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Cell Biology of T Cell Receptor Expression and Regulation

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Abstract

T cell receptors (TCRs) are protein complexes formed by six different polypeptides. In most T cells, TCRs are composed of αβ subunits displaying immunoglobulin-like variable domains that recognize peptide antigens associated with major histocompatibility complex molecules expressed on the surface of antigen-presenting cells. TCRαβ subunits are associated with the CD3 complex formed by the γ, δ, ε, and ζ subunits, which are invariable and ensure signal transduction. Here, we review how the expression and function of TCR complexes are orchestrated by several fine-tuned cellular processes that encompass (*a*) synthesis of the subunits and their correct assembly and expression at the plasma membrane as a single functional complex, (*b*) TCR membrane localization and dynamics at the plasma membrane and in endosomal compartments, (*c*) TCR signal transduction leading to T cell activation, and (*d*) TCR degradation. These processes balance each other to ensure efficient T cell responses to a variety of antigenic stimuli while preventing autoimmunity.

INTRODUCTION

Antigen-presenting cell (APC): immune cell specialized in antigen processing and presentation (dendritic cells, macrophages, B cells)

Peptide-MHC (pMHC): a complex formed by short peptides associated with MHC class I or class II molecules; antigenic peptides come from the hydrolysis of exogenous or endogenous proteins during antigen processing and presentation (178)

The T cell antigen receptor (T cell receptor or TCR) was first described in the 1980s as a glycosylated αβ heterodimer expressed at the surface of both human and mouse T cells and differentially recognized by monoclonal antibodies specific to T cell clones, i.e., clonotypic (1–4; reviewed in 5). Clonotypic αβ heterodimers were found to be associated with the T3 protein complex, which was later named CD3 and is composed of three polypeptides and expressed in all T cells (6–8). Antibodies to the αβ or the CD3 proteins can block antigen-induced T cell responses but also trigger T cell proliferation and IL-2 production when appropriately cross-linked (1–4, 9, 10; reviewed in 5). TCRαβ proteins were shown to have sequence diversity and homology to immunoglobulins (11, 12). Cloning of TCR genes demonstrated their homology with immunoglobulins by the presence of variable (V), diversity (D), and joining (J) immunoglobulin-type domains that undergo somatic recombination as well as constant (C) domains (13–19). A different type of TCR carrying the TCRγ and TCRδ variable chains (not to be confused with CD3γ and CD3δ subunits) and having a different type of antigen specificity was later discovered (17, 20, 21; reviewed in 22). T cells carrying γδ TCRs are a minor fraction of peripheral T cells in humans, being more numerous within T cells from mucosal compartments and having immune functions distinct from those of αβ T cells (23).

The CD3γ, CD3δ, and CD3ε subunits are genetically related to each other, belonging to the C-type immunoglobulin superfamily. They are encoded by closely linked genes that do not undergo recombination. CD3γ and CD3δ originated by gene duplication in a mammalian ancestor and are tightly linked and oriented head to head (24–28; reviewed in 22). The ζ subunit is genetically and structurally unrelated to the other TCR and CD3 subunits. It has a very short extracellular tail and a long intracellular region, and it is encoded by a chromosome different from that which carries the other CD3 subunit genes (29, 30).

Contrary to immunoglobulins that can recognize antigens in their native form, most αβ TCRs recognize short peptides that are processed by antigen-presenting cells (APCs) and that are expressed on the APC surface bound to major histocompatibility complex (MHC) class I molecules (recognized by $CD8^+$ T cells) or MHC class II molecules (recognized by $CD4^+$ T cells). Some αβ TCRs, expressed by natural killer T cells and others, recognize glycolipids bound to nonclassical MHC class I molecules and those on mucosal associated invariant T (MAIT) cells recognize riboflavin derivatives, whereas the less-characterized ligands for γδ TCRs appear to be more heterogeneous. An additional striking difference between immunoglobulins and TCRs in antigen recognition is their affinity for their respective ligands. Unlike immunoglobulins, the variable domains of αβ TCRs do not undergo somatic hypermutation; after they are selected in the thymus, the TCR amino acid sequences remain unaltered after their first encounters with antigen. Therefore, the affinity of TCRs is several orders of magnitude lower than that of immunoglobulins, when measured using soluble molecules (reviewed in 22). However, increased avidity and proper orientation due to simultaneous interactions between several membrane-bound TCRs on T cells and antigen peptide-MHC (pMHC) and endogenous pMHC complexes on APCs likely stabilize TCR-ligand interactions under physiological conditions (31, 32). Moreover, the coreceptors CD4 and CD8 and adhesion and cosignaling proteins that enhance the tightness of T cell–APC interactions may also influence TCR-ligand interactions in a physiological context. Finally, TCR nanoclusters, facilitated by cytoskeletal networks or local membrane lipid composition, may also strengthen TCR avidity (33–36).

TCR expression and function depend on a number of cell biology processes that control (*a*) synthesis, assembly, and expression of TCR-CD3 complexes at the plasma membrane; (*b*) membrane localization and dynamics of TCR-CD3 complexes at the plasma membrane and

