T-cell antigen-receptor stoichiometry: pre-clustering for sensitivity

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The T-cell antigen receptor (TCR•CD3) is a multi-subunit complex that is responsible for triggering an adaptive immune response. It shows high specificity and sensitivity, while having a low affinity for the ligand. Furthermore, T cells respond to antigen over a wide concentration range. The stoichiometry and architecture of TCR•CD3 in the membrane have been under intense scrutiny because they might be the key to explaining its paradoxical properties. This review highlights new evidence that TCR•CD3 is found on intact unstimulated T cells in a monovalent form (one ligand-binding site per receptor) as well as in several distinct multivalent forms. This is in contrast to the TCR•CD3 stoichiometries determined by several biochemical means; however, these data can be explained by the effects of different detergents on the integrity of the receptor. Here, we discuss a model in which the multivalent receptors are important for the detection of low concentrations of ligand and therefore confer sensitivity, whereas the co-expressed monovalent TCR•CD3s allow a wide dynamic range.

Keywords: conformational change; signal transduction; stoichiometry; T-cell antigen receptor; transmembrane interactions

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Introduction

How transmembrane (TM) receptors respond to ligands with high sensitivity and plasticity has been intensely studied. Particularly intriguing is the T-cell antigen-receptor complex (TCR•CD3). The complex is composed of the TCRαβ, CD3γε, CD3δε and TCRζζ (CD247) dimers (Fig 1), which are non-covalently bound to each other. The invariant CD3 and TCRζ subunits each contain an extracellular part, a TM region with one potential negative charge and a cytoplasmic tail with several signal-transduction motifs (Alarcón *et al*, 2003; Malissen, 2003; Reth, 1989). TCRα and TCRβ each contain a variable immunoglobulin (Ig) domain, which together form the binding surface for the ligand, a constant Ig domain and a TM region

(Fig 1) that is potentially positively charged and couples TCRαβ to CD3 (Blumberg *et al*, 1990a; Call *et al*, 2002; Cosson *et al*, 1991).

The ligand of TCR•CD3 is an antigenic peptide presented by major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APCs). Paradoxically, T cells can be activated by a few antigenic peptides loaded onto the MHC (pMHC; Irvine *et al*, 2002; Purbhoo *et al*, 2004), even though the affinity of the pMHC–TCRαβ interaction is low (1–50 μM; Davis *et al*, 1998). Furthermore, APCs express a 10³-fold to 10⁴-fold higher concentration of MHC/self-peptides than antigenic pMHC. MHC/self-peptides by themselves do not elicit a response, although their affinity for TCRαβ could be only tenfold lower than the affinity of the antigenic pMHC. Therefore, it is difficult to understand how this small difference in affinity results in high specificity when there is a huge excess of self-peptides (Boniface *et al*, 1998). In addition to the extreme sensitivity, T cells still show concentration-dependent responses at 10³-fold to 10⁶-fold higher amounts of ligand (Bachmann *et al*, 1998; Cochran *et al*, 2000; Irvine *et al*, 2002). How T cells display an unsaturated response to such a range of pMHC concentrations is also puzzling considering the properties of the receptor.

Crucial to solving these problems are the stoichiometry (the number of copies of each subunit per complex) and the arrangement of the receptor on the cell surface. This review focuses on the advances made concerning the architecture of the TCR•CD3 complex and tries to answer some of the questions that have been raised above.

The monovalent TCR•CD3 model

The present consensus on the composition of the TCR•CD3 complex is that it comprises the TCRαβ, CD3γε, CD3δε and TCRζζ dimers (Fig 1). The minimal stoichiometry possible is therefore αβγεδεζζ. Of particular importance is the receptor's valency—that is, the number of ligand-binding TCRαβ heterodimers per TCR•CD3 complex.

Early studies that tried to determine the valency of TCR•CD3 used the detergent digitonin to extract TCR•CD3 from the T-cell membrane. Transgenic mice (Punt *et al*, 1994) or human T cells (Hou *et al*, 1994) that simultaneously expressed two distinguishable TCRαβ dimers were used. Specific immunoprecipitation of one dimer did not co-purify the second and vice versa. These data experimentally strengthened the hypothesis that the TCR•CD3 complex is monovalent. It took until 2002 before a complete stoichiometry of all subunits was presented (Call *et al*, 2002). Using an approach involving

