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The role of endocytic trafficking in antigen T cell receptor activation



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ABSTRACT

Antigen T cell receptors (TCR) recognize antigenic peptides displayed by the major histocompatibility complex (pMHC) and play a critical role in T cell activation. The levels of TCR complexes at the cell surface, where signaling is initiated, depend on the balance between TCR synthesis, recycling and degradation. Cell surface TCR interaction with pMHC leads to receptor clustering and formation of a tight T cell-APC contact, the immune synapse, from which the activated TCR is internalized. While TCR internalization from the immune synapse has been initially considered to arrest TCR signaling, recent evidence support the hypothesis that the internalized receptor continues to signal from specialized endosomes. Here, we review the molecular mechanisms of TCR endocytosis and recycling, both in steady state and after T cell activation. We then discuss the experimental evidence in favor of endosomal TCR signaling and its possible consequences on T cell activation.

Like all the components of multicellular organisms, T lymphocytes constantly communicate with other cells and their extracellular environment. The plasma membrane plays an essential role in this communication since it expresses a plethora of receptors that are capable of detecting extracellular signals, either by binding soluble mediators, or by recognizing ligands expressed by other cells. The expression of these

various receptors at the plasma membrane is precisely controlled by endocytosis, an active process by which small regions of the plasma membrane are internalized and form vesicles that undergo homotypic fusion to generate early endosomes (EE). EE are the main sorting hub for internalized plasma membrane receptors, which can be either recycled back to the plasma membrane, or delivered to lysosomes for

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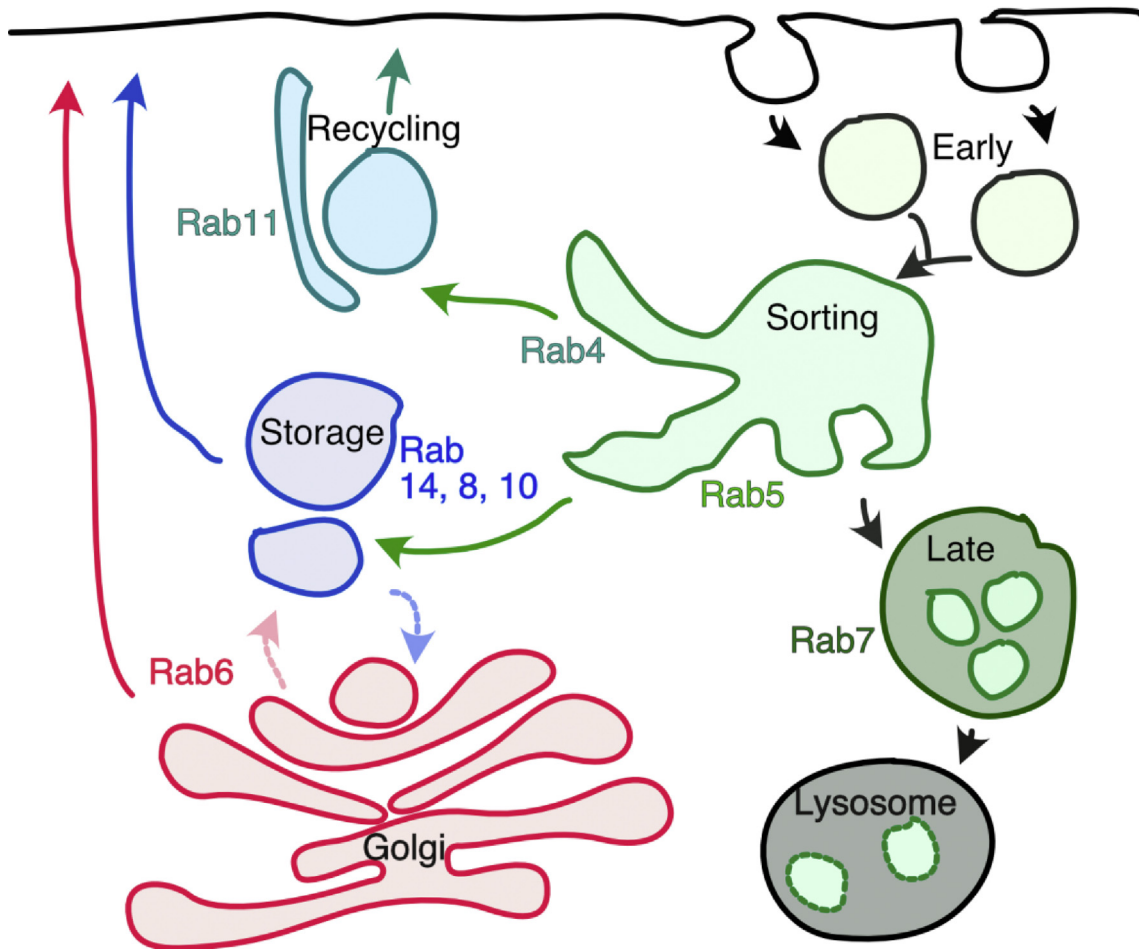


Fig. 1 Overview of endosomal trafficking. Plasma membrane derived vesicles undergo homotypic fusion and form early endosomes, which by further fusion generate the sorting endosome (Rab5). In the sorting endosome, the cargos are selected for lysosomal degradation (Rab7), for retrograde transport to the trans-Golgi network (Rab6) or for recycling. Constitutive recycling can be rapid (Rab4) or slow (Rab11), while regulated recycling (Rab14, 8 and 10) is slow in basal conditions, but sensitive to regulation by cell-specific signal transduction pathways.

