A Response Calculus For Immobilized T Cell Receptor Ligands

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Abstract

To address the molecular mechanism of T cell receptor (TCR) signaling we have formulated a model for T cell activation, termed the 2D-affinity model, in which the density of TCR on the T cell surface, the density of ligand on the presenting surface and their corresponding two-dimensional affinity determines the level of T cell activation. When fitted to T cell responses against purified ligands immobilized on plastic surfaces the 2D-affinity model adequately simulated changes in cellular activation as results of varying ligand affinity and ligand density. These observations further demonstrated the importance of receptor cross-linking density in determining TCR signaling. Moreover, it was found that the functional two-dimensional affinity of TCR ligands was affected by the chemical composition of the ligand-presenting surface. This opens for the possibility that cell-bound TCR ligands, despite their low affinity in solution, are of optimal two-dimensional affinity thereby allowing effective TCR binding under physiological conditions, i.e. at low ligand densities in cellular interfaces.
Introduction

Most T cells present αβ T cell receptors (TCR) on their surface. The natural ligands for these receptors are small antigenic peptides bound to MHC molecules (pep-MHC) on the surface of other cells with which T cells interact (1). Antigen recognition can result in various protective functions including release of cytokines to cause local inflammation and specific killing of virus-infected cells (2).

The TCR comprises the α/β subunits which recognize pep-MHC and the signal transducing subunits δ, ε, γ and ζ (CD3-ζ complex) which contains the immunoreceptor tyrosine-based activation motifs (ITAMs) (3). Signaling of the TCR/CD3-ζ complex can be viewed as a dynamic phosphorylation/dephosphorylation equilibrium of ITAMs where the steady-state levels of phosphorylated ITAMs is low in un-stimulated T cells (4). The molecular mechanism by which ligand-bound TCR perturbs this equilibrium is unknown.

One way to help identify the molecular features underlying TCR signaling is to develop mathematical models capable of simulating TCR signaling. Describing TCR signaling as a dynamic equilibrium indicates that it should be possible to model T cell behavior using mathematical expressions involving the binding constants of TCR/ligand interactions. Moreover, as recently discussed (5), such models are attractive because they allow for simulation of T cell responses and thus help guide future research, and because they can assist in optimizing clinical immuno-modulatory strategies.

Previous mathematical analysis of T cell responses have been successful in modeling specific features such as fast ligand dissociation kinetics (6), peptide antagonism (7,8), rate of receptor internalization (9) or the effect of co-stimulation on proliferation (10). Current models favor a discrimination between the potency of TCR ligands based on the life-time of the interaction,
i.e. the off-rate (6,7,9). In Support, recent studies suggest that TCR signaling correlates to ligand dissociation rate (11-13). This is, however, still a matter of controversy since conflicting reports exists, which favor ligand affinity as the determining factor for TCR signaling (14-20).

Recently, we have compared the stimulatory efficacy of a panel of anti-TCR antibodies and a panel of superantigens varying 10,000-fold and a 150-fold in TCR affinity, respectively, with corresponding changes in binding kinetics as well (20). Stimulation of T cells with ligand-coated plastic surfaces revealed that the biological activity primarily matched the affinity of the TCR/ligand interaction. It therefore appeared that it was the density of bound receptors in the contact area, which determined the strength of the T cell stimulus. This prompted us to develop a theoretical framework in which T cell responsiveness was expressed in mathematical terms. We here present a mathematical model, termed the 2D-affinity model, which allows for direct computation of T cell responses based on receptor and ligand densities and their corresponding solution affinity.

**Experimental procedures**

*Lymphocytes and T cell lines.* A5 T cell hybridomas expressing the 14.3.d TCR (I-E^d/HAA 110-119; Vα4.2, Vβ8.2) and carrying the reporter gene plasmid were grown in the presence of 0.5 mg/ml Hygromycin (Calbiochem, La Jolla, USA) and cultured in RPMI 1640 medium supplemented with penicillin, 2x10^5 U/L (Leo Pharmaceutical Products, Ballerup, Denmark), streptomycin, 50 μg/ml (Merck, Darmstadt, Germany), and 10% (v/v) FCS (Life Technologies, Paisley, U.K.) at 37°C in 5% CO2.

*Protein expression and purification.* Antibodies and I-E^d chimeras were produced in *Drosophila* cells basically as previously described (21). The recombinant protein was purified from culture
supernatant using affinity chromatography followed by ion exchange chromatography. Superantigen were produced in E coli and isolated from the periplasm as previously described (22).

