1. Introduction

The antigen receptor of T cells (TCR) has a critical role in the recognition of antigen, the initiation of signaling events determining T-cell fate, and providing immunity or tolerance. The ligand of the TCR is a composite surface of a peptide bound to a major histocompatibility complex molecule (pMHC) on the surface of an antigen presenting cell (for helper T cells) or a target cell (for cytotoxic T cells) [1]. The TCR binds to foreign peptides on MHC with moderate affinity (1–50 µM), resulting in phosphorylation of the TCR and other signaling molecules triggering T-cell activation. In contrast, the affinity of the TCR to self peptide-MHC is lower, and such engagement does not stimulate the T cell, thereby avoiding autoimmunity [2]. To understand how the TCR can distinguish between different ligands, it is crucial to study its structure and the molecular mechanisms through which it transmits the information of antigen binding into the cell. Unfortunately, the three-dimensional molecular structure of the complete TCR is not known. However, biochemical and imaging studies have provided insight into the organization of the TCR on the cell surface. On resting T cells, the TCR is not randomly distributed in the plasma membrane, but forms multimeric structures. In this review, we discuss how the models for the distribution of the TCR on the cell surface before antigen binding have changed, along with the development of microscopic and biochemical techniques. We discuss how TCR activation in pre-clusters might happen, and the biological consequences of TCR cluster formation.

2. The subunit composition of the TCR

The TCR complex consists of distinct type I transmembrane polypeptide chains for antigen recognition and for transmitting the information to the intracellular signaling molecules. The TCRα and TCRβ chains each contain a clonotypic variable immunoglobulin (Ig) domain, which together form the antigen-binding site, in addition to a constant Ig domain in each chain. Potentially positively charged transmembrane segments connect these extracellular domains to the short intracellular tails (Fig. 1a). The signaling chains are the CD3γδ and CD3εζ heterodimers and the ζζ homodimer. Each CD3 subunit has an extracellular Ig domain followed by a stalk region, whereas the extracellular part of ζ consists of only nine amino acid residues. The transmembrane regions of the signaling chains are potentially negatively charged (Fig. 1a). In their intracellular tails the CD3 subunits contain one, whereas ζ contains three immunoreceptor tyrosine-based activation motifs (ITAMs)
For biochemical analyses, the TCR is extracted from the membrane with detergents. This procedure might disrupt weak associations, especially those taking place within the membrane. The minimal stoichiometry of the TCR complex extracted by detergents is $\alpha\beta\gamma\delta\epsilon\zeta$, although one report suggested a $\alpha\beta\gamma\delta\epsilon\zeta\zeta$ composition. Using mutagenesis and an erythropoietin receptor-based proximity assay, it was shown that CD3 heterodimers are docked in tandem orientation on one side of the TCR. TRIM homodimer, which is a dispensable part of the TCR, is loosely associated. (b) The putative structure of the TCR from the top view according to Kuhns et al. The TRIM homodimer, which is a dispensable part of the TCR, is loosely associated.

3. TCR clustering upon ligand engagement

For the initiation of T-cell activation, sustained TCR/pMHC engagement is necessary. It has not been easy to mechanistically explain how this happens, given the low amount of MHC molecules displaying the cognate peptide on the surface of an APC, and the short half-life of the TCR–pMHC interaction ($t_{1/2} \approx 15–0.1$ s). Receptor oligomerization upon ligand engagement plays a role in the initiation of the signaling of several membrane receptors (e.g., the EGFR family), and this mechanism was similarly proposed for the TCR. This model requires that the TCRs are individually expressed on the cell surface (Fig. 2a). The fact that soluble oligomeric, but not monomeric, pMHC activates the TCR makes it probable that TCR oligomerization is a prerequisite for signal initiation. In the two dozen structures of complexes between the ectodomains of TCR$\alpha\beta$ and soluble pMHC, no dimers could be observed. The same holds true for the ectodomains of TCR$\alpha\beta$ alone. However, the complete TCR in its native membrane-bound environment was not studied, thus these data do not resolve whether the native TCR forms dimers or oligomers before or after pMHC binding.