degradation (Fig. 1) [1]. The balance between lysosomal degradation and endocytic recycling is essential to preserve a sufficient receptor pool for an optimal ability of cells to adapt to environmental changes. Even if the receptor pool is constantly replenished by newly synthesized molecules, the rate of protein synthesis cannot substitute for the endocytic recycling, which is a rapid and massive process. It is estimated that mammalian cells recycle at least half of their plasma membrane within an hour [2], which probably also applies to T cells. In comparison, protein half-life is much longer varying from 0.5 to 35 h in dividing mammalian cells [3]. Thus, the role of constitutive endocytic recycling on receptor expression at the cell surface must be major. Beside its important role in regulating the cell surface receptor amounts, accumulating evidence supports the concept that endocytosis also promotes signaling amplification and signal diversification for several receptors that recruit signaling adaptors at the endosomal level [4].

This review discusses how endosomal recycling and endosomal signaling platforms contribute to T cell activation, especially by regulating receptors trafficking and signaling. Although several receptors important for T cell function, such

as co-stimulatory or chemokine receptors, are subject to regulation by endocytosis, we focus on the antigen T cell Receptor (TCR) and only briefly mention other receptors of T cells whose signaling involves endocytic platforms.

Overview of endocytosis mechanisms

Depending on the molecular mechanisms that are involved, endocytosis can be roughly divided in two main pathways: clathrin dependent endocytosis (CDE), and clathrin independent endocytosis (CIE) [5]. The proteins involved in CDE were studied in depth and their identification allowed a comprehensive analysis of the CDE “interactome” that confirmed clathrin and its AP2 adaptor as the two major players in CDE [6]. Cells defective for CDE are still capable of endocytosis by CIE [7]. CIE designates several different processes of endocytosis that are poorly understood and which all have as a common feature their dependence on the cholesterol content of the membrane. Based on specific protein association with CIE, five CIE pathways are currently proposed to exist (Fig. 2).

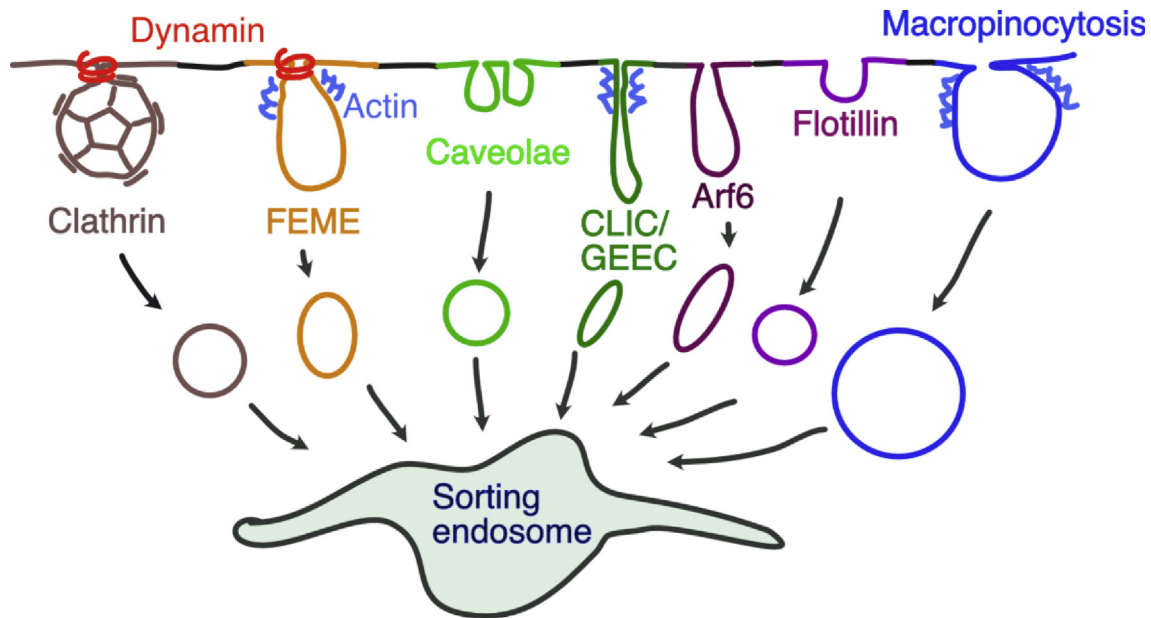


Fig. 2 The main mechanisms of endocytosis. CDE is triggered by binding of the AP-2 clathrin adaptor to the cytosolic tail of the receptor. This initiates the formation of a clathrin-coated pit that is closed by the action of dynamin, a large GTPase that mediates the vesicle fission at the neck of the plasma membrane invagination. FEME is triggered by ligand–receptor interactions, is regulated by endophilins and requires actin polymerization and dynamin-mediated scission from the plasma membrane. Caveolae formation requires caveolin and caveolin-associated cargoes overlap with those of CLIC/GEEC pathway (clathrin-independent carrier (CLIC)/GPI-AP-enriched early endosomal compartment (GEEC)). CLIC/GEEC endocytosis is a constitutive process that does not involve receptor–ligand interactions, but clustering of extracellular galectins that drive vesicle formation and cargo incorporation. The mechanisms of Arf6-mediated and flotillin-mediated endocytosis are unclear, but they seem to be different from the other CIE pathways. Macropinocytosis is in general stimulated by growth factors and is controlled by actin dynamics. Regardless of the mechanism of internalization, early endosomes fuse with sorting endosomes, from where various cargoes are directed towards recycling or degradation.