Preparation of ligand coated surfaces. Maxisorb™ microtiter plates (NUNC A/S Denmark) were termed surface A and treated with 50 µl of a PBS containing 10µg/ml protein A (Pharmacia, Sweden) over night at +4°C prior to use with antibodies. Plates were blocked for >1 hours with PBS containing 2% Bovine Serum Albumin (Sigma, USA). Antibodies at 10 µg/ml in PBS + 0.2% BSA were diluting against a non TCR-binding antibody at similar concentration in order to keep the level of protein A binding constant and thereby secure that the dilution factor was also represented on the surface. Antibody dilutions were incubated together with immobilized protein A for >2 hours after which excess antibody was removed by washing. Alternatively, surface A was prepared by coating maxisorb plates with serial dilutions of each superantigen (SEC3) variants. To ensure uniform coating at different concentrations SEC3 molecules were diluted into PBS containing 5 µg/ml BSA. Surface B was prepared by coating antibodies directly on the surface of nunclon microwell™ plates (NUNC A/S Denmark). As above, antibodies were diluting against a non TCR-binding antibody in order to keep the total concentration of protein (10 µg/ml) constant. TCR binding to Surface A and B was compared by ELISA (Figure 3A). Antibody at 10 µg/ml was incubated with protein A-coated surface A or coated directly onto surfaces B. Serial dilutions of soluble TCRβ (Vβ8.2+) (23) were thereafter added for and left to bind for >1 hours. Bound TCRβ-chain was detected using a biotinylated antibody against the Cβ mixed with excess mouse serum to compete for unwanted binding to protein A. The assay was developed using HRP-conjugated strepavidin and the TMB-plus peroxidase substrate (Kem-En-Tech, Copenhagen, Denmark)

T cell stimulation. A5 T cell hybridomas, expressing green fluorescent protein (GFP) upon activation of the IL-2 promotor, were stimulated for 4.0-4.5 hours and cellular activation was
determined by the presence of intracellular GFP. Fluorescent cells were detected by FACS and recorded as positive.

Mathematical fitting of T cell responses. Two-dimensional affinities are best expressed as molecules per \( \mu \text{m}^2 \). The previously determined affinity constants ((20) and P.S.A. and K.K. unpublished results) were therefore rescaled from moles per liter to molecules per \( \mu \text{m}^3 \) prior to use in the fitting procedures (see table).

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<tr>
<th>Ligand</th>
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<td>Peptide/MHC</td>
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The average surface area of A5 T cell hybridomas were calculated from the cell diameter in growth medium (4\( \mu \text{m} \)) and multiplied by a correction factor of 1.8 to compensate for the roughness of the cell surface (Cell surface area = \( 4 \times \Pi \times (4\mu \text{m})^2 \times 1.8 = 360 \mu \text{m}^2 \)) (24). The total amount of TCR was determined by a calibrated FACS analyses using PE-conjugated 2C11 Antibody and calibrated PE-conjugated beads (BD Biosciences, NJ, USA). Receptor density was determined by dividing the average surfaces area with the average number of receptors per cell ([TCR]_{total} = 9200 molecules\text{cell}^{-1} / 360 \mu \text{m}^2 = 25 \text{molecules/\mu m}^2). Ligand densities were determined by radio-
immuno assays, as previously reported (20). Curves were fitted using the Sigma Plot software (SPSS inc., Chicago, USA). Fits were done using the least-square Marquardt-Levenberg algorithm. T cell stimulation experiments were fitted directly using a combined expression of equation 5 or equation 5 and 6, as indicated in the text. Background values from un-stimulated cells were subtracted prior to the fitting analysis. As an alternative to $f_{ac}$, fits could also be improved by assuming the density of TCR ($[TCR]_{total}$) as variable (data not shown). However, these fits were not considered further because 1) fitted values of $[TCR]_{total}$ were significantly larger than measured values and 2) $K_{stim}$ varied between different data sets and showed a strong mathematical dependency on $[TCR]_{total}$ in the fitting procedure.

**Results**

*Modeling T cell responsiveness by laws of mass action.*

T cells are activated when brought into contact with surfaces displaying ligands that interact with the TCR. The natural stimulus is other cells displaying antigenic pep-MHC complexes on their surface. However, purified TCR ligands coated onto surfaces composed of materials such as glass, agarose or plastic (polystyrene) are generally found to be effective mimics of the antigen presenting cell (APC) and thus able to activate T cells. Regardless of surface, it appears reasonable to assume that laws of mass action guard initial contacts between TCR and ligand. Only few and relative specialized methods exist for measuring binding constants between molecules attached to surfaces (25-27). To bypass this difficulty, we propose to use affinities, i.e. equilibrium dissociation constants ($K_d$), measured in solution as basis for estimates of affinities in cellular interfaces.