Ligand binding-induced TCR dimerization was studied using the soluble extracellular parts of TCR$\alpha\beta$ and pMHC; again not representing the membrane-bound complete TCR. Based on light scattering measurements, it was suggested that the ectodomains of the TCR$\alpha\beta$ from murine 2B4 T cells oligomerize in solution and form supramolecular structures following pMHC binding. It was proposed that, at limiting antigen concentrations, preferential oligomerization of specific TCR$\alpha\beta$–pMHC complexes occurs on the cell surface, thus maintaining T-cell sensitivity for low-abundance ligands. Surface plasmon resonance measurements using the soluble TCR fragment of mouse OT-1 T cells and its ligand showed that
agonist but not antagonist pMHC binding to the TCR\(\alpha\)\(\beta\) led to dimerization [18]. However, these results could not be repeated using the human A6 TCR, suggesting that the multimerization of soluble TCR/pMHC complexes is not a general phenomenon [19]. These controversies suggested that the multimerization of the TCR preceeding or following antigen binding should be better studied on the cell surface, using fully assembled TCRs.

The development of microscopy indeed had a great impact on our understanding of the mechanism of T-cell activation. It was discovered more than two decades ago that T cells form long-lived cell–cell contacts with APCs and this process required the specific TCR–pMHC interaction [20]. Using immunofluorescence microscopy, it was shown that TCR redistributes on the cell surface and accumulates at the contact zone with the APC, forming the immunological synapse [20]. Within the synapse, signaling and adhesion molecules form supramolecular activation clusters (SMACs) [21]. Real-time imaging revealed that the TCR/pMHC complexes accumulate in the outer ring of the synapse at the initiation of the T cell–APC interaction and migrate to the center of the SMAC [22]. After pMHC binding, TCR forms microclusters 300–800 nm in diameter, containing approximately 20–70 receptors. Signaling is initiated in these supramolecular organizations, which also contain the tyrosine kinase ZAP70 and the adaptor protein SLP-76. As initiated in these supramolecular organizations, which also contain the tyrosine kinase ZAP70 and the adaptor protein SLP-76, the TCRs form microclusters, which have a size comparable to the previously discussed activation-induced microclusters observed earlier [23,24]. These results were also confirmed by immuno-gold electron microscopy [27].

Using two-color coincidence detection (TCCD) to define the self-association quotient of TCRs, it was found that the TCR is present on the cell surface mostly in monovalent form [28]. On the other hand, the self-association quotient is greater than zero, suggesting that some TCRs are pre-associated. In a related study, a significant portion of TCRs (10%) were found to be associated, using the dynamic single-molecule co-localization (DySCo) method [29]. However, the cells used in these experiments expressed 10-fold less TCR than normal T cells. This difference in concentration might result in a dramatic change in dynamic associations, thus underestimating the number of pre-clustered receptors in cell expressing physiological levels of the TCR.

4. Preformed TCR clusters on non-activated T cells

It was shown that in cells expressing two TCRs with different specificities, stimulation of one TCR resulted in the internalization of the other. This was the first experimental indication, albeit not direct evidence, of physical association of at least two TCR\(\alpha\)\(\beta\) [25]. Further analysis of naïve, resting T cells from double transgenic mice expressing two different TCRs showed that at least a fraction of the TCRs might be multivalent, i.e. contain two antigen-binding TCR\(\alpha\)\(\beta\) dimers in one pre-formed cluster [9]. The two different TCR\(\alpha\)\(\beta\) could be co-immunoprecipitated [25]. An EndoH digest of the carbohydrate groups proved that these TCRs had passed the Golgi complex, indicating that they were correctly assembled TCRs and not misfolded proteins from the endoplasmic reticulum. Co-purification of two distinguishable TCRs was also observed in a study using Jurkat transfectants [8]. Fluorescence resonance energy transfer (FRET) between the two TCRs on living T cells from the double transgenic mice confirmed this result [9]. On this basis, Antonio de la Hera and Balbino Alarcon’s groups demonstrated for the first time that two antigen-recognizing TCR\(\alpha\)\(\beta\) heterodimers associate in at least some of the TCRs on the surface of resting primary T cells [9].