The existence of these multiple endocytosis pathways complicates the study of the impact of endocytosis on receptor trafficking and signaling, especially since the same receptor often employs more than one pathway for internalization, depending on experimental conditions, such as cell type or ligand affinity and concentration. These aspects have been extensively studied for the epidermal growth factor receptor (EGFR) whose internalization by different endocytic mechanisms has dramatically different consequences in signaling. Thus, low EGF concentration induces EGFR internalization through CDE, whereas high EGF concentration induces EGFR internalization through FEME (Fast Endophilin-Mediated Endocytosis), a CIE pathway that involves dynamin and endophilins [8]. CDE-mediated EGFR endocytosis amplifies signaling and receptor recycling. Conversely, CIE-mediated endocytosis targets the receptor to lysosomes, reducing EGFR signaling [9]. Thus, endocytosis pathways regulate receptor traffic and signaling in several cell types, including the T cells and their receptors, which will be discussed in this review.

Antigen T cell receptor structure and assembly

An activated T cell, powered by cytokines and cytotoxic granule secretion, is a major player in the immune system, and its

specificity must be well controlled to avoid collateral damage. The specificity of T cell activation is entirely based on its TCR, which recognizes the antigenic peptides bound to the major histocompatibility complex (pMHC) molecules. The strength of pMHC – TCR interaction shapes the signaling downstream the TCR and its impact on the fate of the T cell. Thus, low affinity interactions, usually with self pMHC complexes, drive T cell selection and maturation in the thymus and T cell survival in peripheral organs, while higher affinity interactions with non-self pMHC initiate effector T cell responses during infections or tumor development [10]. Therefore, the TCR is able to discriminate between a variety of pMHC complexes, to integrate this diverse information and to convert it into the appropriate signaling cascades. To provide this extraordinary ability to discriminate closely related stimuli, the TCR has evolved as one of the most complex receptors in the immune system, made up of 8 different protein chains: the clonotypic $\alpha\beta$ heterodimers, two CD3 ϵ , a CD3 γ , a CD3 δ and a CD3 ζ homodimer (Fig. 3) [11]. The $\alpha\beta$ heterodimers ensure TCR specificity to the antigen, but are devoid of signal transduction motifs. On the contrary, the CD3 chains have no role in the specificity of TCR interactions with the antigen, but are crucial in signal transduction, providing, all together, 10 ITAM (Immunoreceptor tyrosine-based activation motif) signaling units to the TCR complex. While each of the CD3 ϵ , CD3 γ and CD3 δ chains bears only 1 ITAM, the CD3 ζ homodimer has 6 ITAMs.