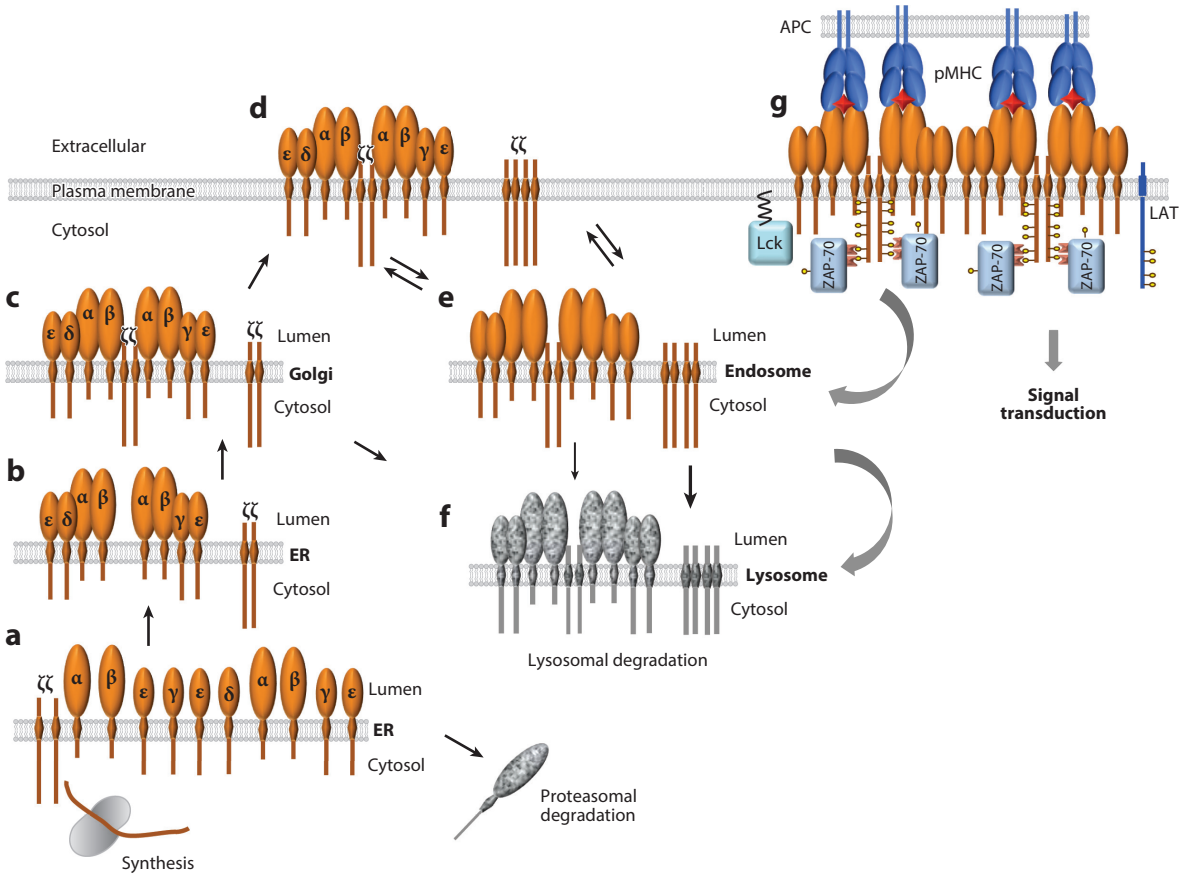

Figure 1

Schematic representation of the cell biology of TCR expression and regulation. (*a–d*) Synthesis and assembly. (*a*) TCR-CD3 subunits, with the exception of CD3ζ, are synthesized in excess and translocated to the ER membrane, where they are retained, owing to ER retention motifs, until they assemble with other subunits. Unassembled TCRα, TCRβ, CD3γ, CD3δ, and CD3ε subunits are degraded by the proteasome, without reaching the Golgi apparatus or the plasma membrane. (*b*) Partial complexes comprising TCRαβ and CD3εδ or CD3εγ are formed in the ER and can progress to the Golgi apparatus when CD3ζ joins them. (*c,d*) Full complexes having all of the six different subunits assembled in the Golgi apparatus can be delivered and stably expressed at the plasma membrane. (*d,e*) TCR dynamics between the plasma membrane and endosomal compartments: Once at the plasma membrane, TCR-CD3 complexes undergo cycles of endocytosis and recycling. (*f*) A minor proportion of partial complexes are degraded during every cycle of endocytosis. Moreover, in the absence of CD3ζ, TCRαβ and CD3εδ and CD3εγ partial complexes are degraded in lysosomes. CD3ζ likely traffics, at least in part, independently of the rest of the complex, since it is more detectable in endosomes and has a shorter half-life. (*g*) TCR signal transduction. After encountering peptide-MHC complexes (pMHC) on APCs, TCR-CD3 complexes form clusters and their intracellular regions become accessible to phosphorylation by the tyrosine kinase Lck. The latter initiates signal transduction via phosphorylation of ITAMs, leading to the interaction of ZAP-70 with CD3ζ. In turn, ZAP-70 phosphorylates the signaling adaptor molecule LAT. Tyrosine phosphorylation of CD3ζ, ZAP-70, and LAT is depicted by yellow circles in the intracellular regions (detailed in **Figure 4**). Abbreviation: ITAM, immune receptor tyrosine-based activation motif.

within intracellular vesicular compartments; (*c*) the ability of the TCR to transduce signals; and (*d*) TCR degradation (**Figure 1**). The balance between these processes ensures TCR expression and the dynamics needed for T cells to respond to a large variety of antigenic stimuli such that immune responses are efficient and do not cause autoimmunity. This review summarizes these cell biology processes, focusing on the best-characterized αβ TCRs.

STRUCTURE OF THE TCR-CD3 COMPLEX

Immune receptor tyrosine-based activation motif (ITAM): peptide sequences characterized by two regularly spaced tyrosine residues (YxxL/Ix (6–8)YxxL/I) present in the intracellular regions of various immune receptors and phosphorylated during immune cell activation (40)

Complementary determining region 3 (CDR3): hypervariable regions that form a loop in the TCRα and TCRβ subunit variable domains and are involved in pMHC interactions

TCR α and β subunits have extracellular domains formed by variable and constant immunoglobulin-like domains covalently linked by a disulfide bond between two cysteine residues. A long stalk sequence of unknown structure connects immunoglobulin-like domains with the transmembrane regions, which are key to maintaining TCR complex association. Finally, intracellular regions are short, apparently nonstructured, and not involved in signal transduction. TCRαβ subunits are noncovalently associated with the CD3γ, CD3δ, and CD3ε subunits, which are formed by constant immunoglobulin-like extracellular domains, a connecting peptide, transmembrane domains, and intracellular regions much longer than those of the TCRαβ subunits (reviewed in 22, 37, 38). The CD3ζ subunit is composed of a short extracellular region, a transmembrane domain, and a long intracellular segment (29). CD3ζ forms disulfide-linked homodimers and may form heterodimers with CD3η, which is highly homologous to CD3ζ and expressed at much lower levels in thymocytes and T cells (30). Finally, the FcRγ subunit, also structurally related to CD3ζ and usually associated with the high-affinity Fc receptor, may also form heterodimers with CD3ζ (39). All CD3 subunits display signal transduction motifs also present in other immune receptors, e.g., B cell and Fc receptors. These motifs, named immune receptor tyrosine-based activation motifs (ITAMs), are present as a single copy in CD3γ, CD3δ, and CD3ε and three copies in CD3ζ (40).

Low-resolution negative staining and electron microscopy of detergent-isolated TCR complexes established their maximum length and width to be 12 nm × 6.5 nm (41). Moreover, an extensive panel of X-ray and nuclear magnetic resonance structures of the TCRαβ ectodomains, in isolation or in complex with pMHC, has been reported (42). Finally, there is high-resolution structural information on the arrangement of the ectodomains of the CD3γε and CD3δε dimers, of the transmembrane domain of the CD3ζ homodimer, and of the cytoplasmic tail of CD3ε (43–46). However, a high-resolution structure of the entire TCR complex is still lacking, and the arrangement of the ligand-binding TCRαβ relative to the signaling CD3γε and CD3δε subunits, which is key to understand mechanisms of signal transmission, remains unknown.