On theoretical grounds, Bell suggested that the affinity in solution ($K_{d(3D)}$) and the affinity in a cellular contact area ($K_{d(2D)}$) are related by a constant ($\sigma$) which is proportional to the height of the confined region in which binding occurs (28).
\[ K_{d(2D)} = \sigma \cdot K_{d(3D)} \]  \hspace{1cm} (1)

Theoretically, the confinement region height has been predicted to be in the 1-10nm range (29) and furthermore recently demonstrated experimentally by Dustin and colleagues (24,30).

Having determined a relationship between the affinities measured in solution and the affinity between molecules attached to apposing surfaces, we are able to compute the density of bound receptors using the law of mass action (eq. 2) and the law of mass conservation (eq. 3a and 3b),

\[ [\text{TCR}] \cdot [\text{L}] / [\text{TCR}/\text{L}] = K_{d(2D)} \] \hspace{1cm} (2)

\[ [\text{TCR}]_{\text{total}} = [\text{TCR}] + [\text{TCR}/\text{L}] \] \hspace{1cm} (3a)

\[ [\text{L}]_{\text{total}} = [\text{L}] + [\text{TCR}/\text{L}] \] \hspace{1cm} (3b)

where \([\text{TCR}]\), \([\text{L}]\) and \([\text{TCR}/\text{L}]\) are the density of free TCR, free ligand and bound TCRs in the contact area, respectively. The total density of TCR and ligand ([L]_{\text{total}} and [TCR]_{\text{total}}) can be determined experimentally independent of the biological experiments. Equation 1-3 can be solved with respect to \([\text{TCR}/\text{L}]\) and thus used to estimate the density of bound TCR in the contact zone. To translate binding events into response units we assumed that subsequent cellular responses were directly proportional to the density of bound receptors expressed as

\[ R = K_{\text{stim}} \cdot [\text{TCR}/\text{L}] \] \hspace{1cm} (4)

where \(R\) is the response level and \(K_{\text{stim}}\) is the proportionality constant expressing the increase in stimulation per TCR/ligand complex in the initial contact area. Thus, equation 1-4 allows us to make a single equation (eq. 5), which describes the relationship between ligand density, receptor density, solution affinity and cellular response.

\[ R = K_{\text{stim}} \cdot 0.5 \cdot \sqrt{([TCR]_{\text{total}} + [L]_{\text{total}} + K_{d(3D)} \cdot \sigma) + \sqrt{([TCR]_{\text{total}} + [L]_{\text{total}} + K_{d(3D)} \cdot \sigma)^2 - 4 \cdot [TCR]_{\text{total}} \cdot [L]_{\text{total}}}} \] \hspace{1cm} (5)

*Direct fitting of T cell responses against immobilized TCR ligands*
As the 2D-affinity model promises the ability to fit cellular responses directly, initial analysis could be done using existing datasets, which described activation of T cells as a function of ligand affinity and ligand density (20). The TCR ligand was a panel of antibodies of predetermined affinity against the variable domain of the TCR β-chain and T cell activation was determined by activation of the transcription factor of activated T cells (NFAT) (31). In order to become biologically active, the antibodies were immobilized on polystyrene plastic surfaces (surface A) coated with protein A. Ligand and receptor densities were determined independently of the biological assays by radio immuno-assays and FACS, respectively. Results in Figure 1A demonstrate that antibody-coated surfaces were potent activators of T cells. Response curves showed that high TCR affinity activated most cells with a gradual drop in potency as the affinity of the antibody decreased. For antibodies of highest affinity an optimum was reached after which the response started to decline. This decline was only evident at unphysiologically high ligand densities (>200 sites/µm²) and it therefore appeared reasonable to exclude the declining phase in order to simplify the modeling.