Further evidence for TCR pre-clusters on the surface of non-activated T cells was provided using biochemical techniques and electron microscopy [8]. Using Blue Native PAGE of detergent-extracted TCRs from resting, primary T cells as well as from T cell lines, higher-ordered complexes could be detected. In addition, monovalent receptors were also present. The pre-clustered TCRs were most likely dimers and multimers of the basic TCR\(\alpha\)\(\beta\) stoichiometry and did not contain comparable amounts of other surface proteins. Multimeric forms of detergent-extracted TCRs were also observed in other studies [26]. According to immuno-gold labeling for electron microscopy, more than 55% of the TCRs were organized in oligomeric complexes, the majority of which were found to bind two or three and a small proportion bound four or more anti-CD3\(\varepsilon\)-specific gold particles [8]. However, due to the low staining efficiency of the immuno-gold labeling, and the partial disruption of TCR oligomers by detergents used for the biochemical analysis, the extent of TCR multimerization could be underestimated.

Recently, modern microscopic techniques have allowed imaging the surface of T cells with even better resolution. Using high-speed photoactivated localization microscopy (hsPALM), molecules can be located with a mean accuracy of ~25 nm. Using this technique, it was shown that, on the surface of live T cells on non-activating fluid lipid bilayers, TCRs form islands of 70–140 nm diameter, containing 7–30 molecules each [27]. On bilayers containing pMHC, the protein islands coalesce, so that 5–15 islands form clusters, which have a size comparable to the previously discussed activation-induced microclusters observed earlier [23,24]. These results were also confirmed by immuno-gold electron microscopy [27].

If the TCRs do not form stable oligomers based on strong protein–protein interactions, but rather cluster as a result of co-localization in small regions of the cell surface, the term “oligomer” might be misleading and the use of “pre-formed assemblies or clusters” is more appropriate.
6. Triggering models for TCR pre-clusters

The existence of pre-clustered TCRs in resting cells (Fig. 2b and c) questions the importance of antigen-induced clustering in TCR triggering. Several models of receptor activation upon antigen binding can be applied to the TCR. However, there are only two models that do not exclude a role for pre-formed TCR clusters: the segregation model and the conformational change model. However, in principal both models would also be compatible with individually expressed TCRs (Fig. 2a).

The segregation model explains TCR phosphorylation by antigen binding-induced spatial separation of the TCR from phosphatases based on the size differences of the molecules [32]. TCR and pMHC molecules are relatively small (both have a ~7 nm long ectodomain), and the cell membranes of the T cell and the APC or target cell have to come close to allow engagement. The larger phosphatases (e.g. CD45) are thus excluded from the contact zones, where TCR becomes phosphorylated by kinases. This model is supported by findings that increasing the distance of the pMHC molecules from the plasma membrane leads to inefficient TCR triggering, since in this case the phosphatases do not segregate from the TCR-pMHC contact zone [33].

On the other hand, according to the conformational change model, antigen binding induces structural rearrangement within the TCR complex, including the cytoplasmic tails of CD3, leading to TCR phosphorylation [34]. The first evidence for ligand-induced conformational changes in the TCR complex was the exposure of the proline-rich sequence in the cytoplasmic tail of the CD3ε upon dimeric ligand binding [35,36]. Consequently, it was proposed that bivalent binding of pMHC to two TCRs changes their respective orientation towards each other, resulting in CD3 rearrangements and receptor activation status (permissive geometry model) [35] (Fig. 3).

Besides this scenario, two other possible mechanisms of how antigen binding of TCRεβ leads to conformational changes in CD3 were recently published. First, a structural rearrangement in the AB loop of the constant Ig domain of TCRε was found in a soluble TCRεβ upon binding of pMHC [37]. From the AB loop, this structural change could spread to the CD3 subunits. However, this model does not explain why dimeric antigen binding is needed for T-cell activation. Furthermore, it has recently been shown that the CD3 dimers are situated on the opposite side of the TCRεβ from the AB loop, and that the AB loop is likely to have a role in TCR dimerization upon triggering [10]. Second, a point mutation in the stalk region of CD3ε prevented the ligand-induced conformational change in CD3, suggesting that ligand binding to the ectodomains of CD3 leads to a stiffening of the CD3 structure including the cytoplasmic tails [38]. In agreement with the finding that the conformational change is required for TCR activation [35], the CD3ε mutant inhibited signaling through the TCR. Interestingly, the mutant CD3ε was also able to inhibit signaling via the wild type CD3ε, although it was expressed in much lower levels. This dominant effect can be explained, by a model involving TCR pre-clusters, such that one mutant CD3ε inhibits signaling via all TCRs within the pre-cluster [38].

