Correlation of a dynamic model for immunological synapse formation with effector functions: two pathways to synapse formation

Sung-Joo E. Lee, Yuko Hori, Jay T. Groves, Michael L. Dustin and Arup K. Chakraborty

During antigen recognition by T cells different receptors and ligands form a pattern in the intercellular junction called the immunological synapse, which might be involved in T-cell activation. Recently, a synapse assembly model has been proposed, which enables the calculation of the propensity for synapse assembly driven by membrane-constrained protein binding interactions. We bring together model predictions of mature synapse assembly with data on the dependence of T-cell responses on T-cell receptor (TCR)–MHC–peptide (pMHC) binding kinetics. Predictions of mature synapse assembly, based on TCR–pMHC binding kinetics, correlate well with observed cytokine responses by T cells bearing the relevant TCR but not with cytotoxic T lymphocyte-mediated killing. We discuss the suggested different role for the synapse in pre- and post-nuclear activation events in T cells. The view of immunological synapse assembly given here emphasizes the importance of both the on and off rates for the TCR–pMHC interaction and in this context recent data on a positive role for analogs of self-peptides in synapse assembly is considered.

T-cell activation leading to cytokine production or cell killing requires an appropriate interaction of the T-cell receptor (TCR) with a limited number of foreign MHC–peptide (pMHC) complexes present among the vast array of self-pMHC [1–4]. Focus has been on defining what properties of the TCR–pMHC interaction are required. Events associated with full T-cell activation include the formation of a geometrically patterned collection of receptors and ligands in the immunological synapse, a specialized cell–cell junction composed of supramolecular activation clusters (SMACs) [5,6]. SMACs are defined as micrometer-scale domains of receptors and ligands in the intercellular junction. The nascent immunological synapse appears as a ring of TCR–pMHC interactions surrounding a central region of integrin-mediated adhesion [6,7]. The mature immunological synapse shows an inverted pattern with a ring of integrin-mediated adhesion (pSMAC) surrounding a central cluster of engaged TCRs (cSMAC) [5–7]. The nascent synapse evolves into a mature synapse in 5–15 min but the mature synapse can be sustained for >2.5 h, which is required for T-cell commitment to proliferation [7]. A similar pattern of receptors is formed by cytotoxic T cells (CTLs), although their generation and structure are not well characterized. The job of killing the target cell is accomplished in only 5–10 min [8].

Most T-cell activation models are based on initial TCR triggering by ligand binding, a fast step that is not well defined and is difficult to study [9]. A complementary approach to this problem is consideration of the physical requirements for immunological synapse formation, a slower process that is readily studied by fluorescence microscopy and that correlates with functional T-cell responses [5,6,10]. Immunological synapse formation has been modeled as a biophysical process [11] depending on specific kinetic parameters of ligand binding by the TCR and integrins, receptor mobilities and the mechanical properties of the membranes in which these molecules reside. Here we elaborate on the key parameters of this model and compare the predictions of this model with data relating the kinetics of the TCR–pMHC interaction to either cytokine production by T cells or T-cell hybridomas or target lysis by CTLs. Mature synapse assembly predicted for particular TCR–pMHC binding interactions is highly correlated with cytokine responses by T cells bearing the relevant TCR. However, no correlation between the activation of target lysis by CTLs and mature synapse assembly was found. The discussion of these results focuses on the important role of TCR–pMHC interaction kinetics (both on and off rate) in immunological synapse formation and T-cell activation and suggests a different role for the immunological synapse in the pre- and post-nuclear functions of T cells.

The synapse assembly model

A framework for quantitative analysis of the mechanisms underlying immunological synapse assembly has been recently developed [11]. This model uses partial differential equations to describe the binding interactions of receptors and ligands, with the constraint that they are embedded in apposed deformable membranes linked to a cytoskeletal complex. First, it considers the specific binding interactions of the surface molecules involved and their lateral mobility in the plasma membrane. Second, it accounts for the differing molecular dimensions of these receptor–ligand pairs – when short and tall molecular pairs reside near one another there are energetic costs owing to deformation of the membrane. TCR downregulation and serial

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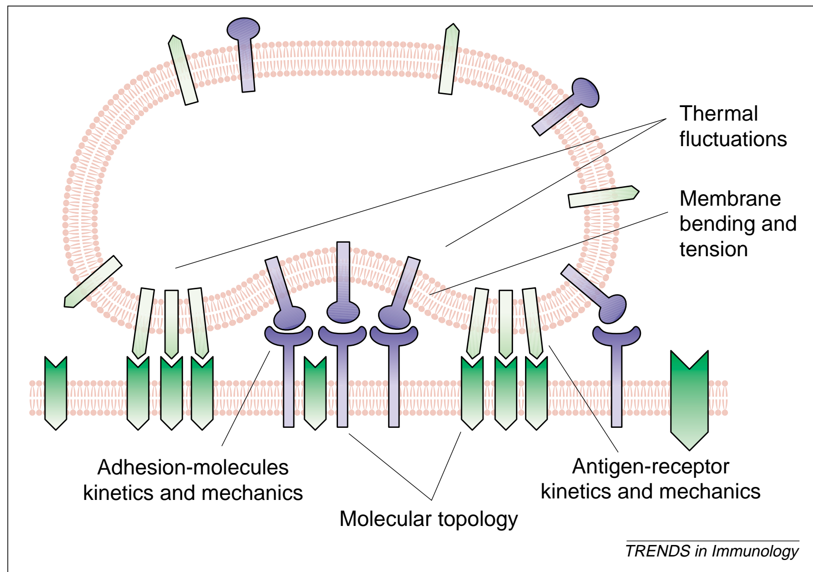