The TCRαβ ectodomains adopt a structure resembling that of immunoglobulin Fab fragments, with the hypervariable loops of TCRα and TCRβ oriented toward the α1 and α2 helices of MHC class I or II molecules loaded with antigenic peptide. The third hypervariable loops of TCRα and TCRβ, named complementary determining region 3 (CDR3α and CDR3β), occupy a central position on the pMHC surface, being mostly responsible for peptide antigen binding, whereas the germline-encoded CDR1 and CDR2 hypervariable loops mostly interact with amino acids of the α1 and α2 MHC helices (**Figure 2**). The affinities of natural TCRs for their pMHC ligands span a wide range, from 0.5 μM to >100 μM (47), although TCRs with an affinity less than 10 μM are uncommon. Interestingly, the CDR3 loops in TCRs with the highest affinities have been reported to undergo conformational changes to fit the pMHC surface (48–50). Another conserved feature is that the plane formed by the six CDRs of the αβ TCR usually forms an angle of ~30° with the plane of the pMHC surface (50). This angle seems to be important for signal transduction: An artificial TCR with high-affinity binding to the pMHC but parallel planes does not signal (51). The surfaces of interaction Vα-Cα and Vβ-Cβ are quite big, suggesting a rigidity that might be important for downward signal transduction. Interestingly, the stalk sequences of the αβ ectodomains are longer than those of the CD3 subunits, and it has been proposed that such a feature may indicate that the αβ ectodomains rest on the CD3 ectodomains (52) (**Figure 2**). This notion is supported by the presence of a large FG loop connecting the Vβ and Cβ domains that is proposed to cover the ectodomain of the CD3εγ dimer. This possible layered arrangement of the CD3ε ectodomains could be important for a proposed piston-like movement of αβ

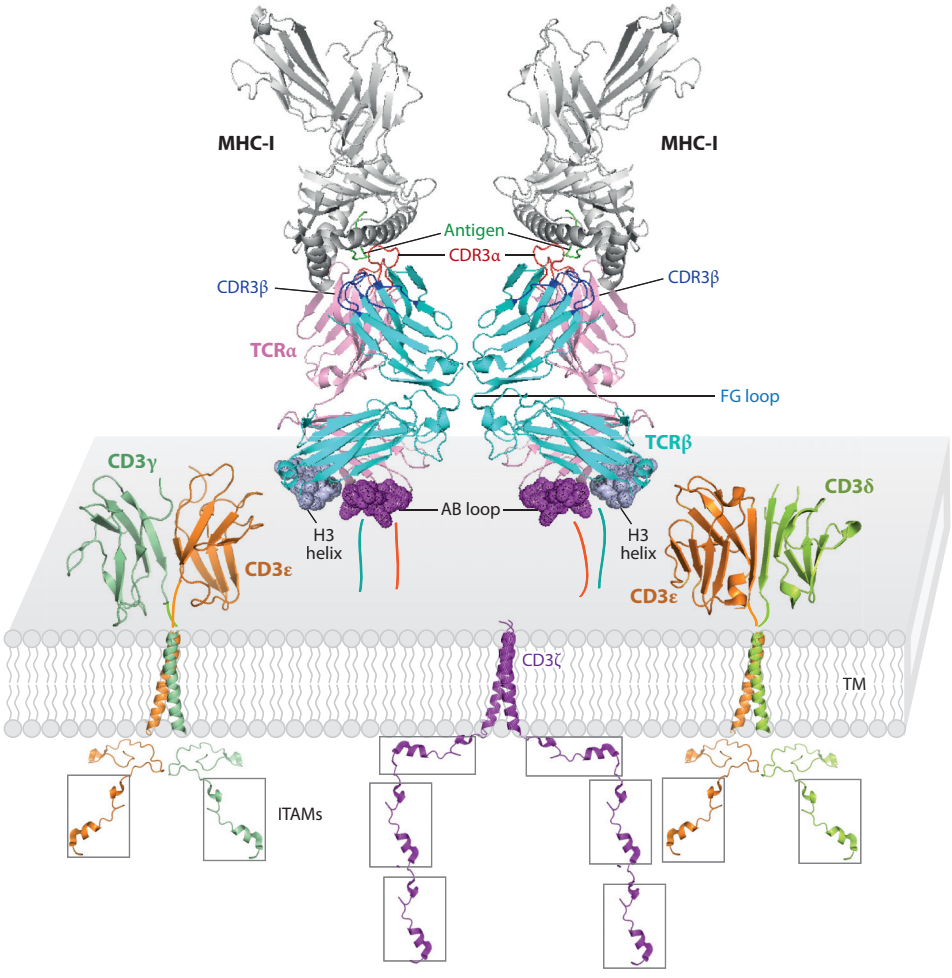

Figure 2

Structural model of the TCR-CD3 complex. Ectodomains of the αβ heterodimer bound to peptide–MHC-I and associated with the CD3γε, CD3δε, and CD3ζζ dimers embedded in the plasma membrane. The TCR-CD3 complex is represented as a dimer of αβ dimers each associated with CD3εγ or CD3εδ and CD3ζζ dimers. Features represented here include the CDR3 loops (*dark blue*) for TCRβ (*teal*) and TCRα (*pink*), the antigen peptide (*green*), the FG loop of TCRβ, and the AB loop of Cα (*purple spheres*) and the H3 helix of Cβ (*gray spheres*). The transmembrane domains of CD3ε, CD3γ, and CD3δ have been modeled from the CD3ζ transmembrane structure, and the CD3 cytoplasmic tails have been modeled from that of CD3ε. Ribbon structures were generated from Protein Data Bank entries 5IVX (B4.2.3 αβ TCR bound to P18–110/H2-Dd) (48), 1JBJ (CD3γε) (53), 1XIW (CD3δε) (43), 2HAC (CD3ζ TM) (44), and 2K4F (CD3ε cytoplasmic tail) (46). Abbreviations: ITAM, immune receptor tyrosine-based activation motif; TM, transmembrane.

ectodomains on CD3 subunits as a possible triggering event (53). The use of optical tweezers to artificially trigger the TCR complex indicates, however, that a lateral displacement relative to the plasma membrane rather than a vertical one triggers the TCR (54). This suggests that a lateral arrangement of the CD3 ectodomains in regard to the αβ ectodomains might be preferred. Such a lateral arrangement is supported by extensive mutagenesis data (55).

The CD3δε and CD3γε ectodomains are formed by cylindrical β barrels firmly connected by interactions between the G β strands of CD3ε and CD3δ or CD3γ (45, 53) (**Figure 2**). The rigidity of those interactions has been suggested to be important for the transmission of signals to the transmembrane and cytoplasmic domains of the CD3 subunits. In fact, mutations in the stalk sequence of CD3ε and the end of the G strand have been shown to abrogate TCR signaling (56, 57). Likewise, residues in the stalk domain of CD3δ have been shown to be important for transmitting structural changes to the cytoplasmic tail (58). The ITAM sequences of CD3ε and CD3ζ have been shown to form helical structures of one and two turns connected by a coiled-coil sequence (46, 59) (**Figure 2**). Interestingly, the ITAM helices have been proposed to be in tight contact with the inner side of the plasma membrane in the resting TCR and to detach from the membrane when the TCR is triggered (60).