Response curves for the whole antibody panel were fitted globally to equation 5 using the predetermined values for \( K_{d(3D)} \), TCR density and ligand densities. Plotting the fitted lines together with the experimental data points (figure 1B) showed that the 2D-affinity model was able to model many of the observed features of the response curves. First, it described the relative ranking in potency of the ligands of different TCR affinity. Second, it described the increase in steepness of the response curves as the affinity of the ligand increases in contrast to the flatter profile of the low affinity response curves. In addition, statistical analysis (Table I) of the fits showed that the coefficient of determination \( (R^2) \) was relatively close to unity and that the probability of being wrong was low \( (P<0.05) \) which demonstrated that fits were of good quality and that the fitted values, \( \sigma \) and \( K_{stim} \), were statistically well defined.
On closer examination, the residuals (i.e. the difference between the theoretical and measured values; see bottom of figure 1B) showed that the 2D-affinity model was less capable of fitting the potency of high affinity antibodies, notably at low ligand density. One possible complication was that ligand density on the plastic surfaces was measured using soluble TCR molecules. Roughness of the plastic surface could exclude potential binding sites from T cell contact and thereby lower the density of biologically active ligand. Thus, we included an additional variable, $f_{ac}$, that described the fraction of active ligand on the plastic surface.

$$[L]_{ac} = [L]_{total}f_{ac} \quad (6)$$

Where $f_{ac}$ describes the fraction of active ligand. Substituting the total ligand density, $[L]_{total}$, with the density of active ligand, $[L]_{ac}$ results in a new version of equation 5 that was able to compensate for functional variability of immobilized ligands. Fitting of cellular responses revealed a significant improvement in the fits in the high affinity range (Figure 1C) and ~57% of the plastic bound antibodies were estimated to be biological active (Table I). Residual plots showed that antibodies of high affinity were now fitted with equal quality as those of low affinity. The increase in fitting quality was also evident by statistical analysis where the $R^2$ became closer to one while P values were still significantly small (Table I). Inclusion of $f_{ac}$ had no effect on $K_{stim}$ and caused a marginal 2-fold decrease of $\sigma$ to 1.4 $\mu$m.

A $\sigma$ value of 1.4 $\mu$m suggests that binding is confined to a relative large volume, several times the height of an antibody or a TCR. This contrasts theoretical predictions and the few experimental estimates of $\sigma$ that were in the 1-10 nm range (discussed above). Regardless, the antibody-coated plastic surfaces were able to stimulate T cells with only a few ligands per $\mu$m$^2$ demonstrating the high sensitivity of the experimental approach.

*Direct fitting of cellular responses of superantigen stimulated T cells*
To address the role of ligand topology and to test the generality of the 2D-affinity model we tested the fitting procedures on T cells stimulated by bacterial superantigens. The superantigens were variants of *Staphylococcus* enterotoxin C3 (SEC3), which had been selected for enhanced binding to TCR (20). The cellular assays were the same as used with the antibodies: T cells were stimulated by serial dilutions of superantigens coated onto surface A and cellular activation was determined by NFAT activity (Figure 2A). The completeness of the data set was not sufficient to allow estimates of $f_{ac}$ (data not shown). However, fitting of response curves using equation 5 gave nice fits as judged by graphical (Figure 2B) and statistical (Table I) analysis. As for the antibodies, $\sigma$ was determined to be relative large and $K_{stim}$ was almost identical for the two sets of ligands (Table I). It therefore appeared that immobilized anti-TCR antibodies and superantigens were equal in their ability to activate T cells. The 10-fold higher $\sigma$ values of the SEC3 variants relative to the antibodies could be explained by the inability of the relative hydrophobic surface A to co-align with the negatively charged cell surface. Precise membrane alignment has previously been suggested to be an important factor in enhancing the affinity between membrane proteins (24). The smaller size of SEC3 relative to the antibody would therefore make the interaction more sensitive to poor alignment of the apposing surfaces and hence increase $\sigma$.

*Change in surface chemistry enhanced the functional affinity of TCR ligands*

In their attempt to develop theoretical models for cellular adhesion, Bell and colleagues predicted that the two-dimensional affinity of surface-bound proteins could be affected by electrostatic forces acting on the apposing surfaces (32). Also, a recent study has demonstrated that membrane roughness can modulate the effective affinity of membrane proteins (33). Taken together with the unexpectedly high $\sigma$ values for antibodies and SEC3 variants on surface A, it appeared possible that chemical changes of the ligand-presenting surface could enhance the 2D-affinity of immobilized
ligands. Accordingly, another plastic surface (surface B) was tested for its ability to present ligands to T cells. Surface B was chemically equivalent to the surface A but subjected to stronger irradiation (i.e. stronger oxidation) in the manufacturing process and therefore less hydrophobic and more electro negative. To exclude that surface charge had any direct effect on antibody affinity the two surfaces were compared by a sandwich ELISA approach. Antibody variants of medium affinity were immobilized on the two plastic surfaces and assayed for binding to soluble TCRβ chain (Figure 3A). TCR binding of the two surfaces were similar and fitted well to equations for one-to-one interactions demonstrating the intactness of the binding sites. To address the functional affinity, serial dilutions of each antibody panel were coated directly on surface B and used to stimulate T cells (Figure 3B). The most prominent difference to the previous results was the relative small difference in potency among the high-affinity antibodies indicating that ligand binding was approaching a plateau. Thus, making the ligand-presenting surface more electronegative clearly affected the functional affinity of the antibodies and the appearance of a potency limit at lower affinity indicated an overall increase in 2D-affinity of the antibodies attached to surface B (see figure 5).