Fig. 1. The synapse assembly model. Schematic representation of a living T cell interacting with a supported bilayer mimic of an antigen presenting cell (APC) illustrating the forces involved in synapse formation.

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engagement are also included. This model is still in early stages of development, yet it is successful at predicting the stages of immunological synapse formation observed experimentally [11]. Many of the parameters in the model can be estimated based on measurements taken from live cells and from receptors whose physical interactions have been studied in great detail (see associated web only article). Once these parameters are set to reasonable values, the TCR–pMHC kinetics are the only parameters that are varied. The model does not include any parameters for signaling as yet and only considers the process of synapse assembly once T-cell movement has stopped. Thus, the only signaling implied is a stop signal [12] and those signals that set the values of certain parameters, such as receptor mobility and membrane stiffness. Qi *et al.* [11] demonstrated that when the cellular environment sets specific values for the magnitudes of forces owing to binding, receptor–ligand mobility and the mechanical response of the deformable membrane, large scale synaptic patterns, similar to those observed using living cells, can be generated by self-organization processes. Early signaling events are probably responsible for setting the magnitudes of the various forces involved in synapse formation [7,13]. A key factor driving the evolution of synaptic patterns is the coupling between size differences of the receptor–ligand pairs [~42 nm for leukocyte function-associated molecule-1 (LFA-1)–intercellular adhesion molecule-1 (ICAM-1) [14–16] and 15 nm for TCR–pMHC [17,18]] and the mechanics of the cell membrane [11].

Figure 1 depicts the situation in which one membrane is deformable, representing a T-cell membrane, and the other is flat, corresponding to a supported bilayer, which mimics an inflexible antigen-presenting cell (APC) [6]. This corresponds to the experimental model described by Grakoui *et al.* [6] that has provided some of the most detailed quantitative information on synapse formation. The receptor proteins on the deformable membrane can bind to complementary ligands on the supported bilayer (rate coefficient, k_{on}) if the two membranes are sufficiently close at that location. The receptor–ligand complexes can also dissociate (rate coefficient, k_{off}). Adhesion molecule and antigen-receptor–ligand pairs are often different in size and it is a feature of the model that the larger receptors interact first, as has been suggested previously [19].

Segregating receptor–ligand pairs with different sizes into separate regions minimizes membrane deformation in the contact zone. The energetic cost of membrane deformation has a central role in sorting TCR–pMHC and integrin–integrin ligand pairs into distinct zones in the forming synapse. The membrane shape changes, in the absence of binding interactions, are determined by the free energy cost of stretching and bending deformations [20]. Interfacial tension (or stiffness) resists new area creation and a bending rigidity determines the extent to which local deviations from the spontaneous curvature are tolerated. When a receptor–ligand complex exists at a given location in the intercellular junction it exerts a force on the deforming membrane that counters membrane movement away from the resting length of the unstrained molecular pair; this effect is modelled using a harmonic approximation. Thus, binding interactions are limited to membrane areas that are close enough for receptors to reach their ligands and the receptor–ligand pairs, once formed, influence the membrane spacing in a manner consistent with the physical properties of the interactions [21].

Receptors and ligands can move in the plane of the membrane that contains them, which can be a result of diffusion driven by concentration gradients of the free proteins. For T cells there is also experimental evidence that the cortical actin cytoskeleton moves during synapse formation [22–24], which could lead to directed motion of the TCR molecules (characterized by a velocity, V). These mechanisms are included in the general form of the model and corresponding effects on large-scale spontaneous pattern formation can be studied (see associated web only article).

Serial engagement occurs naturally in this model and TCR downregulation is included, with TCR internalization incorporated in the simplest way [25–27]. TCRs bound to a particular pMHC for greater than a critical time (τ) are downregulated (i.e. internalized by the cell), whereas others return to the T-cell surface as available TCRs. The physico-chemical processes described can be represented in mathematical terms using non-linear stochastic differential equations. Protein mobility and binding are described in the current model by Smoluchowski equations [28] and shape changes of the deformable membrane are described by a time-dependent Landau-Ginzburg (or Langevin equation for continuous fields) description [29]. The relevant mathematical equations are provided in an associated web only article.