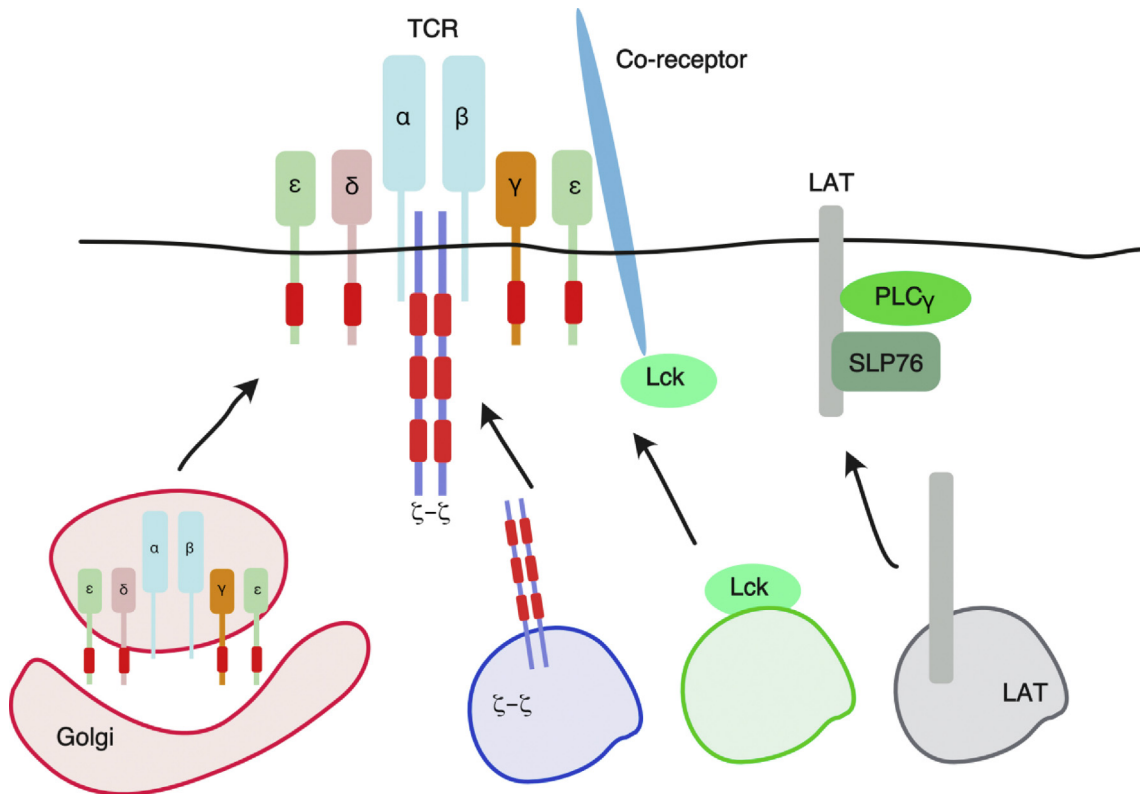


Fig. 3 TCR complex and intracellular pools of its signaling components. At the plasma membrane the TCR consists of the antigen-binding α and β chains, and the associated CD3 γ , ϵ , δ , and ζ chains that contain ITAMs (red boxes) crucial for signal transduction. Inside the cell, the majority of α , β , γ , ϵ and δ chains are found in the endoplasmic reticulum, which they leave as an unstable complex. The ζ chain, which is found in a separate vesicular pool (see text for details), stabilizes the TCR complex at the plasma membrane. In resting conditions, in addition to the ζ chain, essential pools of TCR signaling components, such as the Lck kinase and the LAT adaptor, are mainly located in intracellular vesicles. Under TCR stimulation, all these intracellular pools are polarized towards the synapse. The signaling pathways that trigger this polarized exocytosis are still not well understood.

TCR ITAMs are essential for signal transduction upon TCR interaction with pMHC [12]. Briefly, following TCR activation, TCR ITAMs are phosphorylated by Lck, whose recruitment to the TCR complex is facilitated by CD8 or CD4 specific coreceptors. Phosphorylation of both tyrosines of a TCR ITAM generates a binding site for the kinase ZAP-70, which is stabilized in an active conformation after phosphorylation by Lck. Active Zap-70 phosphorylates then the linker for activation of T cells (LAT) on Y¹³², Y¹⁷¹, Y¹⁹¹ and Y²²⁶. Phospho-Y¹³² is involved in PLC γ 1 recruitment followed by Ca²⁺ increase and Ras-MAPK activation. The phosphorylation of Y¹⁷¹, Y¹⁹¹ and Y²²⁶ is important for the recruitment of SOS and SLP-76 via Grb2 and Gad adaptors. SLP-76 leads to activation of Rac and Ras Rho GTPases. In parallel, TCR activation triggers class I PI3K signaling, either directly, or through the costimulatory receptor CD28. The phosphatidylinositol (3,4,5)-trisphosphate (PIP3) produced by PI3K is bound by several proteins including ITK and SLP-76, which are thus recruited to the membrane and will amplify PLC γ 1 signaling, Ca²⁺ increase and Ras-MAPK activation.

Inactivation of several ITAMs in the TCR complex has suggested that they are functionally redundant [13–17]. Nevertheless, the number of ITAMs was shown to be

important for the control of the signaling threshold during T cell selection in thymus [18], for the development of follicular T cells [19] and for the ability of T cells to respond to low affinity or low amounts of antigen [17,20]. The number of ITAMs also regulates the function of mature, peripheral T cells in which a low ITAM number is sufficient to trigger TCR signaling events leading to cytokine secretion, but a high number of functional ITAMs is required for TCR-driven proliferation, suggesting that ITAM multiplicity is important for fine regulation of TCR signaling [21]. Moreover, not only the number of motifs matters for the regulation of TCR signaling, but it seems that each motif has its own features since downstream signaling molecules bind preferentially, or even exclusively, to a particular ITAM, among the 10 ITAMs of the octameric TCR complex [22–24]. Functional specialization among TCR ITAMs is also suggested by major defects in T cell development in transgenic mice having a unique ITAM sequence for all CD3 chains of the TCR [25]. Overall, it seems clear that ITAM multiplicity gives exceptional plasticity and diversity to TCR signaling, in which the CD3 ζ chain is supposed to play a major role since it provides 6 out of the 10 TCR ITAMs.

In addition to its role in signal transduction, the CD3 ζ chain also plays an important role in TCR stability at the plasma

membrane, as demonstrated by the phenotype of CD3 ζ -deficient mice that have very low TCR expression and in consequence develop very few T lymphocytes [26]. For T cell development, the role of CD3 ζ in TCR stability seems even more important than its role in signaling, as T cell development in CD3 ζ deficient mice can be almost perfectly restored by the expression of a CD3 ζ chain without any functional ITAM. However, this signaling-incompetent CD3 ζ chain was not able to drive T cell activation in suboptimal conditions [20], which supports the double role of the CD3 ζ chain, in controlling both the TCR expression at the plasma membrane and the amplitude and quality of TCR signaling.