SYNTHESIS, ASSEMBLY, AND CELL SURFACE EXPRESSION OF TCR-CD3 COMPLEXES

TCR-CD3 Assembly

From their synthesis to their expression on the plasma membrane, TCR-CD3 subunits undergo a finely regulated process of assembly and secretion via the endoplasmic reticulum (ER) and the Golgi apparatus that ensures the expression of TCR complexes containing the complete set of subunits (**Figure 1*a–d***). TCRαβ, CD3γ, CD3δ, and CD3ε subunits are synthesized in excess and retained in the ER. In the case of the TCRα subunit, it was estimated that 90% of the synthesized protein is degraded before being incorporated into the complex (61). Retention occurs via defined amino acid motifs that are distinct in sequence and localization depending on the subunits. They are localized in the extracellular, the transmembrane, or the cytosolic region (62–66). Unassembled subunits or partial complexes do not progress to the Golgi apparatus and are extruded from the ER and degraded (61, 67–69) (**Figure 1*a***). Degradation of some of the subunits (e.g., TCRα) takes place in the proteasome, whereas partial CD3 complexes are degraded in lysosomes, with the involvement of di-leucine– and tyrosine-based amino acid motifs for lysosomal localization (70) (**Figure 1*b–f***). Subunit assembly within the ER occurs through the generation of intermediary CD3γε or CD3δε complexes and single TCRα or TCRβ subunits, or TCRαβ dimers (71) (**Figure 1*b***). Although all the subunits have ER retention signals, once they are in a partial complex, some signals are neutralized, while other ER retention signals remain active and are dominant (e.g., signals in CD3ε dominate over those of CD3γ when CD3εγ dimers are formed) until the entire complex is formed. This is consistent with an ordered sequence of association events that inactivate retention signals by the association of additional subunits (66). Through their secretory pathway via the ER and the Golgi apparatus, TCRαβ, CD3γ, and CD3δ (but not CD3ε or CD3ζ) subunits undergo glycosylation.

Contrary to the other subunits, CD3ζ is neither synthesized in excess nor retained in the ER, and it can reach the Golgi apparatus independently of the other subunits. In the absence of CD3ζ, the TCRαβ and CD3γε and CD3δε partial complexes may associate, but they do not reach stable plasma membrane expression and are degraded. Thus, TCR-CD3 complexes can be detected at the cell surface in the absence of CD3ζ, although to a much lesser extent than entire complexes. This indicates that CD3ζ is necessary for both expression of the complex at the plasma membrane and its stability at the cell surface. Consistently, expression of variable amounts of CD3ζ modulates cell surface levels of the entire TCR-CD3 complex (72–75).

Interestingly, the predicted transmembrane segments of all the TCR-CD3 subunits contain positively or negatively charged amino acid residues, which are necessary for TCR-CD3 complex

association and cell surface expression. These complementary charges are necessary to stabilize the complex for complete assembly (44, 76–78).

TCR-CD3 Complexes at the Plasma Membrane

Although at least six different subunits are necessary for the expression of TCR-CD3 at the plasma membrane, the complex does not behave as a unit but displays remarkable heterogeneity. Thus, the associations between different partial complexes are of a distinct nature. While TCRαβ and CD3ζζ dimers are covalently linked via disulfide bonds, CD3γε and CD3δε are not covalently linked yet are stably associated. In contrast, the interaction between TCRαβ and CD3γε, CD3δε, or CD3ζζ appears weaker and displays different sensitivity to nonionic detergents (e.g., NP40, Triton X100, or digitonin). Furthermore, TCRαβ and CD3γε and CD3δε have much longer half-lives than CD3ζ. In addition, contrary to the other subunits, CD3ζ can be independently expressed at the plasma membrane. Therefore, newly synthesized CD3ζζ is likely incorporated into longer-lasting TCRαβCD3γεCD3δε complexes (74). This could occur at the plasma membrane or within intracellular compartments, like endosomes or the Golgi apparatus, through which the complex may be dynamically trafficked. Finally, CD3γ and CD3δ are partly redundant and have been shown to form alternate complexes in patients lacking CD3γ and in murine γδ T cells lacking CD3δ, and to be expressed at different relative levels in a variety of T cell lines (79–81). Altogether, these findings indicate that TCR-CD3 complexes are dynamic structures formed by several partial complexes that may be exchanged during the lifetime of the receptor.

The stoichiometry of TCR-CD3 complexes is still a contested matter. Although it is generally accepted that two copies of CD3ε are present in a single TCR-CD3 complex, the presence of one or two αβ heterodimers has been intensely debated. The presence of the αβ heterodimer in one versus two copies has functional consequences, because it defines the minimal functional unit of the TCR complex—whether TCR signaling can be triggered by a monovalent TCRαβ-pMHC interaction or instead requires cross-linking of two αβ TCRs in the same complex. The most accepted stoichiometry is one in which a single TCRαβ dimer is associated with one CD3εδ dimer, one CD3γε dimer, and one CD3ζζ dimer (78). This monovalent stoichiometry was opposed by early copurification, density centrifugation, and FRET (fluorescence resonance energy transfer) experiments that indicated the presence of at least two TCRαβ dimers in close proximity in the TCR-CD3 complex (82, 83). However, the finding that the TCR complex is present in the membrane of resting T cells as preorganized oligomers of up to 20 TCR-CD3 complexes, termed nanoclusters, complicated the interpretation of results in favor of a bivalent stoichiometry. Various techniques support the existence of nanoclusters: electrophoresis under native conditions, high-resolution single-molecule confocal microscopy, and several electron microscopy approaches (33–36, 84). These clusters of oligomeric TCRs are enriched in antigen-experienced memory T cells (85) and form preferentially at microvillus tips (86). TCR stoichiometry, clustering, and topology likely improve TCR recognition and sensitivity to low amounts of pMHC complexes on the surface of APCs (87, 88).

TCR engagement by pMHC molecules on APCs changes the topology of TCRs, inducing the formation of microclusters at the T cell–APC interface, named the immunological synapse. TCR microclusters are dynamic structures that form at the periphery of the synapse and then coalesce in the center, forming a large cluster called a supramolecular activation cluster (SMAC). At this point TCRs appear segregated from adhesion (i.e., integrins) and cytoskeleton-associated molecules (e.g., filamentous actin, talin, ezrin) (89–93). Although initially reported to be an activation domain (91, 92), the central TCR cluster was later proposed to be a domain devoted

to signal extinction. This was characterized by the presence of late endosomal compartments involved in ubiquitin-dependent protein degradation, by the enrichment of the tyrosine phosphatase CD45 (94, 95), and by the presence of TCR-enriched extracellular vesicles (96). Therefore, TCR plasma membrane distribution and dynamic reorganization are likely a mechanism by which T cells improve antigen recognition and then balance TCR triggering and signal termination (97–99).

DYNAMICS OF TCR-CD3 COMPLEXES BETWEEN THE PLASMA MEMBRANE AND ENDOSOMAL COMPARTMENTS

TCR-CD3 complexes do not stably remain at the plasma membrane but continuously traffic between the plasma membrane and endosomal compartments, undergoing constitutive rapid cycles of endocytosis and recycling before eventually being degraded in lysosomes (100–103) (**Figure 3**). This equilibrium between the plasma membrane and endosomes is controlled by the