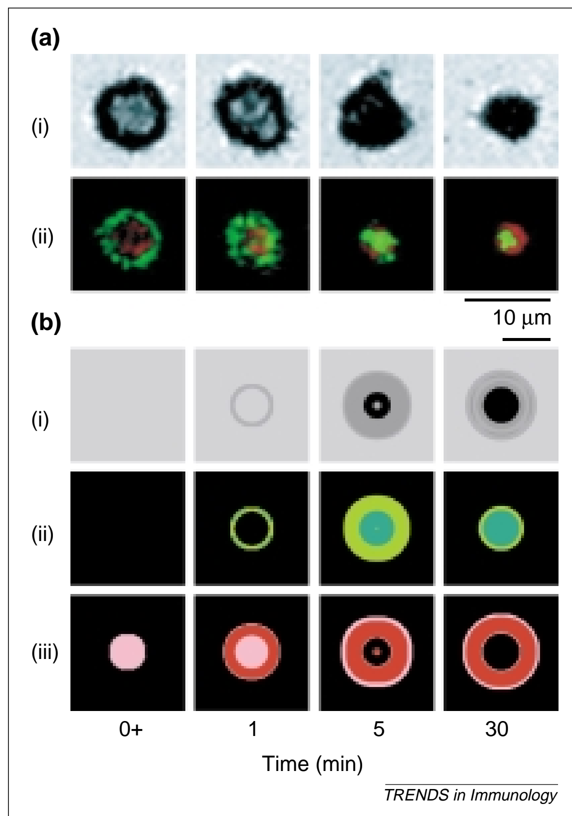

Fig. 2. A comparison between model predictions and experimental data, obtained using interference reflection microscopy (IRM) and fluorescence microscopy [6], on immunological synapse assembly and model predictions [11]. (a) Panels (i) and (ii) are experimental data and (b) panels (i–iii) represent model predictions. The top panel and the third one show the evolution of T-cell shape; the darker the color, the closer the apposition between the T cell and the supported bilayer at that location in the intercellular junction. The different shades of red [for intercellular adhesion molecule 1 (ICAM-1)] and green [for MHC–peptide (pMHC)] for the model predictions represent different concentration levels in the intracellular junction; darker shades imply larger concentrations.

The parameters in the equations reflect the magnitudes of various forces involved when populations of receptors and ligands interact across membranes. The magnitude of forces resulting from phenomena that occur on small length scales (e.g. receptor–ligand binding, membrane mechanics and protein mobility) are described by parameters that can be measured in the cellular environment. Many microscopic cellular processes (e.g. those involving the cytoskeleton) determine the magnitudes of the physico-chemical parameters in the model. Some parameters, such as protein binding kinetics, are intrinsic molecular properties; others, such as protein mobility and membrane tension, are regulated by various microscopic cellular processes (see associated web only article). Using the model, we can ask, given that cellular processes regulate the various individual forces involved, such that they acquire certain values, how, and under what conditions, is the complex system of interacting components poised to form synaptic patterns? Previously [11], it was demonstrated that for membrane and receptor characteristics relevant to experiments [6] with agonist TCR–pMHC interactions, spontaneously emergent receptor patterns and cell shape predicted by the synapse assembly model are in close agreement with experimental observations (Fig. 2).

The role of self-pMHC in synapse assembly

Different peptide ligands and TCR mutants exhibit different binding kinetics and affinities [30]. Using the model described and by Qi *et al.* [11] the relationship between synaptic pattern assembly in T cells and the binding kinetics of the TCR and pMHC was determined. The propensity for forming a synapse over a range of values of k_{off} and k_{on} that spans four orders of magnitude was examined. Mature immunological synapse patterns (ICAM-1 outside, pMHC inside) form only over a narrow window of values of k_{off} and k_{on} (Fig. 3). Outside this range of values the patterns that result from the membrane constrained receptor interactions do not resemble the concentric ring patterns of SMACs observed in a variety of imaging experiments. In these ranges of TCR–pMHC binding kinetics the time constants associated with individual processes, such as protein mobility, binding and dissociation, membrane shape changes and TCR downregulation are not related in the synchronous manner required for mature synapse formation. For example, when k_{on} becomes very large [as for killer cell inhibitory receptors (KIRs) in NK cells] a ring of TCR–pMHC forms at the periphery of the synapse and does not evolve into the cSMAC–pSMAC pattern because the time constant associated with binding is too large. This is also the observed synapse pattern for NK cells [31]. The qualitative shape of domains corresponding to stable synapse formation does not change with peptide density.

There are two distinct domains where synapse formation is predicted, one corresponding to interactions with relatively slow TCR–pMHC binding kinetics characteristic of agonist pMHC and another corresponding to relatively fast TCR–pMHC binding kinetics characteristic of null pMHC interactions. The result that TCR–pMHC interactions with fast kinetics might contribute to synapse assembly was unexpected because both Monks *et al.* and Grakoui *et al.* found that these interactions did not trigger formation of SMACs or a mature immunological synapse on their own. Interestingly, recent experiments [5,6,10] have shown that null peptides with no *in vitro* biological activity, when spiked with small amounts of agonist peptide, contribute positively to synapse assembly.