To test if the 2D-affinity model could simulate this phenomenon, response curves were fitted globally to equation 5 and 6 using predetermined values for $K_{d(3D)}$, [TCR]_{total} and [L]_{total} (Figure 3C). Fits were similar in quality to those obtained by the SEC3 variants as judged by the residuals (Figure 3C) and the statistical analysis (Table I). $\sigma$ was estimated to 98 nm which corresponds to a 15-fold increase in 2D-affinity relative to surface A and the theoretical curves also showed signs of approaching an affinity limit. $K_{\text{stim}}$ matched the previous results indicating that the biological activity of each receptor/ligand complex was the same on the two surfaces. The active fraction of antibodies dropped to ~20% which was 3-fold lower than on surface A. Excluding protein A by attaching the antibodies directly to surface B would lead to a more random orientation.
of antibodies thus explain the drop in activity. Nevertheless, the results showed that the 2D-affinity was dependent on the chemical composition of the presenting surface.

Direct fitting of cellular responses of pep-MHC stimulated T cells

To further test the generality of the 2D-model, T cells were stimulated with purified pep-MHC. I-E<sup>d</sup> was expressed as bivalent molecules fused to human FcγI domains and subsequently loaded with HA peptide. The Fc domains made it possible to repeat the comparison of surface A and B using pep-MHC as immobilized ligand (figure 4A). As with the antibodies, I-E<sup>d</sup>/HA complexes were more potent on surface B relative to surface A. Fitting of the 2D-affinity model to the cellular responses gave estimates of the confinement region heights of 1.5µm and 46nm which matched the estimates for the antibodies. Thus, the surface-induced change in 2D-affinity seemed general and not linked to one particular ligand.

Estimating the limits of TCR-ligand affinity

To further understand what role surface chemistry played in presenting ligands to T cells it was of interest to compare the studies above to a T cell response against pep-MHC on a cell surface. Using the above estimates of confinement region heights for surface A and B and an independent estimate of the confinement region height of TCR/MHC interactions in a cellular interface (30), T cell responses were calculated corresponding to a panel of ligands ranging from $10^{-3}$ M to $10^{-9}$ M in TCR affinity. Three conditions were simulated representing the surface of the APC, surface B and surface A corresponding to $\sigma$ values of 1.2, 46 and 1400 nm, respectively (figure 5A). As observed experimentally on surface B, the modeling demonstrated that beyond a certain limit additional increase in affinity did not lead to further stimulation due to saturation of the ligand. The modeling further predicted that the strongest binding antibody ($K_d = 2.3\text{nM}$) on surface A was close to the
experimental limit as previously suggested (20). Interestingly, as $\sigma$ moved into the physiological range the solution affinity limit got close to $10^5$-$10^6$ M. The importance of this became evident when the theoretical limits were compared to the experimental limits observed for the solution TCR affinity of pep-MHC and bacterial superantigens (Figure 5B). The limits predicted for the T cell/APC interface closely matched the observed limits for native TCR ligands. Thus, the 2D-affinity model implies that the low solution affinity of pep-MHC and superantigens to TCR equals maximal binding in the contact zone between the cell membranes.

Discussion

The important role of ligand binding strength in TCR signaling and hence T cell activation is well documented (1,2). Central events in T cell development such as positive and negative selection in the thymus are in part determined by the binding strength of TCR ligands (14). Moreover, modified TCR ligands of sub-optimal binding have been found to act as partial agonist or antagonist (36,37). The TCR is therefore capable of responding to a wide range of binding affinities resulting in differential cellular responses.