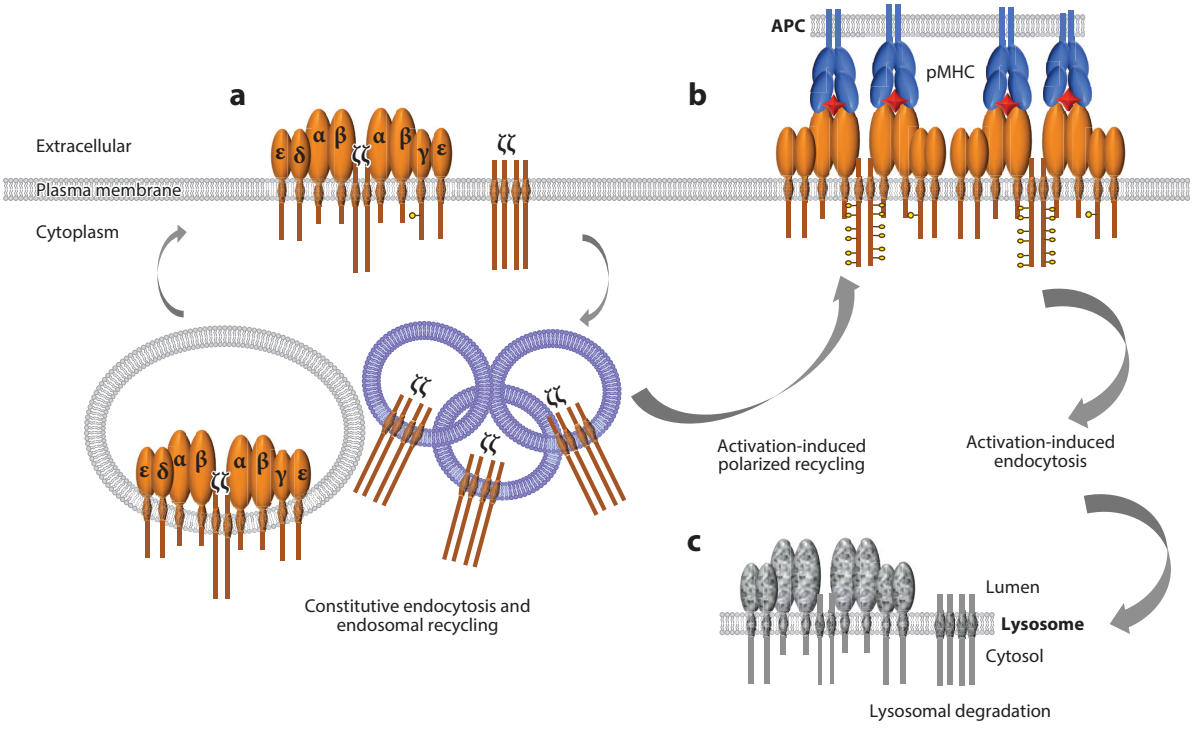

Figure 3

Schematic representation of TCR-CD3 complex dynamics between the plasma membrane and endosomal compartments. (*a*) In nonstimulated cells, TCR-CD3 complexes, and likely individual CD3ζζ dimers, undertake cycles of constitutive internalization and endosomal recycling (*thin arrows*). CD3ζ is more readily detected in endosomes and may follow a different route (depicted by different vesicle colors). (*b*) Soon after TCR engagement by peptide-MHC complexes on APCs, microtubules reorient and endosomal traffic is redirected toward the T cell–APC contact site (*thick arrow*), contributing to TCR-CD3 clustering at the immunological synapse. (*c*) Engagement of TCR increases the endocytosis and degradation (*thick arrow*) of both TCRαβ-CD3γδε and CD3ζ subunits in lysosomes. Degradation of CD3ζ is faster, again suggesting that its trafficking is different from other TCR-CD3 subunit trafficking. Tyrosine phosphorylation of CD3ζ is depicted by yellow circles at the intracellular regions. Abbreviations: APC, antigen-presenting cell; pMHC, peptide-MHC complex.

phosphorylation of a serine residue located close to a di-leucine endocytosis motif present in the intracellular region of the CD3γ subunit (104–106). In addition, several other endocytosis motifs present in other CD3 subunits cooperate to maintain TCR-CD3 endocytosis. Some motifs are dominant over the others (107). The presence of CD3ζ modulates the exposure of some of those domains (i.e., the CD3γ di-leucine–based motif). Hence, TCR-CD3 is more rapidly internalized in CD3ζ-deficient cells (75, 108).

Although all TCR-CD3 subunits constitutively traffic via endosomes (109–112), under the microscope CD3ζ is more readily observed in endosomal compartments than are other TCR and CD3 subunits (A. Alcover, J. Bouchet & I. del Rio-Iñiguez, unpublished observations). This suggests that CD3ζζ subcomplexes undergo slower endosomal traffic, retaining CD3ζ in endosomes during longer transit periods. This, together with the fact that CD3ζ has a shorter half-life (74), indicates that the intracellular trafficking and fate of CD3ζζ are at some stage independent of the rest of the TCR-CD3 complex, and CD3ζζ is in continuous exchange with the other TCR-CD3 partial complexes (**Figure 3**). The functional relevance of these differences in membrane versus endosomal localization and of differences in half-life of the TCR-CD3 subunits is at present unknown, but these factors may be important for regulating TCR-CD3 stability at the plasma membrane and improving T cell sensitivity to antigen and TCR signal control.

The endosomal traffic of TCR-CD3 is redirected to the sites of TCR engagement at the T cell–APC contact site (113, 114). This contributes to rapid changes in TCR-CD3 complex localization and to initiation of the dynamic clustering characteristic of immunological synapse formation (109, 111, 112). Polarized TCR-CD3 endosomal traffic is finely controlled by microtubule dynamics and endosomal traffic regulators, involving, as in neural synapses and ciliated cells, various vesicle transport, docking, and fusion regulatory molecules, e.g., GTPases, intraflagellar transport proteins, and SNAREs [soluble NSF (*N*-ethyl-maleimide-sensitive fusion protein) attachment protein receptors] (109, 111, 112, 115, 116). Moreover, at least two signaling molecules, the tyrosine kinase Lck and the adaptor LAT, also traffic through recycling endosomes and need this transport to cluster at the synapse (**Figure 3**). Interestingly, the endosomal compartments transporting TCR-CD3, CD3ζ, Lck, and LAT signaling molecules are distinct, using different traffic regulators, such as Rab GTPases or SNARE complex components (110, 117–119).

Engagement of TCR by pMHC, superantigen, or monoclonal antibodies to TCR or CD3 subunits increases the endocytosis and degradation of TCR and CD3 subunits (**Figure 3**), leading to the downregulation of TCR-CD3 cell surface expression (1, 74, 100, 101, 120, 121). TCR downregulation also involves nonengaged receptors (122–124). This may reflect the stoichiometry and/or nanoclustering organization of plasma membrane TCR-CD3 complexes, as well as possible lateral interactions and cooperativity between TCR complexes (57, 125, 126). Interestingly, internalization and traffic of bystander receptors appear to be differentially regulated (124, 127). TCR-CD3 downregulation involves various internalization mechanisms, including endocytic and phagocytic pathways (100, 107, 124, 128), and depends on tyrosine and serine phosphorylation events (105, 129, 130). Although TCR and CD3 appear to be downregulated simultaneously, the kinetics of the partial-complex internalization and their fates may be different. In fact, separation of CD3ζ from the rest of the complex has been reported (74, 131–133). Finally, TCR-CD3 complexes may be released to the extracellular milieu via vesicles that bud in response to TCR stimulation and accumulate at the immunological synapse (96, 134), also contributing to TCR-CD3 cell surface downregulation. Therefore, the central supramolecular activation cluster at the immunological synapse may be not only an endocytic and exocytic site for TCRs (95, 96) but also a site for inactivation of signaling molecules; e.g., it might bring together tyrosine kinases and phosphatases (94).