Since CD3 ζ is a major component of TCR that is essential for its function, it is quite unexpected that this chain has a distinct intracellular trafficking. Thus, only a small proportion of the CD3 ζ chain, estimated at 25%, is in the TCR complex at the plasma membrane [27]. The remaining ζ chain is located in vesicles, distinct from the endoplasmic reticulum, where the other TCR chains, α , β , ϵ , γ and δ are mainly located. The α , β , ϵ , γ and δ interact in the ER and traffic towards the plasma membrane through the constitutive secretory pathway, while CD3 ζ vesicle translocation to the plasma membrane can be regulated by several stimuli, such as TCR ligation by pMHC, or cytosolic Ca²⁺ increase by thapsigargin and ionomycin [28]. The nature of CD3 ζ containing vesicles has been extensively studied and different markers were reported to partially colocalize with CD3 ζ . Thus, depending on the study, CD3 ζ colocalized partially with: the small GTPases Rab3d, Rab8b [28], Rab35 [29], Rab4 [27,28,30], Rab5 [28,30–33] and Rab11 [31–33], the SNAREs Vamp3 [31], Vamp7 [28] and Stx6 [30], the type II transmembrane protein IRAP (Insulin Responsive Aminopeptidase [30]) and the lipid raft proteins flotillins [33,34]. Given the multitude of fusion and fission events in the endocytic system, it is not surprising that so many typical vesicular trafficking proteins, such as Rabs and SNAREs, have been reported as markers of CD3 ζ vesicles. It is obvious that current data do not allow to build a unitary model of CD3 ζ trafficking and new approaches, possibly by fast multicolor time-lapse microscopy using spectral imaging or *in situ* biotinylation [35,36], will succeed to simultaneously investigate the dynamics of Rab and SNARE proteins associated to the CD3 ζ at steady state and after TCR activation. Despite their limitations, the data available give precious indications about TCR internalization and recycling possibilities, both in basal conditions and after cell activation.

Constitutive versus activation-induced TCR endocytosis

TCR constitutive and activation-induced internalization take place by different mechanisms. The CDE constitutive endocytosis of the TCR is not surprising since the complex bears 22 AP-2-binding motifs, 20 of which are tyrosine-based (Yxx θ) and 2 with Di-leucine sequences. A systematic mutagenesis of each tyrosine-based AP-2 binding motif in the TCR complex demonstrated that although several of them interact with AP-2, the membrane distal Yxx θ motif in the CD3 δ chain has the

highest contribution to TCR internalization [37]. The same study, performed in the 293 cell line, analyzed also the Di-Leucine motifs of CD3 γ and CD3 δ chains and revealed that only the CD3 γ Di-Leucine motifs mediate TCR internalization, as previously reported in T cells [38–41]. The internalization driven by the CD3 γ Di-Leucine motif is amplified by the PKC-mediated phosphorylation of the Ser¹²⁶ residue in the γ chain [40,41] and is prevented by the CD3 ζ chain, which, when integrated in the TCR complex, masks the cytosolic domain of CD3 γ , blocking its interaction with AP-2 [42,43].

An intriguing finding of the systematic analysis of TCR Yxx θ motifs is that the tyrosine-based motifs of CD3 ζ alone were able to induce partial TCR internalization, although their interaction with AP-2 was undetectable [37]. This can be explained by a very weak interaction between monomeric CD3 ζ and AP-2, which might be strengthened in the case of CD3 ζ dimerization. Alternatively, CD3 ζ might regulate TCR internalization by recruiting other internalization partners. Such a partner is the aminopeptidase IRAP that we recently detected as interacting with the CD3 ζ chain [30]. IRAP is a type II transmembrane protein having a cytosolic domain of 110 aa that contains several trafficking motifs, required for the endosomal localization of the protein [44]. Among them, DED^{64–66} and the Di-Leucine^{76,77} [45] are essential for IRAP endocytosis, which is dependent on AP-2 and Dynamin2. In the absence of AP-2 or Dynamin2, the intracellular pools of IRAP and CD3 ζ are completely depleted and these proteins are localized exclusively at the plasma membrane [30]. In the absence of IRAP, the CD3 ζ chain is also entirely localized at the plasma membrane, which indicates that IRAP is required either for the internalization of the CD3 ζ chain, or for its retention in the intracellular pool [30].

TCR activation induces internalization of both antigen-triggered and non-triggered, bystander TCRs. The internalization of bystander TCRs is clathrin-dependent and requires protein tyrosine kinase (PTK) activity, while the internalization of antigen-triggered TCRs is independent of clathrin and PTK activity [46–48]. CDE directs the bystander TCR in endosomal recycling pathways [42,49], whereas CIE seems to target, at least partially, the engaged TCR in lysosomes for degradation [49,50]. The amplitude of activated TCR endocytosis and the balance between recycling and lysosomal degradation appears to be regulated by the strength of TCR activation. Thus, weak TCR activation leads to clathrin-dependent internalization of bystander TCR, while strong activation levels trigger clathrin-independent internalization of the engaged TCR [46]. The precise CIE mechanisms driving antigen-triggered internalization are not well understood and, for the moment, this TCR internalization cannot be assigned to any of the CIE pathways depicted in Fig. 2. However, two Ras family GTPases, TC21 (Rras2) and RhoG, were shown to participate in at least two CIE processes involving TCRs. One concerns the internalization of activated TCR at the immune synapse, and the other, called trogocytosis, involves the internalization of TCR together with peptide-MHC complexes coming from antigen presenting cells (APC) [51].