Using plastic-immobilized anti-TCR antibodies and superantigens of varying affinity and binding kinetics, we have previously reported that ligand-coated plastic surfaces were effective stimulators of T cells capable of inducing various responses in different types of T cells (20). Others have shown that many of the morphological responses observed to immobilized antibodies paralleled those observed during the early stages of physiological contact (38). Also, we observed that plastic-bound ligands were able to stimulate T cells at densities as low as 1-10 sites/µm$^2$ which is close to the physiological limit of 0.2 sites/µm$^2$ (30). Thus, immobilized TCR ligands on planar surfaces are able to functionally mimic pep-MHC complexes on the surface of APCs. Moreover, the use of planar surfaces coated with purified ligands is advantageous when addressing the role of
TCR ligand affinity in TCR signaling since the simplified nature of the stimulus excludes the contribution of co-stimulatory receptors. However, one important difference between ligand-coated surfaces and the native ligand-presenting surface is the inability of plastic-immobilized ligands to diffuse laterally (discussed below).

Previous studies have indicated that the potency of pep-MHC complexes on the surface of APCs correlates to TCR affinity (14-16,19) and that stimulation of T cells follows the law of mass action (17,18). It therefore appears possible that TCR signaling to a large extend is determined by the density of ligand-bound TCR in the contact zone. Based on the laws of mass and the concepts of 2D-affinity (26) we here propose a response calculus for T cell activation called the 2D-affinity model capable of modeling many of features of the T cell responses using predetermined values of TCR density, ligand density and their corresponding affinity. The ability to simulate changes in ligand potency based solely on experimentally determined changes in solution affinity supports the assumption that changes in solution binding are translated into the cellular interface (see eq. 1). Furthermore, the assumption that the response was directly proportional to the density of ligand-bound TCR in the contact area allowed for an estimate of the specific potency of each ligand/receptor complex. Fitting of T cell responses against three types of immobilized ligands (i.e. antibodies, superantigens and pep-MHC) indicated that the likelihood of cellular activation increased with 2-3% for each additional receptor/ligand complex formed per $\mu$m$^2$ of contact.

Using ligands attached to phospholipid bilayers, Dustin and colleagues demonstrated that cell adhesion mechanisms of low solution affinity could produce contact areas of high physiological affinity presumably through precise alignment of the apposing membranes (24,26). Such mechanism might explain that the potency of immobilized TCR ligands (antibodies and pep-MHC) was improved when increasing the negativity charge of the presenting surface. The chosen polystyrene surface was relative hydrophobic in contrast to the cell surface and the phospholipid
bilayer which are covered with negatively charged sugar and phosphor groups, respectively. Making the polystyrene surface more electronegative thus made it more similar to the biologically relevant surfaces. Better alignment of the T cell membrane with surface B relative to surface A could therefore explain the estimated 15 to 30-fold increase in two-dimensional affinity. In a more recent study, Dustin and colleagues have reported a confinement region height of 1.2 nm for cellular interfaces involving 2B4 T Cells and I-E\(^k\)/MCC complexes inserted into phospholipid bilayers. Relative to our estimates of ~1.5 μm and ~50 nm on plastic surfaces this suggests an even better alignment of the T cell surface with the lipid bilayer (30).

The ability of the 2D-affinity model to fit different experimental conditions allowed us to simulate T cell responses assuming different ligand-presenting surfaces. In agreement with our experimental data on surface B, the simulations show that for each simulated condition an affinity limit existed. Enhancing the affinity beyond that limit did not cause any further increase in stimulation due to lack of free ligand. Interestingly, we observed that when the confinement region height became small, representing the APC, the theoretical affinity limit of 10\(^{-5}\)-10\(^{-6}\) M matched the binding constants found for most pep-MHC ligand. It therefore appears reasonable to assume that the low affinity in solution of native TCR ligands (i.e. pep-MCH and superantigens) does not represent the physiological interaction. Rather, the affinity would be close to maximal in cellular interfaces and hence optimal for TCR signaling.

APCs need to carry in the range of 100-400 pep-MHC complexes in order to stimulate T cells (35,39,40). Since only a fraction of the cell surface participate in the initial contact with the T cell, this indicates that few pep-MHC complexes are sufficient to start the signaling process. Taken together with the low solution affinity, this lead to the formulation of the low-affinity - high sensitivity paradox (41): how could receptors of low affinity bind ligands at low density? The 2D-
affinity model offers an explanation to this problem by indicating that TCRs are indeed of high intrinsic affinity in cellular interfaces and hence capable of recognizing low levels of ligands.

