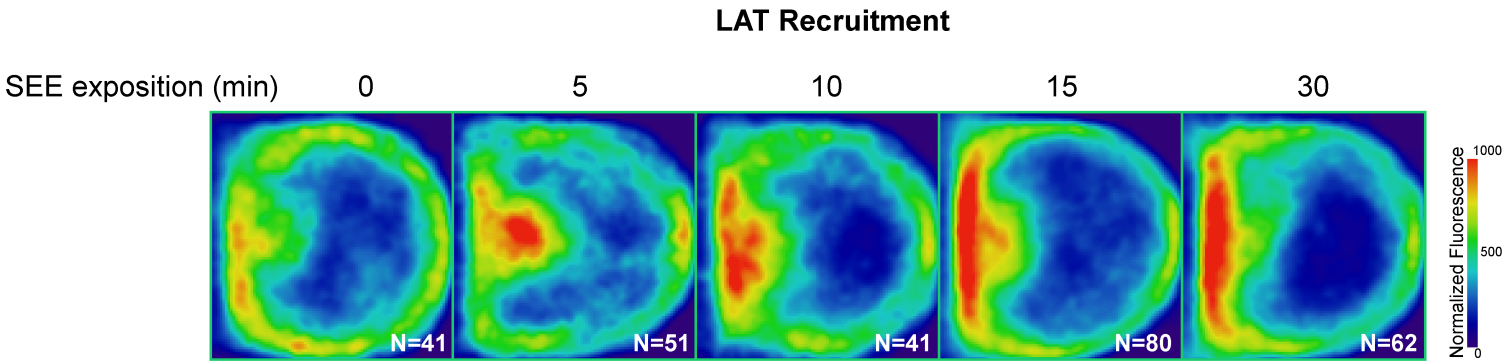
and cytosolic soluble GFP were used to measure the maximal lifetime of GFP in the absence of GFP-mCherry interaction. (B) The CD3ζ FRET reporter used in (A) is localized in intracellular vesicles and in the TGN stacks labelled by anti-Syntaxin 6 antibodies.

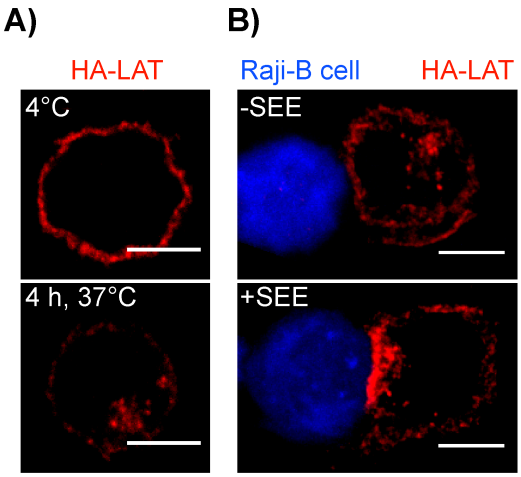
Figure 5 (A): **Characterization of membrane fractions obtained on mechanically broken T lymphocytes.** JCAM2.5 cells (LAT deficient Jurkat cells) expressing a chimeric LAT-Twin-*Strep*-Tag (LAT-TST) molecule were mechanically disrupted and the membranes were subjected to a floatation gradient. After ultracentrifugation, ten fractions were collected. Fraction 1 corresponds to the top and fraction 10 to the bottom of the tube. The different fractions were immunoblotted to reveal the distribution of GM-130 (Golgi), Calnexin (E.R), the t-SNARE STX16, LAT-TST, the v-SNARE VAMP7 and Rab5 (endosomes). (B**)_Distribution of P-CD3ζ in the different fractions after T cell activation.** JCAM2.5 cells expressing LAT-TST were left unstimulated or were stimulated with anti-CD3 (12.5µg/ml) and anti-CD28 (25µg/ml) antibodies for the indicated times. Following stimulation the cells were mechanically disrupted and the membranes were subjected to a floatation gradient as in (A). The different fractions were immunoblotted to reveal on the same membrane both the distribution of phospho-CD3ζ (black arrow) and total CD3ζ (open arrow). The amounts of protein in fractions 1 to 5 were quantified and the ratio of P-CD3ζ (p-z)/total CD3ζ (z) is shown below each fraction. Molecular weights (M.W) are indicated in kilo Dalton.