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Fig 1 | The T-cell antigen receptor (TCR•CD3) complex consists of the $\alpha\beta$, $\gamma\epsilon$, $\delta\epsilon$ and $\zeta\zeta$ dimers. Schematic pictures of the individual components of the TCR•CD3 complex are shown. The variable immunoglobulin domains of TCR $\alpha\beta$ (V) bind to the ligand, whereas the cytoplasmic tails of the CD3 subunits ($\gamma\epsilon$ and $\delta\epsilon$) and TCR $\zeta\zeta$ interact with cytosolic-signalling proteins. Disulphide bridges are shown by thick black lines. Potential charges in the transmembrane regions are also depicted.

the co-purification of TCR•CD3 complexes expressed in an *in vitro* translation system into endoplasmic reticulum (ER)-derived microsomes (Call *et al*, 2002, 2004), the $\alpha\beta\gamma\epsilon\delta\epsilon\zeta\zeta$ stoichiometry was experimentally confirmed for digitonin-extracted TCR•CD3s (Fig 2A). However, as the ζ subunit might only assemble with the other subunits in the Golgi apparatus *in vivo* (Dietrich *et al*, 1999), and as TCR•CD3 is post-translationally modified in the secretory pathway, it was unclear whether data obtained in the *in vitro* translation system reflected the stoichiometry at the plasma membrane of T cells or just the possible stoichiometry in the ER.

The multivalent TCR•CD3 model

Early on, it was noticed that the $\alpha\beta\gamma\epsilon\delta\epsilon\zeta\zeta$ complex would have an unfavourable imbalance of three potential negative charges in the TM region (Fig 1). This problem could theoretically be solved by adding another TCR $\alpha\beta$ heterodimer to the complex, resulting in a bivalent $\gamma\epsilon\alpha\beta\zeta\zeta\alpha\beta\delta\epsilon$ receptor with a neutral TM region (Fernandez-Miguel *et al*, 1999; Green, 1991; Rojo & Portoles, 1991). Alternatively, the $\alpha\beta\gamma\epsilon\delta\epsilon\zeta\zeta$ stoichiometry of the TCR•CD3 complex led to the idea that two acidic amino acids contact one basic amino acid (Call *et al*, 2002).

The first experimental evidence for a multivalent stoichiometry (that is, more than one TCR $\alpha\beta$ dimer) came from hydrodynamic measurements of the TCR•CD3 size after octylglucoside extraction, which revealed that the complex was larger than $\alpha\beta\gamma\epsilon\delta\epsilon\zeta\zeta$ (Exley *et al*, 1995). Furthermore, studies of the TCR•CD3 stoichiometry, performed without disrupting the plasma membrane, suggested multivalency (Fernandez-Miguel *et al*, 1999). Fluorescence resonance-energy transfer (FRET) between two TCR $\alpha\beta$ s on intact primary double-TCR $\alpha\beta$ -transgenic murine T cells proved that at least two TCR $\alpha\beta$ dimers are present in one complex. Furthermore, when solubilized with the detergents nonidet P-40 (NP40) and Brij96, two TCR $\alpha\beta$ heterodimers could be co-purified. Endoglycosidase H treatment of the receptors showed that it was the surface form that contained at least two TCR $\alpha\beta$ s (Fernandez-Miguel *et al*, 1999). These data clearly showed that multiple TCR $\alpha\beta$ s constitute the receptor, although they did not prove a $\gamma\epsilon\alpha\beta\zeta\zeta\alpha\beta\delta\epsilon$ stoichiometry. So, how could the discrepancy between the monovalent and multivalent models be resolved?

A unifying model of TCR•CD3 valency

The TM domains have a fundamental role in the interactions between TCR $\alpha\beta$ and the CD3 subunits (Blumberg *et al*, 1990a; Call *et al*, 2002; Cosson *et al*, 1991). Therefore, the choice of detergent is crucial for maintaining TM interactions. Conversely, the detergent should be effective enough to dissolve the membrane around the protein complex. Also, the assumption that only one defined complex exists might not be true. Therefore, the experimental approach used to study a multiprotein complex should be able to discriminate between complexes of different stoichiometries.

Blue-native polyacrylamide-gel electrophoresis (BN-PAGE; Schägger & von Jagow, 1991; Schamel & Reth, 2000) with different detergents was used to study the size and stoichiometry of TCR•CD3 (Hellwig *et al*, 2005; Schamel *et al*, 2005). In contrast to co-purification and FRET, BN-PAGE determines the relative abundance and number of different complexes that are formed by a given protein. Furthermore, the complexes are formed in their native environment, which allows post-translational modifications and interactions with other proteins, such as chaperones, to occur.