Experiments were carried out [6] with 2B4 TCR T cells and supported bilayers where the MHC molecule I-E^k was loaded with the agonist moth cytochrome C (MCC) peptide 88–103 mixed with a null peptide variant in which the lysine residue at position 99 was changed to an alanine residue (K99A). This pMHC does not elicit a biological response from 2B4 T cells when presented alone by APCs or planar bilayers. They found, however, that a small amount of the agonist pMHC mixed with a 99–999 fold excess of null pMHC did lead to the formation of a mature synapse and T-cell activation (Fig. 3). The high apparent sensitivity of the T cells to agonist pMHC was remarkable. However, the identity of the pMHC molecules that accumulated in the cSMAC was not determined. Recently, experiments have shown that

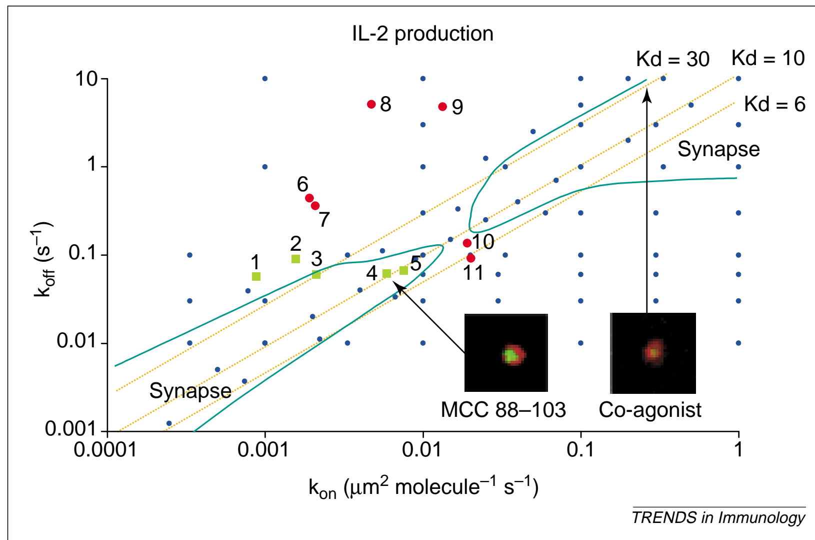

Fig. 3. The sensitivity of synapse assembly to T-cell receptor (TCR)–MHC–peptide (pMHC) kinetics. Blue points represent conditions for which calculations have been done using the synapse assembly model. Green curves enclose ranges of conditions that lead to mature immunological synapse formation [intercellular adhesion molecule 1 (ICAM-1) outside, MHC inside]. The two images, for the agonist peptide MCC88–103 and the co-agonist variant show results of experiments [6,10] visualizing synapse assembly; in these images, red corresponds to ICAM-1 and green to pMHC. The arrows point to the binding kinetics to which they correspond. Green squares correspond to the binding kinetics of pMHC mutants that lead to efficient IL-2 production. Red circles correspond to binding kinetics of pMHC mutants, which do not activate IL-2 production. The labeled points correspond to: 1, MCC (amine coupling) [45]; 2, PCC [42]; 3, MCC (cystein coupling) [45]; 4, MCC88–103 [6]; 5, Hb (64–76) [47]; 6, MCC (T102N) [40]; 7, MCC (T102S) [45]; 8, MCC (T102G) [45]; 9, MCC (K99R) [45]; 10, MCC (E72T) [47]; 11, MCC (E73D) [47]. The diagonal lines correspond to constant values of K_d.

2B4 TCR T cells interact with a B-cell lymphoma APC expressing an I-E^k–GFP fusion protein or a planar bilayer mimic of the APC [10]. Again mixtures of agonist pMHC and excess null pMHC were presented. Digital microscopy showed that I-E^k molecules loaded with the null peptide accumulated at the T-cell–APC or T-cell–bilayer junction and enhanced T-cell proliferation was also observed. Control experiments demonstrated that this accumulation of null pMHC in a mature synapse is not the result of lateral clustering with agonist pMHC–TCR complexes [10]. When lysine 99 in the MCC88–103 peptide was changed to glutamic acid (K99E) the complex formed with I-E^k is also characterized as a null pMHC. However, in this case no synergism was noted with the agonist pMHC and this null pMHC did not cluster in the center of the synapse. Thus, the null pMHC complexes can be separated into two groups. We propose the term co-agonist pMHC to describe the fraction of null pMHC that are positively synergistic with agonist pMHC. The second group lack activity of any kind and are correctly defined as null pMHC. Co-agonist is more suitable than supra-agonist, which is used to describe a related, but less thoroughly characterized, class of ligands that contribute to CD8$^+$ T-cell activation [32].