A)

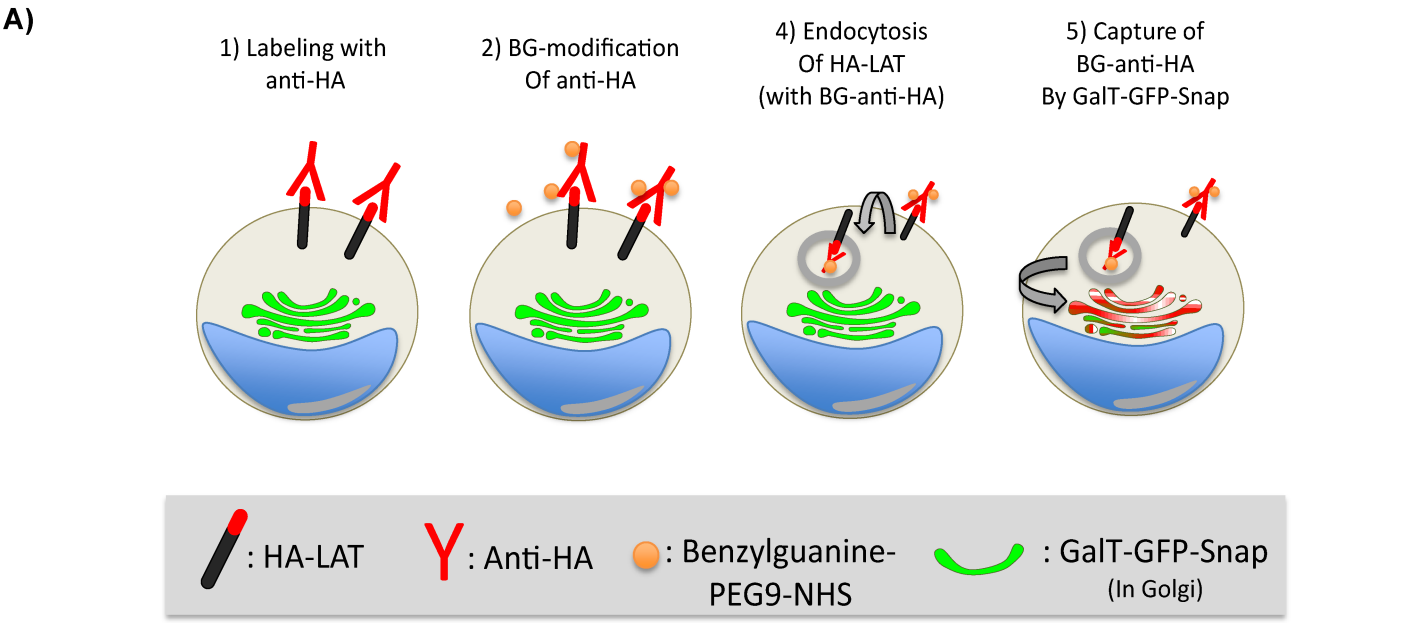

Conjugates Raji-Jurkat
LAT
Raji
From 1 z plane
Crop and rotate
Normalization for size
and fluorescence Intensity
(Stack Creation)
Projection
(Average Intensity)
Synapse

B)