When the TCR•CD3 complex was extracted in digitonin from murine and human primary T cells, as well as T-cell lines, a single monovalent complex with the $\alpha\beta\gamma\epsilon\delta\epsilon\zeta\zeta$ stoichiometry was detected by BN-PAGE (Hellwig *et al*, 2005; Schamel *et al*, 2005; Zapata *et al*, 2004). By contrast, the detergents Brij96 and Brij98 allowed the separation of a variety of complexes of different sizes (Hellwig *et al*, 2005; Schamel *et al*, 2005). Control experiments excluded the possibility that Brij96 caused an artefactual aggregation of TCR•CD3 (Schamel *et al*, 2005). The large TCR•CD3 complexes were located at the plasma membrane as they became tyrosine-phosphorylated on pMHC stimulation. For confirmation, one TCR β was co-purified with another from Brij96 lysates, indicating that the Brij96-solubilized TCR•CD3 complexes have at least two TCR β chains (Schamel *et al*, 2005). Co-purification, however, was not possible using digitonin extraction.

The co-existence of TCR•CD3 complexes with different valencies identified by BN-PAGE in Brij96 and other detergents is in accordance with the previously mentioned FRET data on intact T cells. Furthermore, electron microscopy (EM) of immunogold-labelled primary human T cells, as well as human and murine T-cell lines, showed that before stimulation, monovalent TCR•CD3 complexes are co-expressed with a variety of multivalent TCR•CD3s (Schamel *et al*, 2005; Fig 2B). The same conclusion has been reached by using a distinct EM technique (B. Lillemeier & M.M. Davis, unpublished data). At their largest, these multivalent TCR•CD3 complexes consist of around 20 CD3 and TCR $\alpha\beta$ dimers (Schamel *et al*, 2005). The pre-formed clusters are too small to be seen by confocal microscopy, and are therefore different from the so-called TCR•CD3 patches or microclusters that can be detected by light microscopy after TCR•CD3 stimulation and subsequent receptor aggregation (Mossman *et al*, 2005; Yokosuka *et al*, 2005).

In summary, multivalent TCR•CD3s are co-expressed with monovalent receptors on the resting T-cell surface (Fig 2). As expected, different detergents have different effects on the integrity of the TCR•CD3 complexes (Fig 3). Brij96 is the mildest detergent, extracting all TCR•CD3s while leaving the multivalent and monovalent receptors intact. By contrast, digitonin disrupts the multivalent receptors, yielding only the monovalent stoichiometry. Taking into account the different effects of the detergents, the co-existence of monovalent and multivalent TCR•CD3s is in agreement with all published data (Blumberg *et al*, 1990b; Call *et al*, 2002, 2004; de la Hera *et al*, 1991;

Fig 2 | Arrangement of the T-cell antigen receptor (TCR•CD3) complexes on the cell surface before stimulation. The old model shows monovalent receptors with a stoichiometry of $\alpha\beta\gamma\epsilon\delta\epsilon\zeta\zeta$ distributed evenly on the plasma membrane. The new model has monovalent receptors and a variety of multivalent receptors that co-exist in the intact membrane of unstimulated T cells, and that have been detected by fluorescence resonance-energy transfer and electron microscopy. Due to the lack of certainty, the stoichiometries of the monovalent and multivalent TCR•CD3s are not shown. The monovalent complexes, however, might have the $\alpha\beta\gamma\epsilon\delta\epsilon\zeta\zeta$ stoichiometry. The multivalent TCR•CD3s might be present in a different lipid context (dark grey). The cell surface also contains large areas that are devoid of any TCR•CD3. TCR $\alpha\beta$ is shown in green and CD3 is shown in various shades of blue/turquoise.

Fig 3 | Influence of different detergents on the integrity of the multivalent T-cell antigen receptor (TCR•CD3) complex. The detergents Brij96, Brij98, lubrol and octylglucoside keep the multivalent TCR•CD3 intact. Digitonin disrupts the multivalent complexes, resulting in the $\alpha\beta\gamma\epsilon\delta\epsilon\zeta\zeta$ stoichiometry. In contrast to murine TCR•CD3, the human receptor is disassembled into its dimeric constituents by nonidet P-40 (NP40) and TritonX-100 (TX-100; San Jose *et al*, 1998). Sodium dodecyl sulphate (SDS) treatment leads to unfolding of the subunits and subsequent destruction of all non-covalent interactions.

Exley *et al*, 1995; Fernandez-Miguel *et al*, 1999; Hou *et al*, 1994; Punt *et al*, 1994; Schamel *et al*, 2005; Zapata *et al*, 2004).

Architecture of the multivalent TCR•CD3 complexes

In the 2B4 T-cell line, approximately one-half of the TCR $\alpha\beta$ chains are present in monovalent TCR•CD3 complexes, whereas the others are present in multivalent complexes (Schamel *et al*, 2005). This ratio varies between different cell types for a given TCR $\alpha\beta$ dimer (Hellwig *et al*, 2005), indicating that factors other than TCR•CD3 determine the degree of multimerization before stimulation.