The half-life characterizing TCR binding to the co-agonist pMHC used in the experiments is <0.1 s [10]. In at least one case it is known that >50% of the TCR–pMHC binding energy can be attributed to TCR–MHC contacts [33]. Therefore, the binding energy of I-E^k loaded with the null peptide used in the experiments is assumed to be 50% of the value for the agonist MCC88–103 and the entropy loss associated with binding is the same for the homologous binding interactions. Thus, the value of two-dimensional (2D) K_d for the null peptide can be estimated to be of the order of 30 molecules μm^{-2} (for the agonist MCC88–103 it is 10 molecules μm^{-2} [6]). Figure 3 predicts that a system with this value of K_d is capable of forming a classical synapse even if the half-life of the interaction is <0.1 s. ($k_{off} > 7\ s^{-1}$). The assumption that only 50% of the binding energy is owing to TCR–MHC contacts is a conservative one as higher values [33] would make synapse formation more probable. Calculations using this model have been carried out for mixtures of the agonist pMHC and co-agonist pMHC lying in the region enclosed by the green curves in Fig. 3. At the dilutions used in the experiments described, classical synapses form with accumulation of the null peptides in the intercellular junction, provided the TCR transport rate and other forces involved acquire the appropriate values (see associated web only article) and cell migration is arrested.

The following explanation is suggested for the experimental results previously reported [6,10]. Binding of the agonist pMHC to the TCR leads to a stop signal and signaling events that set the value of effective TCR mobility and other forces to the values appropriate for synapse formation. Once this is established, binding kinetics of the co-agonist pMHC variant are such that forces owing to mobility, binding and membrane constraints are synchronous and self-organization processes enable the assembly of a mature immunological synapse with I-E^k molecules loaded with the co-agonist peptide. The contribution of the synergistic pMHC to synapse assembly is sufficient to enable full T-cell activation in response to very low levels of agonist pMHC. The results of these calculations support the hypothesis [10,34] that endogenous pMHC that show no biological activity on their own can accumulate in the mature immunological synapse without need for lateral aggregation with agonist pMHC or any other mechanism.

The peptide ligands with very fast kinetics, which are predicted to form stable synapses, exhibit values of k_{on} and k_{off} that are similar to the kinetics of self peptide interactions [35]. Synergistic self-pMHC could provide an explanation for the ability of T cells to form a synapse and respond to an APC bearing very small amounts (~30 molecules) of foreign pMHC. An important question for future study is to determine the frequency of co-agonist and null pMHC in the self-peptide repertoire and whether APCs differ in the proportion of their self-pMHC that have co-agonist activity. The levels of co-agonist pMHC could be a predisposing factor in autoimmunity.

Synapse assembly and T-cell activation

TCRs recognize a spectrum of pMHC that span a biological range from strongly stimulatory (agonist), weakly stimulatory (weak agonist), inhibitory (antagonist), synergistic with agonists (co-agonist) and non-stimulatory (null) [10,35–37]. These peptide ligand families are generated either, by starting with an agonist peptide and mutating key TCR contact residues to effect subtle changes in TCR–pMHC binding kinetics and biological efficacy, or by maintaining the peptide in its original state and varying residues in the peptide-binding domain of the MHC molecule [38–40]. The diverse self-peptides complexed to MHC on APCs also interact with the TCR, although thymic negative selection mandates that these interactions are typically in the co-agonist or null categories [35]. The binding kinetics of TCR–pMHC and biological efficacy can also be changed by mutations in the complementarity determining regions of the TCR [41].

A significant amount of experimental data are now available concerning the relationship between T-cell activation potency and extracellular binding kinetics of TCR–pMHC [41–50]. The results shown in Fig. 3 provide us with relationships between mature synapse assembly and TCR–pMHC binding kinetics. How do the relationships involved in the dependence of mature synapse assembly and T-cell activation on TCR–pMHC binding kinetics compare?

Several attempts have been made to correlate T-cell activation with avidity of TCR–pMHC binding or k_{off}. Although a stronger correlation has been found with k_{off}, and explanations based on serial triggering and kinetic proofreading models have been provided [6,42,47,51,52], exceptions to a simple and universal correlation with k_{off} have been noted [41,46,48]. For mature synapse assembly both k_{on} and k_{off} are important and the same could be true for signaling leading to T-cell activation. Thus, it might be inappropriate to seek universal correlations with either just the affinity or the k_{off} of the TCR–pMHC interaction.