TCR SIGNAL TRANSDUCTION

Particular features distinguish TCRs from other receptors able to induce cellular activation. First, TCR ligands are not soluble molecules; they are membrane anchored (i.e., pMHC complexes expressed at the surface of APCs). Second, TCRs have low affinity for their cognate ligands, yet they have enough sensitivity to recognize low-abundance pMHC complexes on the surface of APCs. Third, although positively selected in the thymus to recognize self-pMHC complexes, in the periphery TCRs should discriminate self–peptide antigens from those of foreign origin, so as to mount specific T cell immune responses without developing autoimmunity.

TCR engagement occurs in the context of the interaction between T cell and APC, which triggers formation of the immunological synapse. This involves actin and microtubule cytoskeleton structures as well as several organelles, including endosomes, the Golgi apparatus, mitochondria, and lysosome-related organelles (91, 92, 135–140). Immunological synapses control T cell activation as well as helper and cytotoxic effector functions, like polarized secretion of cytokines or cytotoxic granules.

TCR signal transduction is a complex process that likely involves a combination of nonexclusive molecular mechanisms summarized below (reviewed in 141–143). It is well established that soon after TCRαβ engagement by agonist pMHC complexes, TCR-CD3 complexes cluster and recruit the *src* family protein tyrosine kinase Lck, which phosphorylates CD3 subunits on their ITAMs, initiating T cell activation cascades (**Figure 4**). However, the earliest physical events that connect TCR engagement with CD3 phosphorylation and further signal transduction are not fully understood.

The crystal structures of most characterized TCRαβ dimers have not revealed differences in the conformation of the dimer when it is bound to its pMHC ligand, other than in the CDR loops directly contacting the pMHC surface (42). This led to the proposal that the top-down transmission of information from the ligand-binding CDRs to the CD3 subunits does not involve conformational changes (49). However, a ligand-induced change in the AB loop of the TCRα subunit was detected in the LC13 TCR (144), and more recently, a ligand-induced change in the H3 helix of the TCRβ subunit of the B4.2.3 TCR has also been detected (48) (**Figure 2**). Interestingly, both the AB loop of TCRα and the H3 helix of TCRβ are placed at the bottom of the TCRαβ dimer, where it is supposed to contact the CD3 ectodomains. Mutagenesis of both the AB loop and the H3 helix has shown that both structures are required for signal transmission (48, 145). The occurrence of a conformational change in the TCR-CD3 complex as a primary mechanism for the outside-in transmission of information was first evidenced as the ligand-induced exposure of a polyproline sequence in the cytoplasmic tail of CD3ε that resulted in its binding to the N-terminal SH3 domain of the adapter protein Nck (146). Support for such change in the conformation of the cytoplasmic tail of CD3ε was later reinforced by FRET studies indicating movement of the cytoplasmic tail of CD3ε and the inner juxtamembrane sequence of CD3ζ away from the plasma membrane, provoked by pMHC ligand binding to the TCRαβ ectodomains (46, 147). The movement of the cytoplasmic tails of the CD3 subunits away from the plasma membrane and their juxtaposition are in agreement with their acquisition of resistance to protease attack in the triggered TCR (148).

A possible way to transmit conformational changes from the ligand binding αβ CDRs to the cytoplasmic tails of the CD3 subunits is via the conformational changes mentioned above detected at the bottom of the Cα and Cβ domains, but those changes have been detected in a minority of TCR-pMHC crystals. Therefore, it is possible that the resolution of all the other crystals did not allow detection of such changes, or that the changes in the AB loop and H3 helix represent a more generalized mechanism of transduction that occurs within the entire