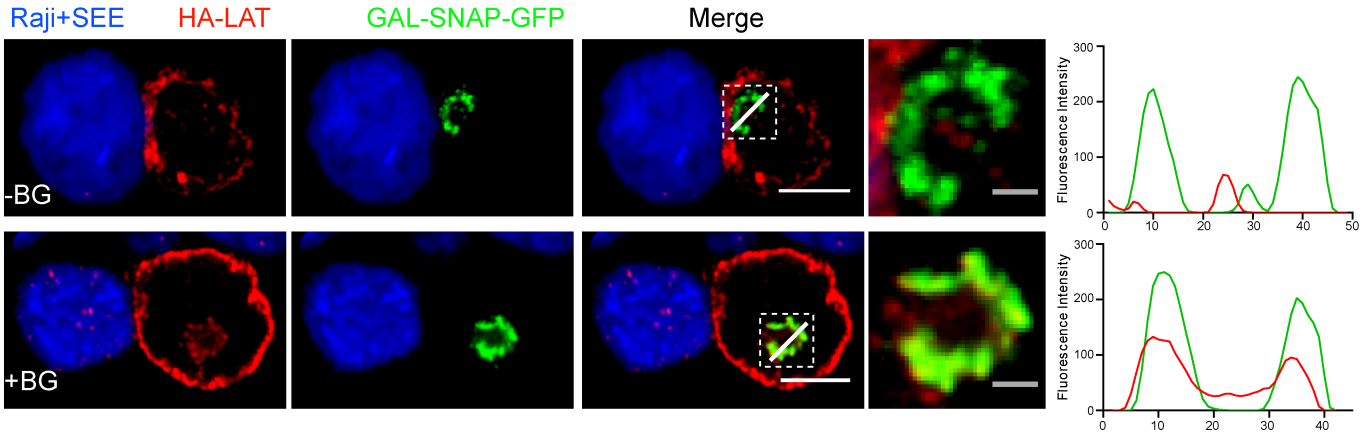

LAT Recruitment
SEE exposition (min)
0
5
10
15
30
N=41
N=51
N=41
N=80
N=62
Normalized Fluorescence
1000
500
0


A)
B)
HA-LAT
Raji-B cell
HA-LAT
4°C
-SEE
4 h, 37°C
+SEE

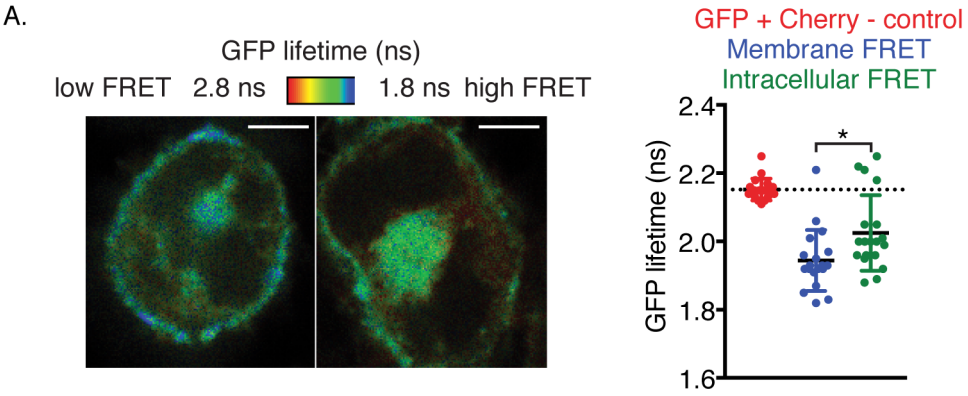
A)


1) Labeling with anti-HA
2) BG-modification Of anti-HA
4) Endocytosis Of HA-LAT (with BG-anti-HA)
5) Capture of BG-anti-HA By GalT-GFP-Snap
: HA-LAT
: Anti-HA
: Benzylguanine-PEG9-NHS
: GalT-GFP-Snap
(In Golgi)

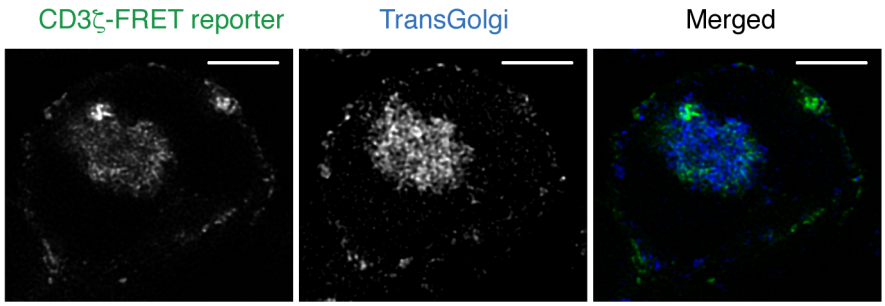
B)