How can the increase in two-dimensional affinity be understood? Binding at equilibrium (the affinity) is equally determined by the rate by which molecules attach (the on-rate) and the rate by which the separate (the off-rate). Comparison of off-rates of adhesion molecules attached to membranes of in solution showed little difference (42). Any increase in affinity as a consequence of reduction in confinement region must therefore be linked to the on-rate. As the volume in which binding occurs gets smaller the encounter frequency of two independently moving molecules increases. This also increases the chance of complex formation and thereby increases the on-rate. On the contrary, the off-rate is independent of concentration of the reactants and should therefore be insensitive to changes in confinement region size. Based on these simplified considerations, we propose that the increase in functional affinity presented in this study mainly arises from increasing the on-rate, i.e. the rate by which complexes form in the cellular interfaces.

So, even though TCR ligands are of relative high affinity in cellular contexts, they could still posses’ relative fast dissociation rates thus allowing serial triggering and kinetic proof reading. Nonetheless, as we did not observed any specific dependency on fast dissociation it appears that if serial triggering is involved in TCR signaling it must rely on the general dynamic nature of the immunological synapse (IS) rather than on the half-live of individual interactions. Furthermore, as stimulation did not correlate specifically to the off-rate kinetic proof reading did not appear to be the determining factor. However, the results of the present study and the principles of kinetic proof reading are not mutually exclusive.

The kinetic proof reading model indicates that sub-optimal half-lives of TCR/ligand complexes leads to partial assembly of the initial signaling complex thereby causing a negative signal. The main purpose of the kinetic proof reading model is to explain differential signaling seen
for peptide antagonists and positive and negative selecting peptides in the thymus (7,8). These interactions are characterized by having very short half-lives typically of less than 1s (14,36) and thus below the range of half-lives used in this study. Our results can therefore be explained by assuming a relative short time (~1s or less) for assembly of the full signaling complex. Prolonged contact would not lead to more simulation as such but maintain the signaling machinery in an active state. Higher ligand density or ligand affinity would then lead to stronger stimulation according to an increase in density of TCR/ligand complexes as observed.

Alternatively, as noted above ligands immobilized on plastic surface cannot move laterally and thus cannot cluster in the contact zone as otherwise observed during formation of the IS (30). In fact, the half-life of the ligand/TCR interaction was found to determine the ligand density of the IS which subsequently correlated to the cellular response. Our experimental approach excludes the first step of synapse formation (i.e. active clustering of ligand) and the ligand density in the synapses would therefore not be able to exceed the density of ligand on the plastic surface. Cellular responses would therefore be strictly dependent on ligand density as observed. In support of this, we observed that the cellular response was maximal when the density of immobilized ligand matched the ligand density of ~200 ligands/µm² found in synapses causing maximal cellular responses (see figure 1A).

Since ligands fixed to the plastic surface were able to activate T cells at densities of 1-10 ligands per µm² the distance between each ligand must be relative large, several times the extent of the ligand or the receptor. Lateral interactions, such as specific oligomerization of TCR/ligand complexes, are therefore unlikely to be required for early TCR signaling. Rather, each ligand-bound TCR must be able to transmit a signal independently of neighboring receptors. That TCRs work as independent signaling units is compatible with current hypothesis regarding TCR triggering such as the size-exclusion model (43) which predicts that the TCR signaling cascade is activated by
exclusion of large-sized phosphatases, such as CD45, from the contact zone thereby enhancing ITAM phosphorylation or the raft association model (44) which predicts that ligand-bound TCRs are actively recruited into \textit{lck}-kinase rich membrane mini-domains called rafts. Although we cannot make any discrimination on the bases of our results the former hypothesis seems the most attractive as our results emphasize the importance of precise alignment of the ligand-presenting surface and the T cell membrane in order to achieve high affinity and hence maximal signaling.

**Acknowledgements**

This research was supported by the Danish Medical Research Council (C.G. and S.B), the Danish Cancer Society (C.G.) and the National Institute of Health (R.A.M.). The Basel Institute for Immunology was founded and is supported by Hoffmann-LaRoche Ltd, Basel, Switzerland. P.S.A was supported by fellowships from the Danish Natural Science Research council and the Danish Medical Research Council.
References


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    **132**(3), 465-74


Legends

Figure 1. Direct fitting of T cell responses. (A) Stimulation of A5 T cells with serial dilutions of immobilized antibodies of varying TCR affinity. T cell were scored as positive according to activation of NFAT (see experimental procedures for details) The abscissa gives the number of TCR binding sites per µm$^2$ and the ordinate gives the percentage of activated cells. Kd values are indicated for each response curve. (B) Open symbols represent same data as in (A). Lines represent best fits obtained using equation 5. (C) Fitting of the same data set with a equation 5 and 6 combined, i.e. including the extra parameter $f_{ac}$ that allows and estimate of the fraction of biologically active ligand on the presenting surface. Residuals are shown in lower panels. Numerical results are presented in table 1. Shown is one representative example out of five.