Cytokine production

Cytokine production is a post-nuclear response of T cells because they are thought not to store large amounts of cytokines, rather the release of cytokines requires new transcription and protein synthesis. Quantitative measurements have been made for the binding kinetics of several peptide mutants with TCRs and their potency in activating helper T cells (as measured by IL-2 production). The values of k_{on} and k_{off} for these pMHC variants are represented in Fig. 3. Superimposing this activation data on the model predictions for mature synapse assembly provides an important insight. TCR–pMHC systems that lead to the activation of helper T cells lie in (or very close to) regions that correspond to conditions for mature synapse assembly. The preceding considerations suggest that the activation of helper T cells and mature synapse assembly correlate with TCR–pMHC binding kinetics in the same way. This conclusion is further buttressed by qualitative comparisons between mature synapse assembly and helper T-cell activation data obtained by mutating TCR molecules.

Kalergis *et al.* examined the effects of point mutations in the CDR3 β chain of a K^b-restricted TCR on T-cell activation [41]. Although this is an MHC class I restricted system the parameter measured was IL-2 production by T-cell hybridomas transfected with wildtype and mutated TCRs. R8 cells expressing different variants (A to T mutations) of a K^b-restricted peptide were also studied. TCR mutants with too large or small values of half-life compared with the wildtype exhibit repressed T-cell activation. The effect of a too fast dissociation rate being deleterious for T-cell activation has also been observed using peptide variants [46]. Changes in k_{off} affected by TCR mutations or different peptide ligands are accompanied by changes in k_{on}. Consider the model predictions in Fig. 2, for the effects of changing the values of k_{off} (and concomitant changes in k_{on}), on mature synapse assembly; changes in k_{off}, regardless of whether the corresponding values of k_{on} increase, decrease or stay the same, are predicted to lead to a non-monotonic variation in robustness of mature synapse formation. Too large or too small a value of k_{off} (see Fig. 3) will abrogate synapse assembly. Thus, this correlation between stable synapse assembly and TCR–pMHC binding kinetics is the same as that observed for T-cell activation. Both too short or too prolonged a lifetime for TCR–pMHC bonds disrupts synapse assembly and might contribute to the lower activity observed in these situations.

Table 1 provides a summary of the effects of using different TCR mutants and variants of a K^b-restricted peptide on T-cell activation [41]. Figure 4 and Table 1 (based on results reported in Fig. 3 and previously reported data [41]) demonstrate that, qualitatively, changes in TCR–pMHC binding kinetics that repress T-cell activation also lead to disruption of synapse assembly and vice versa. These observed relationships between T-cell activation efficiency and extracellular binding kinetics [41] are therefore similar to those predicted for stable synapse assembly. Mutations of the K^b-restricted peptide, which lead to either an increase or decrease in k_{off}, repress T-cell activation [41]. This is just a reflection of the non-monotonic dependence of T-cell activation on k_{off}, a characteristic mirrored by the dependence of mature synapse assembly on the half-life (Fig. 3). Experimental observations also show that permanent TCR engagement abolishes signaling (and activation). The synapse assembly model predicts that permanent TCR engagement does not allow a synaptic pattern to emerge.

Based on studies with peptide mutants as well as TCR mutants, this discussion suggests that potent activation of helper T cells and mature

Table 1. Effects of using different TCR mutants and variants of a K^b- restricted peptide on T-cell activation[a]

TCR[b]	K_D	k_{off}	k_{on}	Activity	Effect of peptide change on activity/k_{off}
V98L	=	++	++	No	N/A
G99A	–	–	+	No	N/A
G97A	–	=	+++	Enhanced	++/++
G97-99A	–	–	+	No	++/++

Abbreviations: =, similar; K_D, dissociation constant; K_{off}, off rate for TCR–MHC–peptide (pMHC) binding; k_{on}, on rate for TCR–pMHC binding; –, smaller; +, slightly larger; ++, larger; +++, much larger; TCR, T-cell receptor.
[a]Data from [41]
[b]TCR mutants are described using standard amino-acid code.

synapse assembly correlate with TCR–pMHC binding kinetics in the same way. However, this conclusion needs to be further tested with TCR–pMHC pairs that correspond to a wider range of k_{on} and k_{off} values.

CTL-mediated killing

CTL-mediated killing is a pre-nuclear response of T cells because CTLs store large amounts of perforin and granzymes in cytosolic granules that are then rapidly released towards target cells. CTL-mediated killing does not require new transcription or protein synthesis. There are six systems for which quantitative measurements have been reported for the values of k_{on} and k_{off} for TCR–pMHC binding and their potency in activating CTL-mediated lysis. In Figure 5, these data are superimposed on predictions for mature synapse assembly. Clearly there is no correlation between the way in which mature synapse assembly depends on TCR–pMHC binding kinetics and potency of APC lysis by the corresponding CTL. Thus, in contrast to data on helper T cell activation, CTL-mediated lysis does not appear to depend on TCR–pMHC binding kinetics in the same way as mature synapse assembly.