TC21 or RhoG deficient T cells showed reduced internalization of antigen-triggered TCR and an increase in CD3 ζ chain and ERK phosphorylation, suggesting an increased TCR

signaling. Consistent with an amplified TCR signaling, TC21 deficient cells up-regulated CD69, produced more IL-2 and proliferated better than WT cells when activated with plate-bound or beads-bound CD3 antibodies. Nevertheless, they had reduced proliferation when activated with antigen loaded APC [51]. The reduced proliferation of these cells under physiological TCR activation might be explained by defective PI3K δ activation, since GDP-bound TC21 binds to all the ITAMs of the TCR complex and recruits the catalytic-subunit of PI3K δ . Activation of the PI3K/Akt signaling pathway, including downstream mammalian target of rapamycin (mTOR) complex, is essential for T cell proliferation [52]. This dichotomy between early events following TCR activation, such as CD69 up-regulation, and late events, such as cell proliferation, is an intriguing result which suggests that internalization of activated TCR is absolutely necessary to boost particular signaling modules, such as the PI3K/Akt.

Internalization of the activated TCR is accompanied by TCR ubiquitination and recruitment of endosomal sorting complexes required for transport (ESCRT), leading to lysosomal degradation of the receptor [50]. In contrast, in the same experimental settings, weak TCR activation did not elicit ESCRT recruitment and lysosomal targeting of the TCR [50]. Thus, it seems that under strong activation, TCR internalization limits T cell activation by TCR degradation, in which ubiquitination is a key step [53]. This ubiquitination is performed by two RING finger E3 ubiquitin ligases, c-Cbl and Cbl-b. While the preferred substrate of c-Cbl is the CD3 ζ chain, Cbl-b ubiquitinates also other components of the TCR signaling cascade, such as PI3K, Vav1, PLC γ 1, PKC θ [53]. Deletion or inactivation by mutagenesis of c-Cbl, which is expressed preferentially in the thymus, affects T cell selection [54,55], while deletion of Cbl-b, which is mostly expressed in the periphery, leads to peripheral T cell hyper-reactivity and autoimmunity [53,56]. Therefore, blocking the degradation of activated TCR is definitely deleterious for T cell function, but to our knowledge, there is no experimental proof that shows that 100% of the engaged TCR is rapidly degraded. It is possible that part of the antigen-triggered TCR is diverted from the degradation pathway to other compartments of the endocytic pathway, for recycling, endosomal signaling or both.

Constitutive versus activation-induced TCR recycling

The majority of constitutively internalized TCR is recycled, as demonstrated by TCR trafficking experiments in cycloheximide-treated T cells [57]. Moreover, constitutive TCR internalization is required for TCR accumulation at the immune synapse, as demonstrated by mutation of the di-leucine endocytosis motif of the CD3 γ chain [58]. Masking of this motif by the CD3 ζ chain is necessary for TCR expression at the cell membrane [42]. Nevertheless, the molecular pathway of constitutive TCR recycling has not been investigated in detail and much more attention has been given to recycling pathways that control TCR polarized transport to the immune synapse. The recycling endosomal compartment was shown to be crucial for recruitment of the TCR to the immune synapse and regulated by several components including: Syntaxin-4, the

SNAP-23 t-SNARE and the VAMP-3 v-SNARE [58], Rab35 and its GAP, EPI64C [29]; the intraflagellar transport components IFT20 [59] that directs internalized TCRs from Rab5⁺ endosomes to Rab11, Rab8 and VAMP-3 recycling compartments [31,32]; the EPS-15 homology domain-containing (EHD) proteins that colocalized with all the Rabs associated to TCR recycling [60] and two membrane organizing proteins, flotillin 1 and 2 [34], that coordinate TCR recycling along the Rab5-Rab11 axis [33]. If these factors also affect constitutive TCR recycling, their deletion must also change the TCR complex levels at the plasma membrane. In support to this model, the TCR cell surface amount is significantly decreased in T cells with concomitant deletion of EDH1, 3 and 4 [60]. However, IFT20, Rab35 or flotillins deletion does not change TCR cell surface levels [29,34,59], but their deletion or depletion compromise the recycling of activated TCR, as well as the polarized targeting of the intracellular CD3 ζ pool to the immune synapse. Consequently, IFT20 deficient T cells show decreased phosphorylation of several TCR signaling effectors, such as PLC γ 1, ERK and Vav1, reduced proliferation and reduced effector T cell responses *in vivo* [61]. While the function of IFT20 deficient T cells was thoroughly studied [31,32,59,61], the role of Rab35 in T cell functional response remains to be established. Nevertheless, considering that, similar to IFT20, Rab35 controls cilium function [62], the absence of Rab35 will probably also affect proliferation and effector response of T cells. Analysis of flotillin-2 deficient T cells, which are functionally double ko, since flotillin 1 is not expressed in the absence of flotillin 2, shows a reduced expression of early activation markers, such as CD69 and CD25. The effector function of these T cells has not yet been analyzed [34]. Finally, although the roles of TC21 and RhoG have been mainly analyzed in trogocytosis, the TC21⁺, RhoG⁺ vesicles were shown to contain CD3 ζ originating from internalized receptor from the immune synapse. These vesicles can be rapidly recycled and polarized towards a new APC- T cell contact site [51], which suggests that the two GTPases also play a role in the recycling of activated TCR.