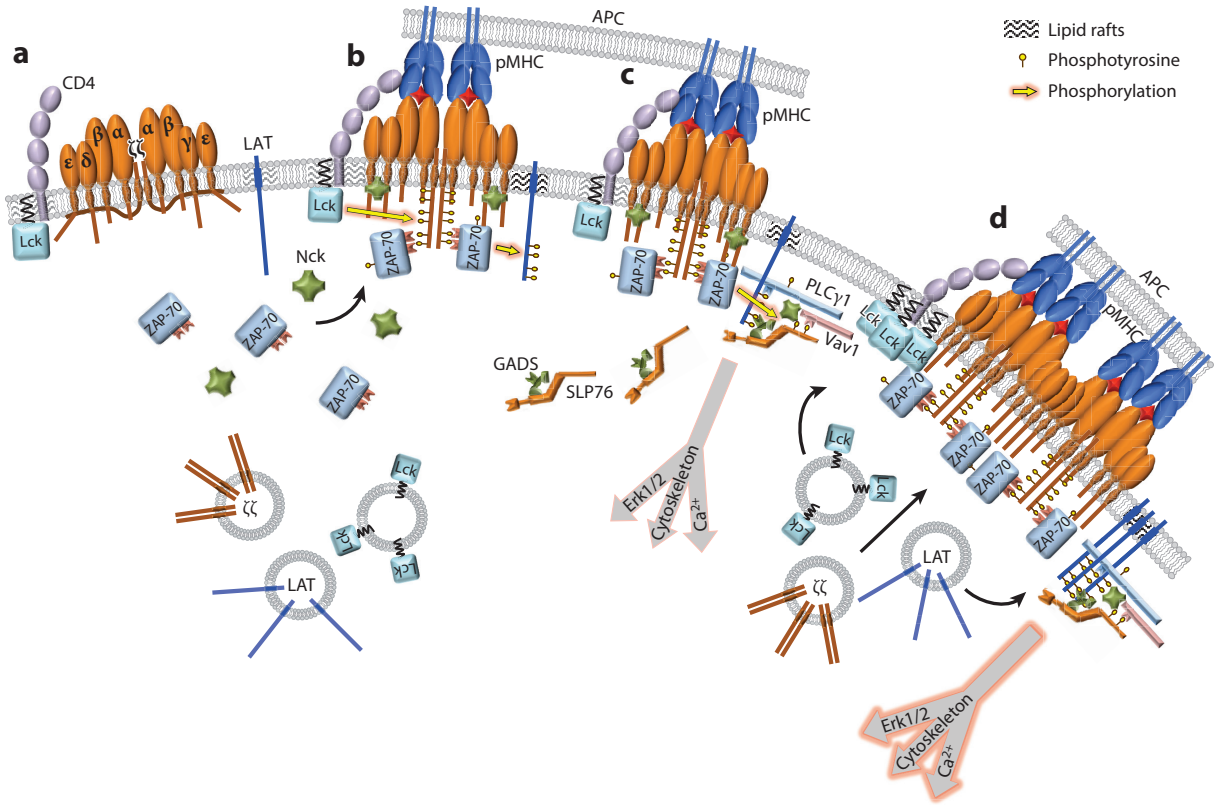

Figure 4

A model for TCR-CD3 complex signal transduction. (*a*) In the unstimulated TCR-CD3 complexes, signaling is prevented by the conformation of the CD3ε and CD3ζ subunit chains, the latter being in close contact with the inner leaflet of the plasma membrane. (*b*) Engagement by cognate pMHC complexes induces conformational or mechanical changes in TCRα and TCRβ chains that are transmitted to the CD3 subunits of the complex, leading to recruitment of Nck to an exposed proline-rich region in CD3ε and to displacement of the intracellular region of the CD3ζ chain from the plasma membrane, making its ITAMs prone to phosphorylation. Lck, which is enriched in lipid rafts and bound to the coreceptor CD4 (or CD8, not shown), is recruited to the engaged TCR-CD3 complex, an event favored by CD4 extracellular interaction with MHC. Then, Lck phosphorylates the ITAMs of the CD3ζ and CD3ε subunits, triggering the recruitment of ZAP-70 to doubly phosphorylated CD3ζ ITAMs. Additionally, Lck can phosphorylate ZAP-70, thus stabilizing its active (open) conformation and upregulating its catalytic activity. In turn, ZAP-70 phosphorylates the transmembrane adaptor LAT, allowing downstream transmission of signaling. (*c*) Phosphorylated LAT recruits the scaffold protein SLP76, via the adaptor GADS, and PLCγ1, controlling the production of second messengers such as diacylglycerol and free intracellular calcium (Ca^{2+}). Other enzymes or adaptors gathered within this complex, including Nck and the small GTPase regulator Vav1, participate in the regulation of actin cytoskeleton. (*d*) Further clustering of TCR-CD3 complexes as well as recruitment of endosome-associated pools of Lck, CD3ζ, and LAT sustain and/or reinforce signaling, thus ensuring productive T cell activation. Abbreviations: APC, antigen-presenting cell; ITAM, immune receptor tyrosine-based activation motif; PLC, phospholipase C; pMHC, peptide-MHC complex.

TCR-CD3 complex and not in the αβ ectodomains in solution. One such possible mechanism is the TCR acting as a mechanosensor: The TCRαβ dimer would act as a lever on the CD3 subunits when T cells are subjected to the mechanical constraints imposed by binding to the membrane of the APC (149). These forces may facilitate the exposure of CD3ε and CD3ζ cytosolic signaling motifs to the priming tyrosine kinase Lck. Lck does not seem to change its activity in triggered

versus nontriggered T cells (150), thus suggesting that TCR regulation must occur by changes in accessibility of the CD3 substrates to the kinase.

Concomitantly with the accessibility of tyrosine residues, CD3 subunit phosphorylation may need Lck to be brought into the proximity of CD3. This may take place via Lck constitutive interaction with the intracellular regions of CD4 and CD8 coreceptors (151, 152). In turn, CD4 and CD8 ectodomain interaction with MHC complexes would bring Lck into the proximity of engaged TCRs (153). CD4 and CD8 could interact with agonist pMHC complexes engaging specific TCRs, or with adjacent endogenous pMHC complexes engaged by surrounding TCRs (32). However, superresolution microscopy revealed that TCR, CD4, and Lck nanoclusters do not coalesce upon TCR stimulation but remain segregated (154). Moreover, not all Lck is associated with CD4 or CD8, and these coreceptors are not always necessary for triggering T cell activation. Therefore, an additional mechanism has been proposed based on the partitioning of TCR complexes into membrane regions enriched in Lck and deficient in CD45, like cholesterol- and sphingolipid-enriched membrane domains or lipid rafts (155–157). However, this raft coalescence model has been challenged (158). Contradictory findings may reflect the fact that membrane micro- or nanodomains are subtly regulated by both lipid composition and the cortical cytoskeleton, together accounting for dynamic proximity between signaling molecules and TCR complexes (33, 34, 155, 159). The distribution of these molecules could occur differently, depending on the strength of TCR interaction with its ligand.

Lck is regulated by the balanced phosphorylation of two tyrosine residues that activate and inhibit Lck kinase activity. Phosphorylation of these residues is controlled by the action of the Csk tyrosine kinase and the CD45 tyrosine phosphatase. A significant amount of active Lck is found in resting T cells, and the amount does not change after TCR engagement (160). This suggests that changes in substrate availability (see above), or Lck localization with respect to the TCR, rather than kinase activation, could ensure TCR signaling. Moreover, the localization of the tyrosine phosphatase CD45 with respect to Lck and the TCR complex may contribute to TCR signaling, as indicated by microscopy imaging and the kinetic segregation model (94, 141). Lck is associated with membrane rafts, but also with the Rab11 endosomal compartment, which controls Lck subcellular localization and its capacity to phosphorylate CD3ζ in resting and activated T cells (119). CD3ζ and LAT are also associated with endosomal compartments that deliver these proteins to the immunological synapse, likely facilitating TCR encounter with Lck at the site of TCR engagement with pMHC molecules from APCs (110–112, 115–118). Therefore, initial TCR interactions with proximal signaling molecules at the plasma membrane likely trigger the initiation of signaling; vesicle traffic may then convey additional TCR-CD3 complexes, Lck, and LAT, fueling the capacity of the synapse to transduce TCR signals and convey sustained T cell activation. Interestingly, CD3ζ is more abundant in endosomes than the other TCR-CD3 subunits, and CD3ζ subunits carrying endosomes are redirected to the immunological synapse (110–116). This may have dual functions: First, it may fuel the synapse with CD3ζ in order for it to be phosphorylated and recruit ZAP-70, thus sustaining TCR signaling; second, the increase in local concentration of CD3ζ may stabilize TCR-CD3 complexes in that area, increasing temporarily and locally their plasma membrane expression by reducing their endocytosis (72, 75, 108). Altogether, these mechanisms could sustain signaling at the immunological synapse.

The use of reductionist experimental systems with a minimal set of components allowed an estimation of the hierarchy of single TCR signaling events (161–163). For instance, the sole intracellular CD3ζ segment bound to a chimeric receptor expressed in a T cell is able to convey signaling leading to ZAP-70 recruitment, calcium flux, and Erk1/2 phosphorylation. Productive signaling requires a certain threshold both of receptor-ligand affinity and of ligand density able to provoke minimal TCR clustering. In turn, TCR clustering ensures enough ZAP-70–CD3ζ

interaction time, likely owing to longer-lasting CD3ζ ITAM phosphorylation. This correlates with productive downstream signaling events (164–166). This is consistent with the results obtained in T cells expressing pMHC-TCR pairs of different affinities (91, 125, 167–169). Moreover, reconstitution on artificial lipid bilayers of a TCR signaling module composed of CD3ζ, Lck, LAT, and CD45 intracellular segments to which one can add several soluble signaling components, such as ZAP-70, Grb2, and Sos1, was sufficient to generate signaling. The addition of phosphorylated LAT, Grb2, and Sos1 was enough to generate LAT microclusters that excluded CD45. Clusters were liquid-like dynamic structures that grew and retracted and could exchange LAT (163). Interestingly, CD45 was excluded from the signaling clusters even in the absence of its extracellular domain and APCs. This finding is somewhat contrary to the kinetic segregation model that proposes that molecules having large extracellular domains, like CD45, would be excluded from the TCR contact, owing to adhesion forces and the proximity of T cell and APC membranes (141). It is likely that these mechanisms—micro- or nanoexclusion of CD45 toward the immunological synapse periphery—coexist and account for initial and later activation needs. Interestingly, a minimal signaling system on lipid bilayers, formed by CD3ζ, Lck, ZAP-70, SLP76, and Gads, can also reorganize the actin cytoskeleton to form from the microclusters of LAT. This requires that the adaptor Nck, actin and the actin regulators N-WASP, and the Arp2/3 complex be provided as components in the in vitro system (163). These findings indicate that phase separation of TCR together with signaling molecules may be the basis for the minimal molecular reactions involved in TCR signal transduction.