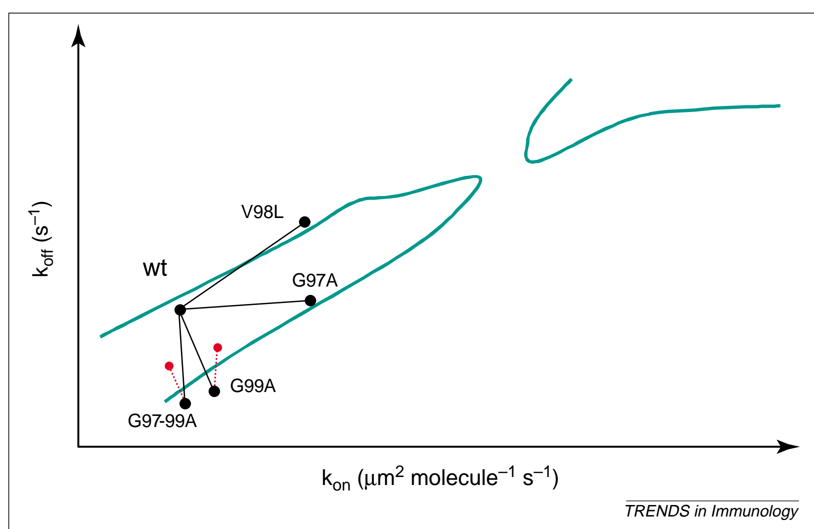

Fig. 4. Qualitative sketches (based on Fig. 3 and data on T-cell activation [41] illustrating that the relationships between stable synapse assembly and T-cell receptor (TCR)–MHC–peptide (pMHC) binding kinetics are similar to those observed for T-cell activation. We assume that the wildtype peptide forms a proper synapse. The red lines indicate what happens when the corresponding TCR mutants interact with a different variant of pMHC than those used for the other experiments. Both stable synapse assembly and T-cell activation efficiency are restored on changing the pMHC molecule.

Comparison of cytokine production and CTL killing

It would be premature to provide an unequivocal explanation for why IL-2 production correlates with TCR–pMHC binding kinetics in the same way as mature assembly of the immunological synapse but CTL lysis does not. However, some observations are appropriate. IL-2 production requires a change in gene expression in the nucleus, which, in turn, demands sustained signaling that requires sufficiently long-lived TCR–pMHC binding interactions. Yet, TCR–pMHC binding interactions are intrinsically short-lived. The assembly of the cSMAC facilitates TCR–pMHC binding interactions because it confines the TCR and pMHC molecules to a small region. The confinement favors the rebinding of the TCR and pMHC and results in an apparent stabilization of TCR–pMHC interactions that might be the result of efficient rebinding or formation of stable complexes [6,53] leading to an effect similar to tonic receptor engagement [54]. This might enable an orderly progression in TCR-mediated information transfer to the nucleus. Recently, Lee *et al.* have shown that tyrosine kinase signaling through the TCR begins mature synapse formation [7]. However, a mature synapse was required for ≥2.5 h before the T cells were committed to robust proliferation, an indication of adequate cytokine production [7]. Because the mature synapse is required for T-cell cytokine production, there is no discrepancy between the synapse assembly model prediction regarding the role of the TCR–pMHC in mature synapse formation and its correlation with IL-2 production. The results of Lee *et al.* also show that the mature synapse follows early TCR signals but is essential for cytokine production and in fact, early signaling events might be necessary for synapse formation [13]. TCR binding properties that lead to synapse formation could be necessary for transcription dependent responses such as IL-2 production. Alternatively, formation of the synapse might modulate intracellular signaling cascades for long periods, necessary for IL-2 production. These could be the reasons why mature synapse assembly and the activation of helper T cells and T-cell hybridomas correlate with TCR–pMHC binding kinetics in the same way.

CTL lysis does not require a change in gene expression in the nucleus and such a pre-nuclear response might not require sustained signaling. In fact, lethal hit delivery by a CTL requires only a few minutes [55]. Thus, the formation of a synapse to facilitate sustained TCR–pMHC interactions might not be necessary. This could explain why mature