As visualized by EM, the small and large TCR•CD3 complexes are located in close proximity to each other on the cell surface, leaving large areas devoid of any TCR•CD3 (Fig 2; I. Arechaga, H.M.v.S., J. M. Valpuesta, W.W.A.S. & B.A., unpublished data). In these experiments, the multivalent complexes were predominantly found as linear assemblies, in which the gap between two adjacent gold particles was not always resolved. As the resolution of the EM was higher than 1 nm and the predicted diameter of one TCR•CD3 unit is larger than the 10 nm gold particles used (Sun *et al*, 2004), it was concluded that the TCR•CD3 units are close enough to establish protein–protein contacts. The organization of the multivalent complexes in linear arrays rather than in random clusters further supports the existence of protein–protein interactions as the driving force for the formation of multivalent complexes. Nevertheless, the disassembly of multivalent TCR•CD3 by cholesterol extraction (Schamel *et al*, 2005) suggests that lipid–protein interactions also have a structural role within the multimers. It is likely that cholesterol confers stability to the multivalent, but not monovalent, complexes. This conclusion is supported by a study indicating that two different TCR•CD3 populations exist on the surface of living cells: one that is sensitive and one that is insensitive to cholesterol extraction (Uhlén *et al*, 2003). Owing to its cholesterol dependence, the multivalent TCR•CD3 could be present in lipid rafts, whereas the monovalent receptor could be excluded. As cholesterol levels are low in the ER membrane (Holthuis & Levine, 2005), it is questionable whether the multivalent TCR•CD3 was present in the *in vitro* translation experiments (Call *et al*, 2002, 2004).

The stoichiometry of the TCR•CD3 is still unresolved

Although the stoichiometry of the TCR•CD3 is most relevant in the intact membrane, at present this information is only available for the complex in detergent micelles. The stoichiometries in detergents milder than digitonin remain unknown. When the mild detergent lubrol, which keeps the multivalent receptors intact (Fig 2; M.S. & W.W.A.S., unpublished data), was used in the *in vitro* translation approach to extract the receptor, a 1:1:1:1 ratio of $\alpha\beta:\gamma\delta:\epsilon:\zeta$ was found (Call *et al*, 2004). As it is not known whether the multivalent complexes form in the ER, it is difficult to interpret the lubrol-derived data. However, they could indicate that the monovalent TCR•CD3 has the $\alpha\beta:\gamma\delta:\epsilon:\zeta$ stoichiometry. Another important question is whether the multivalent receptor clusters are multimers of the monovalent form or contain a different ratio between the individual subunits. An attractive hypothesis is that there is a dynamic equilibrium between monovalent and multivalent receptors on the cell surface.

Until the stoichiometry issue is clarified, it will not be possible to conclude whether each potential positive charge in the TCR $\alpha\beta$ TM region is counterbalanced by one (Fernandez-Miguel *et al*, 1999; Green, 1991) or two (Call *et al*, 2002) potential negative charges. In this context, it is worth mentioning that the extensive mutagenesis study of the charged amino acids in the TM regions of the individual TCR•CD3

Fig 4 | Multivalent T-cell antigen receptors (TCR•CD3s) have higher avidity towards the ligand than monovalent TCR•CD3s. (A) Major histocompatibility complex (MHC)/self-peptide clusters (white or yellow) do not bind with sufficient avidity to either TCR•CD3 form and thus do not activate T cells. (B) Clusters of MHC containing two agonistic peptides bind with sufficient avidity to the multivalent, but not the monovalent, TCR•CD3. Consequently, at low pMHC concentrations, only multivalent TCR•CD3s are activated. (C) In clusters with one antigenic pMHC (orange) and MHC/self-peptides of intermediate affinity (yellow), the average affinity of both interactions will determine whether the multivalent TCR•CD3s can be bound with a sufficiently high avidity for T-cell activation. If the affinity of the MHC/self-peptide is low (upper panel), then TCR•CD3 triggering does not occur. If the affinity of the MHC/self-peptide is above a certain threshold, then, in combination with the agonist pMHC, the avidity might be high enough to trigger multivalent TCR•CD3 (lower panel). This avidity is not sufficient to cluster and activate two monovalent TCR•CD3s. Thus, small amounts of agonistic pMHC presented by an antigen-presenting cell might preferentially trigger multivalent TCR•CD3.

subunits that determined the interaction of the different charges (Call *et al*, 2002) was only conducted in digitonin micelles and therefore might not necessarily reflect the interactions in the intact membrane.