The particular case of trogocytosis

Trogocytosis was first described as a mechanism for lymphocytes to retrieve plasma membrane from APC and was considered to be antigen receptor specific [63]. Later on, trogocytosis was attributed also to other cell types, including non-immune cells [64] and it has been proposed that it is a bidirectional mechanism in which both cells involved exchange plasma membrane domains [65]. Using cell surface markers and flow cytometry, plasma membrane exchange has been observed even in the absence of TCR-MHC interaction [66]. Nevertheless, if this exchange was mediated by trogocytosis or by other mechanisms, such as extracellular vesicles transfer, remains to be established.

Using chemical inhibitors, trogocytosis was shown to depend on Src, Syk and PI3K kinase activities [51,64,67,68], actin-polymerization and, in some conditions, on microtubule motility [67]. However, the best experimental setting to investigate the impact of trogocytosis on T cell function is the inactivation of molecular factors involved in this process. Currently, very few trogocytosis-specific factors have been

investigated by gene deletion, such as the previously mentioned TC21 and RhoG [51].

Functional analyses have shown that trogocytic T cells are more activated than those which did not perform trogocytosis. Thus, following trogocytosis, CD4⁺ T cells produce more IL-2 and proliferate better and CD8⁺ T cells show an increased cytotoxic activity and higher TNF α and IFN γ secretion [69,70]. The stronger activation of trogocytic T cells is supported by the phosphorylation of the ribosomal protein S6, which up-regulates protein synthesis [70]. Since S6 phosphorylation is triggered by the PI3K/Akt pathway, whose activation downstream TCR signaling is controlled by the Ras GTPases TC21 and RhoG [51], it will be interesting to investigate cytotoxic abilities of TC21 and RhoG deficient T cells. Overall, trogocytosis appears to improve effector functions of T cells, but on the other hand it has also been proposed that it promotes fratricidal killing between activated T cells, which, dressed with the pMHC complex stolen from APCs, become false targets for other T cells [71,72]. In a recent study, Boccasavia et al. demonstrated that T cells become themselves presenting cells after trogocytosis of pMHC derived from APCs. Interestingly, antigen presenting T cells acquire a regulatory phenotype while T cells that recognize these presenting T cells differentiate into proinflammatory Th17. Balance between the two phenotypes seems to be regulated by antigen quantity, since low antigen burden promotes Th17 over Treg differentiation [73]. To conclude, it seems that the beneficial or detrimental role of trogocytosis for T cell function is largely dependent on antigen burden and this parameter should be taken into account for future studies.

Endocytic platforms in TCR signaling

The ability of several immune and non-immune receptors to use endosomal signaling platforms has been well established (reviewed in Refs. [4,74]). In contrast, the ability of TCRs to use endocytic signaling platforms has been proposed more recently, although stable association of active TCR signaling components, such as Lck, ZAP-70, LAT and SLP-76, with endosomal membranes has been observed in the past [75–77].

An elegant tool which revealed the intracellular localization of signaling-competent CD3 ζ chain was developed by Yudushkin and Vale [78]. It consists of a FRET reporter in which eGFP and mCherry are inserted between the CD3 ζ chain and the tandem SH2 domains of the human ZAP-70. When the CD3 ζ ITAMs are phosphorylated, the SH2 domains of ZAP-70 bind to them, increasing the FRET efficiency between donor (eGFP) and acceptor (mCherry) fluorochromes. This approach demonstrated that the FRET signal, equivalent to CD3 ζ – ZAP-70 binding after T cell activation, is not only localized at the plasma membrane, but also in intracellular vesicles, where it overlaps with Rab5 and Rab11. Since the generation of intracellular FRET signal was entirely dependent on Lck activity and active Lck was found to colocalize with the FRET reporter, internalized CD3 ζ was proposed to be a direct substrate of endosomal Lck or of an Lck-dependent tyrosine kinase [78]. Interestingly, blocking of endocytosis by chemical inhibition of dynamin strongly reduced the intracellular FRET signal, indicating that endosomal signaling involves CD3 ζ that has

been internalized from the plasma membrane. Therefore, if CD3 ζ phosphorylation occurs directly on endosomes, the receptor activated at the plasma membrane should be sorted from early endosomes towards signaling-compatible endosomes that contain active Lck. This sorting step should involve components of the retromer, the main protein complex that rescues the receptors from lysosome-mediated degradation. This sorting step might be mediated by IRAP which interacts with CD3 ζ [30] and the core of the retromer formed by VPS35, VPS26 and VPS29 [79]. Alternatively, in the full TCR complex, the NPxY motif of the CD3 ϵ chain can bind the sorting nexin 17 (SNX17), which participates in TCR recycling [80], probably in cooperation with EDH proteins [60,81].