7. Functional consequences of TCR pre-clustering

The existence of TCR pre-clusters on resting cells is by now well established, but their role in T-cell triggering has to be further investigated.

According to theoretical calculations, the co-expression of monomeric and pre-clustered receptors provides the cell not only with high sensitivity, but also the ability to respond over a broad range of ligand concentration [39]. In line with this, it was found that at low antigen concentrations, only pre-clustered TCRs are phosphorylated, whereas at high antigen concentrations both TCR monomers and pre-clusters become phosphorylated [8]. This finding explains how pre-clustered receptors could be responsible for the high sensitivity of the T cell to low ligand concentrations [2], whereas monomeric receptors are responsible for distinguishing among different high concentrations upon saturation of the pre-clustered receptors. It was suggested that previously bound and dissociated pMHC can rapidly rebind to another TCR of the same pre-cluster, but not to more distant TCRs, which explains the higher sensitivity of pre-clustered TCRs with respect to monomers [40]. The on-rate of pMHC to a monomeric TCR is much lower than that to a pre-cluster. Thus, the same pMHC concentration stimulates fewer TCRs during a given time period in the monomer compared to the pre-clustered TCR. Rapid rebinding of pMHC to TCRs are the central claim of the serial triggering model [41]. Hence, pre-clustering enhances the avidity of the receptor-ligand interaction, whereas the affinity of the individual TCR-pMHC interaction is the same in TCR monomers and pre-clusters. Affinity is defined as the energy of binding of one receptor to one ligand, whereas avidity is the energy of binding between multiple receptors and multiple ligands.

The effect of increased avidity on the T-cell response can be observed using different cells expressing the same TCR. Activated T cells bind pMHC tetramers better than the corresponding naive T cells [42]. The mathematical analysis of the TCR-pMHC binding data showed that the increased binding was due to higher clustering rather than to an increased intrinsic dissociation constant [42]. This effect is cholesterol-dependent, since cholesterol depletion decreased tetramer binding [42], as it was also shown to reduce the extent of TCR multimerization [8]. Since the MHC molecules on the antigen presenting cell were also shown to be multimeric [43], the avidity of pre-clustered TCRs to pMHC clusters is higher than individual affinities of TCRs to MHCs. Indeed, disruption of MHC pre-clusters on the presenting cell decreased the activation of T cells [44]. This effect was more pronounced at low antigen densities, which also indicates that pre-clustering of MHC and
TCR is important for the high sensitivity of T-cell activation. These observations highlight the importance of testing the pre-clustering state of TCR and pMHC for experiments using T cells and APCs, as variations in this parameter influence the outcome of T-cell stimulation.

If antigen binding to one receptor leads to the phosphorylation of all receptors in the cluster, this would further increase the sensitivity of pre-clustered TCRs [45]. This theory is supported by the above-mentioned study, which shows that a small amount of a mutant, inactive CD3ε can inhibit signaling via the complete pre-cluster [38]. Such cooperativity between the cytoplasmic tails of the TCRs enables T cells to detect small relative changes in the affinity of pMHC in order to distinguish between foreign peptides (moderate affinity) or self-peptides (low affinity) loaded on MHC molecules [45].

The fact that dimeric engagement is necessary to trigger the TCR [14] enforces the stringency for T-cell activation, hence playing another role in discrimination between pMHC ligands with different affinities. Similar to the requirement of double phosphorylation for activation of kinases in the MAP kinase pathway [46], the need for dimeric binding for triggering offers a mechanism to avoid spurious T-cell activation.

8. Conclusions

Full understanding of how pMHC binding translates to TCR phosphorylation, and how T cells discriminate between foreign and self-peptides presented on the MHC molecules is still out of our reach. However, fluorescence microscopy techniques, biochemical methods, protein crystallography and mathematical modelling give us better insight into the molecular mechanisms behind TCR triggering. The newly defined organization of the TCR on the surface of naïve T cells might be important not only for signal initiation, but also for the sensitivity of T cells and their capability to discriminate between antigens. To the arising questions, such as how the TCR is directed in the milieu, where it forms protein islands, what is the driving force of cluster formation, or how is the amount of the clusters in the T cell is regulated, we expect answers from further technical-methodological developments.

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