Figure 2. Fitting of superantigen-mediated T cell responses. (A) A5 T cells were stimulated with serial dilution of immobilized superantigens of varying TCR affinity. Results are presented as in figure 1. (B) Data sets are the same as in (A). Lines represent best fits obtained using equation 5. Residuals are shown in lower panel. The results represent one experiment out of four.

Figure 3. The effect of the ligand-presenting surface on TCR affinity. (A) Test of binding of soluble TCRβ chain to immobilized antibody. Antibody at 10 µg/ml was bound to surface A (open circles) or coated directly onto surfaces B (filled circles). Binding of serial dilutions of soluble TCRβ chain was detected using a biotinylated antibody against the Cβ. Results were fitted as 1:1 interactions (dotted lines and solid lines, respectively) as a test of homogeneity of the immobilized antibodies. (B) A5 T cells were stimulated with serial dilution of antibodies of varying TCR affinity immobilized on surface B. Results are presented as in figure 1 (C) Same data set as in (B). Lines
represent best fits by equation 5 and 6 combined. Residuals are shown in lower panels. The results represent one experiment out of 5.

*Figure 5. Fitting of T cell responses against pep-MHC complexes.* Stimulation of T cells with purified IE4/HA fusion proteins immobilized on surface A (closed symbols) or B (open symbols). Coated surfaces were prepared as in figure 1 and 3 respectively and the response of A5 T cells is shown. Points indicate experimental values and solid lines indicate fits. Numerical results are shown in table 1.

*Figure 5. Simulation of T cell responses.* (A) Calculation of T cell responses against a panel of ligands ranging from $10^{-9}$ M to $10^{-3}$ M in TCR affinity (i.e. from left to right $10^{-9}$, $10^{-8}$, $10^{-7}$, $10^{-6}$, $10^{-5}$, $10^{-4}$, $10^{-3}$) assuming the three experimental conditions for the peptide/MHC presented in figure 4 (i.e. $\sigma = 1400$, 46 and 1.2 nM, as indicated). The exponent of each $K_d$ value is shown next to the graphs. (B) Comparison of binding constants of native TCR ligands and predicted affinity limits for further increase in potency. Equilibrium binding constants of TCR interacting with pep-MHC (solid circles) and bacterial superantigen (open circles) were collected from the literature (14-16,19,22,36,45-52) or the result of own experiments (P.S.A. R.A.M. and K.K. unpublished data). To facilitate comparison, only values recorded by optical biosensors at room temperature are presented. Hatched areas indicate approximate affinity limits for each modeled condition. Corresponding $\sigma$ values are indicated next to each hatched area.
Table 1. Fitting of T cell responses by the 2D-affinity model

<table>
<thead>
<tr>
<th>Ligand Surface</th>
<th>Average of Fitted Parameters</th>
<th>Average Statistics of Fits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>σ (µm)</td>
<td>Kstim [stimulus \cdot [TCR/L]^{-1}]</td>
</tr>
<tr>
<td>Antibody A</td>
<td>2.6 +/- 1.9</td>
<td>1.9 +/- 0.7</td>
</tr>
<tr>
<td>Antibody A</td>
<td>1.4 +/- 1.3</td>
<td>2.0 +/- 0.7</td>
</tr>
<tr>
<td>Superantigen A</td>
<td>18 +/- 14</td>
<td>3.0 +/- 0.4</td>
</tr>
<tr>
<td>Antibody B</td>
<td>0.098 +/- 0.04</td>
<td>2.0 +/- 0.7</td>
</tr>
<tr>
<td>I-Eα/HA A</td>
<td>1.5 +/- 0.4</td>
<td>3.4 +/- 0.3</td>
</tr>
<tr>
<td>I-Eα/HA B</td>
<td>0.046 +/- 0.02</td>
<td>3.0 +/- 0.2</td>
</tr>
</tbody>
</table>

ᵃEach parameter is the average of five or in the case of superantigens four independent estimates.  
bP values indicate the upper limit for the total parameter set. For individual fits P were in most cases <0.0001
Figure 3

(A) Graph showing the relationship between OD_{450nm} and [TCR/β] nM.

(B) Graph showing the percentage of stimulation (% stimulated) plotted against residues.

(C) Graph showing % stimulated and residuals against sites/µm².
Figure 4
Figure 5

A

B

Kd (M)