Another argument in favor of the endosomal TCR signaling hypothesis comes from the study of dynamin 2-deficient T cells [82]. Mice with T cell specific deletion of dynamin 2 have normal T cell development, but reduced numbers of peripheral T cells. Dynamin 2-deficient mature T cells fail to proliferate in response to TCR activation by self pMHC ligands, as well as in response to foreign antigens, as demonstrated by homeostatic T-cell proliferation assays and *Listeria monocytogenes* infection models. Mechanistic studies of dynamin-deficient T cells demonstrated that they activate only transiently mTORC1 upon binding of a ligand to the TCR. The readout of mTORC1 activity was the level of the phosphorylation of the ribosomal protein S6 measured by flow cytometry. Dynamin 2-deficient cells up-regulated phospho-S6 only in the first 8 h after activation, while wt cells showed sustained S6 phosphorylation, up to 20 h. Since the main upstream regulators of mTORC1 activity, such as the activity of PI3K/Akt and AMP-activated protein kinase α , were normal in dynamin 2 deficient T cells, the reduced mTORC1 activity was attributed to reduced expression of c-Myc, a transcription factor that enables the metabolic reprogramming required for T cell proliferation [83]. Thus, dynamin 2 is essential for c-Myc and mTORC1 activation downstream the TCR, presumably by allowing the building of particular TCR signaling platforms at the endosomal level. Future studies, using cell imaging methods [78,84], identification of TCR signalosome composition [35,85] and new tools that can visualize mTORC signaling [86] in the absence of dynamin could be used to directly confirm this hypothesis.

Additional characterization of the role of endosomal TCR signaling in T cell activation and survival is provided by the analysis of IRAP-deficient cells and mice in which constitutive internalization of the CD3 ζ chain is compromised, leading to an increased level of the entire TCR complex at the plasma membrane [30]. Despite the high level of TCR at the plasma membrane, IRAP-deficient Jurkat T cells show reduced phosphorylation of Lck, LAT, ZAP-70, PLC γ and CD3 ζ itself and a strong reduction in IL-2 secretion after TCR stimulation with superantigens or cognate pMHC ligands. This activation defect can be explained by the absence of endosomal signaling in IRAP-deficient cells, as illustrated by the experiments using the FRET reporter that quantifies CD3 ζ interaction with ZAP-70 [78]. While in wt cells the majority of FRET signal equivalent of CD3 ζ -ZAP-70 interaction is found in intracellular vesicles, in IRAP-deficient cells this signal is detected exclusively at the cell surface [30]. The colocalization of the FRET reporter with IRAP and IRAP interaction with CD3 ζ , Lck and ZAP-70 indicate that the ζ chain interaction with ZAP-

70 occurs on IRAP endosomes. Mice with T cell specific deletion of IRAP have some similarities to dynamin deficient ones, such as normal development of T cells in thymus, but low numbers of T cells in the periphery. In contrast to dynamin 2 deletion, IRAP deletion does not block T cell divisions [30,82], although it reduces their survival following suboptimal activation and their ability to control tumor growth [30]. These discrepancies between IRAP-deficient and dynamin-deficient mice must be due to the different effects of protein inactivation on TCR trafficking. Thus, inactivation of both proteins reduces CD3 ζ endosomal signaling measured by FRET [30,78], while endocytosis of activated TCR is blocked by dynamin inactivation [82], but not by IRAP deletion [30]. It is possible that the composition of endosomal signaling complexes varies along the endosomal trafficking pathway. In this case, by blocking TCR trafficking at different stages, IRAP and dynamin can have distinct effects on its signaling.

In conclusion, substantial experimental data indicate that after activation, the internalized TCR is able to signal from endosomes, but the endosomal source of TCR ligands remains unclear. This question is essential since, in general, receptor signaling depends on the presence of its ligand and ligand-independent signaling is only a prerogative of mutated oncogenic receptors. A potential source of TCR ligands in endosomes comes from trogocytosis since pMHC complexes nibbled away from APCs have been showed to colocalize with phosphorylated CD3 ζ , ZAP-70, Lck and ERK [87].

Possible advantages of TCR endosomal signaling

While the available data demonstrates the ability of TCR to signal from endosomes, the “raison d’être” for endosomal signaling is not yet elucidated, but probably, it serves to amplify and diversify TCR signaling. Mathematical modeling demonstrates in an elegant way how endosomal signaling could be beneficial for various cell types. Signals coming from the plasma membrane can be transmitted much more efficiently after association of the receptor with intracellular vesicles, for distances longer than 200 nm which is the limit for simple diffusion of the signal [88]. Moreover and especially in T cells, a signaling amplification mechanism might be the spatial separation of phosphorylated signaling adaptors from phosphatases that can inactivate them. Indeed, T cells express around 50 phosphatases, whose impact on TCR signaling is not determined only by their substrate specificity, but also by their intracellular location [89,90]. Finally, signaling diversification might also be promoted by receptor proximity with downstream signaling modules located on endocytic vesicles, such as mTORC1 [91], mTORC2 [86], Akt [92], MAPKs [93,94] or TRAF endosomal signaling adaptors [95].

The intracellular dynamics of the main TCR signaling components might be investigated in the future using cell imaging approaches that have already been successfully applied in the study of the ζ chain, such as photoactivatable [34] or FRET compatible reporters [30,78] and optogenetic tools [33]. We already identified the ϵ chain in IRAP signaling endosomes using an *in situ* Proximity Ligation Assay [30], but the lack of antibodies recognizing the other TCR chains and detected by immunofluorescence, depicts the necessity of

other approaches to study whether they are involved in endosomal signaling. The components of plasma membrane and endosomal signaling platforms could be identified as previously by tandem affinity purification [85], or by *in situ* biotinylation [35], using cells with defective endosomal signaling. Combined with functional analyses of mice and T cells in which endosomal TCR signaling is impaired, these approaches could elucidate the importance of endosomal TCR signaling on T cell functions and might help to improve T cells-based immunotherapy strategies.

Conflicts of interest

The authors declare no conflicts of interest.

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