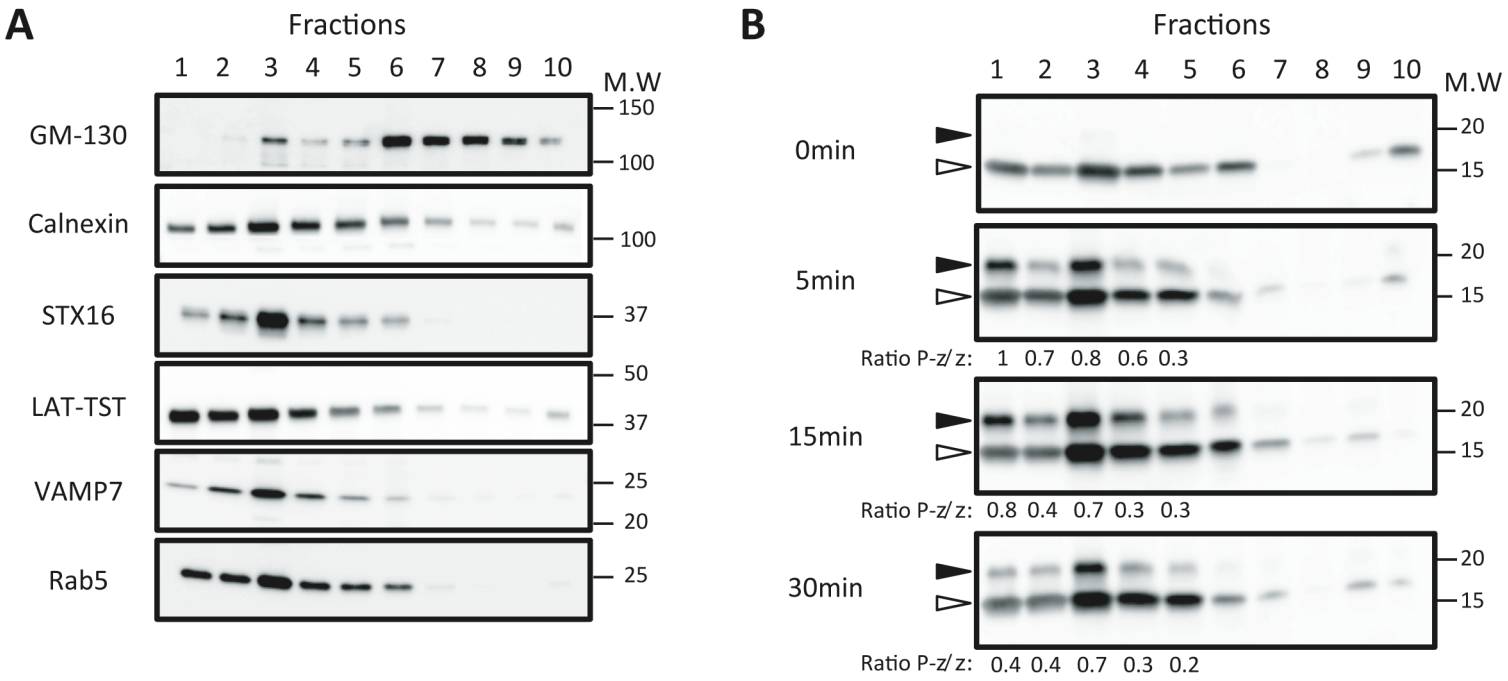

Raji+SEE
HA-LAT
GAL-SNAP-GFP
Merge
-BG
+BG
Fluorescence Intensity
300
200
100
0
0
10
20
30
40
50

A.


GFP lifetime (ns)
low FRET 2.8 ns
1.8 ns high FRET
GFP + Cherry - control
Membrane FRET
Intracellular FRET
GFP lifetime (ns)
2.4
2.2
2.0
1.8
1.6
*

B.


CD3ζ-FRET reporter
TransGolgi
Merged


A
Fractions
1 2 3 4 5 6 7 8 9 10
M.W
GM-130
150
100
Calnexin
100
STX16
37
LAT-TST
50
37
VAMP7
25
20
Rab5
25
B
Fractions
1 2 3 4 5 6 7 8 9 10
M.W
0min
20
15
5min
20
15
Ratio P-z/z: 1 0.7 0.8 0.6 0.3
15min
20
15
Ratio P-z/z: 0.8 0.4 0.7 0.3 0.3
30min
20
15
Ratio P-z/z: 0.4 0.4 0.7 0.3 0.2