TCR DEGRADATION

Degradation of TCR-CD3 complex subunits is a control mechanism operating at different stages of the TCR life cycle. First, it eliminates individual subunits or partial complexes that did not make fully assembled complexes; second, it controls steady-state levels of surface TCR-CD3 in resting T cells; third, it downregulates TCR-CD3 surface expression upon antigen stimulation to moderate further T cell stimulation.

During TCR-CD3 complex assembly, unassembled subunits are retained in the ER and degraded by lysosome-dependent or -independent mechanisms. Information on all individual subunits is not available. TCRα and TCRαβ complexes are degraded after ER retention by a mechanism insensitive to lysosomal inhibitors (61), involving cytosolic proteasomal degradation mediated by ubiquitinated intermediates (170–172). Basic amino acid residues within the transmembrane region of TCRα are key for both TCRαβ assembly and retention and degradation of unassembled subunits (61, 62, 68, 69, 77). CD3δ is also degraded in the ER, when this chain is individually expressed, via ubiquitin intermediates and after undergoing oligosaccharide processing (171). CD3ε and CD3γ are also retained in the ER alone and as partial complexes, although they are degraded much more slowly than the other subunits (64–66).

Once TCRα is associated with CD3γε and CD3δε subunits, the transport of complexes can progress to the Golgi apparatus. Once they have reached the Golgi apparatus, partial complexes containing TCRα and CD3γε or CD3δε are degraded in lysosomes (61). With the use of chimeric molecules, the existence of two distinct amino acid motifs containing di-leucine and tyrosine residues was identified in the CD3γ and CD3δ subunits. These motifs complement each other and also mediate endocytosis of these and other proteins. During TCR-CD3 assembly, they may serve to deviate unassembled partial complexes to lysosomes, preventing their stable expression at the plasma membrane (70).

Plasma membrane TCR-CD3 complexes undergo cycles of constitutive endocytosis and recycling (see above), and a certain percentage of the receptors are degraded per cycle to balance

the newly synthesized receptors arriving at the plasma membrane. The half-lives of TCRαβ and of CD3γε and CD3δε are longer than that of CD3ζζ (74). Therefore, at some point, CD3ζζ has to traffic separately from the other subunits to be degraded independently of the rest of the complex at a faster rate. CD3ζ degradation takes place in lysosomes. CD3ζ ubiquitination and its interaction with a lysosomal protein have been reported (173). Moreover, CD3ζ degradation is also influenced by its Lck-dependent phosphorylation of tyrosine residues (119, 130). Since CD3ζ levels control TCR-CD3 cell surface expression (72), dynamic CD3ζ degradation in response to antigenic stimuli may allow control of TCR-CD3 availability at the cell surface, regulating its endocytosis and thus influencing its degradation (75, 108).

TCR engagement by antigen leads to TCR-CD3 degradation. Degradation of both TCR-CD3γδε chains and CD3ζ is increased, although with different kinetics (74, 120, 174, 175). Degradation occurs in lysosomes and depends on ubiquitination of some of the subunits (176, 177). Interestingly, the balance between sustained signaling and TCR degradation depends on antigen quality, indicating that subtle mechanisms regulate this stage of the process (98, 99).

CONCLUSIONS AND PERSPECTIVES

TCRs are sophisticated molecular machines devoted to coordinated antigen recognition and signal transduction under very diverse stimulatory conditions. Successive quality control mechanisms ensure the synthesis of variable and constant subunits, their controlled assembly to form molecular complexes of six distinct subunits and different stoichiometry, and their controlled expression at the T cell plasma membrane. While it is accepted that degradation of unassembled subunits is a quality control mechanism to allow expression only of entire TCR-CD3 complexes, the evolutionary advantage of synthesizing a large excess of all but one of the individual subunits is a puzzle. This may be important to facilitate fine-tuned control of TCR-CD3 expression under different physiological conditions. Once expressed at the plasma membrane, TCR-CD3 complexes appear dynamic, having continuous exchanges of partial complexes. TCR-complex stoichiometry and clustering are likely variable and may adapt to the state of T cell differentiation and function. Finally, membrane TCR complexes are continuously recycled via endosomes. Altogether, these molecular mechanisms are the basis for functional TCR complexes able to recognize subtle differences in foreign antigens and translate antigen recognition into T cell responses via signal transduction pathways, including the selection process in the thymus. Physiological immune responses should avoid T cell responses to self-antigens, which lead to autoimmunity. TCR complexity may ensure those different functional possibilities.

While very simple molecular setups (e.g., a single CD3ζ intracellular segment and a few signaling components) can account for early T cell signaling events, evolution has selected much more sophisticated molecular machinery with apparent redundancy in signaling motifs and activation abilities. Moreover, the fact that the TCR ligands are expressed on different cells adds layers of complexity, including adhesion and positive and negative cosignaling molecules. Altogether, this orchestrated molecular ensemble ensures controlled T cell responses to a large variety of antigens.

Great efforts have been made to understand how the TCR behaves in time and space at both the ligand interaction and the signaling level. Synthetic biological setups on the APC side (e.g., surrogate APCs made of lipid bilayers presenting different pMHC ligands and a combination of cosignaling molecules, photo-activatable peptide antigens) and the T cell side (e.g., expression of different wild-type or mutated TCRs or cosignaling receptors; replacement of recognition modules by artificial ligands, such as DNA) as well as superresolution microscopy have facilitated more detailed dissection of this complex system and reduced the time frame of responses to a measurable scale.

However, the challenge of understanding the functioning of a complex receptor in the context of an even more complex environment remains. A key link we are still missing is of how antigen recognition is transmitted from TCR to CD3 subunits. Solving the atomic structure of an entire TCR-CD3 complex in its transmembrane environment would help us understand this. However, obtaining crystals of such complexes is not technically feasible at present. Even more difficult would be to have those complexes bound to pMHC complexes, also themselves in the context of the APC membrane. Perhaps cryo–electron microscopy will, at least partially, help to solve more complex and less perturbed TCR-CD3 structures, as it has for some viral proteins. Yet, TCR interaction with its natural ligands, and therefore the resulting conformational changes, is expected to be modulated by T cell–APC interactions involving adhesion and cosignaling receptors, and the resulting cortical cytoskeleton modifications. Therefore, additional high-resolution approaches allowing observation of TCR-CD3 complexes and their ligands in their cellular context, in live cells, will be crucial. The development of superresolution microscopy approaches such as multicolor 3D PALM, combined with the development of fluorescent probes able to detect conformational or protein-protein interactions, will be most valuable to gain deeper insight into TCR structure and function. Finally, an additional level of complexity exists: In vivo, T cells recognize antigens within the crowded environment of lymphoid organs, where APCs are. Improvements in microscopy (e.g., two-photon microscopy) capable of viewing a single molecule penetrating tissue will help us to understand TCR functionality under physiological conditions. Therefore, more than 30 years after it was first characterized at the molecular level, the TCR complex still holds challenging secrets that require even more precise technologies and experimental approaches.

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