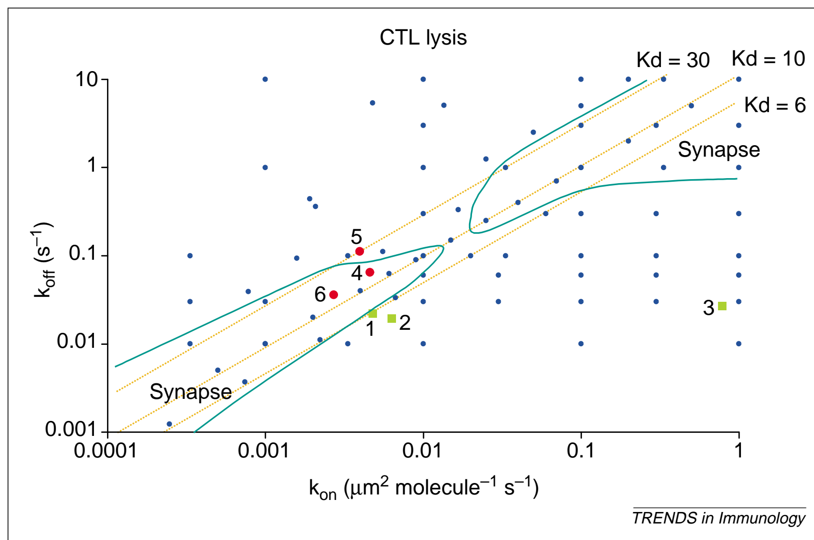

Fig. 5. Cytotoxic T lymphocyte (CTL) lysis data superimposed on model predictions for the dependence of mature synapse assembly on T-cell receptor (TCR)–MHC–peptide (pMHC) binding kinetics. The green squares represent pMHC variants that lead to CTL lysis and the red circles correspond to pMHC variants that do not activate CTL lysis. As in Fig. 2, the green curves enclose the range of conditions (pMHC variants) that lead to mature synapse assembly. 1, OVA/Kb [59–61]; 2, OVA(A2)/Kb [59,61]; 3, p2CL/H-2 L^d [43,62]; 4, OVA(E1)/Kb [59–61]; 5, OVA(R4)/Kb [59,60]; 6, V-OVA/Kb [59,60].

synapse assembly does not depend on TCR–pMHC binding kinetics in the same way as target lysis. Another consideration is that CTLs adhere strongly to target cells and could form pre-organized immunological synapses based on the use of molecules like CD2 and LFA-1 [56,57]. CTLs form an antigen independent immunological synapse as defined by transient adhesion rings in the absence of antigen (K. Somersalo *et al.* unpublished). Thus, TCR–pMHC interactions might not be required to organize an immunological synapse, which self-organize by adhesion molecules in the membrane-cytoskeletal environment of a CTL.

Conclusions

The synapse assembly model shows that mature assembly of the immunological synapse is determined by an interplay between the differential topography of receptors and ligands, membrane mechanics, receptor mobility and kinetics of adhesion molecule and TCR–pMHC interactions in the interface between a T cell and an APC. Under these conditions, the non-equilibrium process of synapse formation depends sensitively on both the value of k_{on} and k_{off} characterizing TCR–pMHC binding. The model also suggests a mechanistic basis for the recent observation of fast dissociating (half-life <1 s) TCR–pMHC interactions that synergize with TCR-agonist pMHC interaction to produce immunological synapses and T-cell activation. We propose that these pMHC, which do not fit into any previously described category, be referred to as co-agonists. Further studies are warranted to explore whether co-agonists contribute mainly because of their effect on synapse assembly, signaling or both.

Measurements and terminology

Measurement of the physical interaction of the T-cell receptor (TCR) and MHC–peptide (pMHC) is performed on the surface of a surface plasmon resonance sensor with one molecule attached to the surface and the other free in solution. This is a three dimensional (3D) affinity with the concentrations expressed in liters.

$K_d = k_{off} \div k_{on}$
The K_d is the inverse of the affinity constant (K_a) and is easy to relate to biological situations because, expressed in molar units, it is equivalent to the concentration at which half-saturation is attained at equilibrium. The K_d is related to the binding energy and is the important parameter in a process that reaches equilibrium. In a non-equilibrium process, such as synapse formation, the rate at which interactions form and break becomes more important than the ratio, so the kinetic rates become the more important parameter to measure. Approaches to measuring solution interaction rates are limited to the development of specific fluorescence assays that are applicable only to very specific combinations of molecules or a more general approach, such as surface plasmon resonance. Surface plasmon resonance measures an interaction at a surface, therefore, there is a possibility of transport effects and self assembly of complexes on the surface, which might need to be taken into account. All methods are very sensitive to aggregation of reagents, which generally leads to both slower on- and off-rates.

Activation of helper T cells or T-cell hybridomas and mature synapse assembly correlate with TCR–pMHC binding kinetics in the same way. However, there is no correlation between the dependence of CTL lysis potency and mature synapse assembly on TCR–pMHC binding. This is intriguing given that IL-2 production by helper T cells is a post-nuclear response and CTL lysis is a pre-nuclear response. Thus, the *raison d'etre* for the immunological synapse could depend on the developmental stage and function of the T cell [58]. The analysis presented and recent findings suggest that the role of the immunological synapse is fundamentally different in cytokine production and cell killing mediated by release of preformed effector molecules. The results are consistent with the proposal that the kinetics of the TCR–pMHC interaction have a crucial role in immunological synapse assembly, sustained signaling and cytokine production. For target killing, we propose that synapse assembly based on TCR–pMHC binding is not essential because the CTLs form synaptic patterns in an antigen-independent manner. Thus, there are at least two pathways to synaptic pattern assembly, one based on TCR–pMHC binding kinetics and the other on pMHC independent mechanisms. The model is still early in development. However, models such as this suggest possible mechanisms and provide predictions suitable for testing in biological systems. Synergistic experimental and theoretical efforts combining chemistry, biology and physics in this fashion, could be valuable for elucidating the underlying principles of T-cell activation.

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