Receptor pre-clustering for higher sensitivity

We have recently shown that multivalent TCR•CD3s are activated at low agonistic pMHC concentrations, whereas monovalent

Fig 5 | Multivalent T-cell antigen receptors (TCR•CD3s) allow spreading of the signal within the multimer. (A) At low agonist–major histocompatibility complex peptide (pMHC) concentrations, the multimeric TCR•CD3 complexes become preferentially activated. This might be due to their higher avidity for multimeric antigenic pMHC (orange). TCRαβs of multivalent TCR•CD3s that are not engaged by antigenic pMHC can bind to MHC/self-peptide (white) and thereby become activated. By amplifying the effect of a few pMHC-binding events to neighbouring receptors within a TCR•CD3 multimer, the T cell might be able to respond to low pMHC doses. (B) At medium agonist–pMHC concentrations, the multimers are saturated. (C) At high agonist–pMHC concentrations, monovalent TCR•CD3s begin to be activated. This allows the T cell to sense high doses of antigen when the multivalent receptors are saturated. (D) Only at high ligand concentrations, when all multivalent and monovalent receptors are engaged, is the T-cell response saturated. APC, antigen-presenting cell.

TCR•CD3s remain unaffected (Schamel *et al*, 2005). This finding could be explained by three mechanisms. First, multivalent TCR•CD3 complexes probably enhance the avidity towards the ligand, which is expressed in clusters on the surface of APCs (Anderson *et al*, 2000; Krishna *et al*, 1992; Kropshofer *et al*, 2002). One consequence might be that at low ligand doses, only the multivalent complexes are bound, whereas monovalent TCR•CD3s require higher ligand concentrations (Schamel *et al*, 2005). This is especially evident when MHC/self-peptides with intermediate affinity to TCRαβ are considered; they cannot activate the TCR•CD3 on their own (Fig 4C), but they can enhance the sensitivity of T-cell activation at low agonist concentrations (Krogsgaard *et al*, 2005; Wülfing *et al*, 2002; Yachi *et al*, 2005). Under these conditions, only multimeric forms of TCR•CD3 might become activated due to their high avidity for the pMHC heterodimers compared with the monovalent form (Fig 4C). Monovalent TCR•CD3 would be activated only at high agonist doses (Fig 5). Second, the multivalent complexes might allow the propagation of the activation signal from ligand-bound TCRαβ to the neighbouring receptors in the same multimer, thereby amplifying the effect of binding and explaining the high sensitivity of T cells. In this model, the endogenous MHC/self-peptides also have a role in occupying the non-agonist-bound TCRαβ within a TCR•CD3 multimer and thereby activating additional receptors (Fig 5). Third, the linear arrays of multivalent complexes could help a single pMHC to serially trigger several receptors (Valitutti *et al*, 1995).

In conclusion, the role of the multimers might be to enable T cells to respond to low concentrations of antigen, for example, at the onset of an immune response (Fig 4). Mathematical modelling has shown that cooperative effects within the receptor multimers amplify small differences in ligand affinity by several orders of magnitude (Schamel *et al*, 2006). Thus, multivalent TCR•CD3s might be more effective in discriminating between distinct pMHC than monovalent TCR•CD3s. This resolves the paradox of the high specificity–low affinity TCR•CD3–pMHC interaction.

Monovalent TCR•CD3s raise the saturation threshold

T cells respond to a wide range of pMHC concentrations (Bachmann *et al*, 1998; Cochran *et al*, 2000; Irvine *et al*, 2002). When the multivalent TCR•CD3 complexes are already saturated, the monovalent receptors can still be activated at increasing ligand doses (Schamel *et al*, 2005). Indeed, theoretical calculations suggest that cells co-expressing monovalent and multivalent forms of a given receptor can show high sensitivity to low concentrations of ligand and concentration-dependent responses to high ligand concentrations (Bray *et al*, 1998). Thus, the presence of monovalent TCR•CD3 might endow the T cell with the capacity to produce a concentration-dependent response even at high pMHC doses when multivalent TCR•CD3s are already saturated (Fig 5).

Conclusions

The controversy over the TCR•CD3 stoichiometry has been resolved by combining advanced biochemical methods using different detergents in combination with non-invasive techniques, such as FRET and immuno-gold EM. The demonstration of the co-existence of monovalent and multivalent TCR•CD3 complexes on the surface of a T cell is a foundation on which new hypotheses can be generated and tested experimentally. Hopefully, we can now take a big step forwards in understanding the TCR•CD3 complex, as well as T-cell biology as a whole.

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