

IDENTIFICATION OF SELF THROUGH TWO-DIMENSIONAL CHEMISTRY AND SYNAPSES

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■ **Abstract** Cells in the immune and nervous systems communicate through informational synapses. The two-dimensional chemistry underlying the process of synapse formation is beginning to be explored using fluorescence imaging and mechanical techniques. Early analysis of two-dimensional kinetic rates (k_{on} and k_{off}) and equilibrium constants (K_{d}) provides a number of biological insights. First, there are two regimes for adhesion—one disordered with slow k_{on} and the other self-ordered with 10^3 -fold faster k_{on} . Despite huge variation in two-dimensional k_{on} , the two-dimensional k_{off} is like k_{off} in solution, and two-dimensional k_{off} is more closely related to intrinsic properties of the interaction than the two-dimensional k_{on} . Thus difference in k_{off} can be used to set signaling thresholds. Early signaling complexes are compartmentalized to generate synergistic signaling domains. Immune antigen receptor components have a role in neural synapse editing. This suggests significant parallels in informational synapse formation based on common two-dimensional chemistry and signaling strategies.

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INTRODUCTION

The definition of self is essential to the integrity of any multicellular organism or organ system. This is true in colonial species formed from relatively few cell types and in vertebrates with specialized immune systems that detect self and enforce self/non-self boundaries. The same concepts can be extended to the cell-cell interactions in development and the elaborate connections within the nervous system. Developmental cell sorting, immune responses, and the appropriate formation of interneuronal synapses are all forms of self/non-self identification, in which self takes on greater complexity owing to the evolution of many cell types and a narrowing of appropriate connections. These cell-cell recognition processes are based on two-dimensional chemistry: the interactions of membrane biomolecules in the nanometer scale spaces between apposed plasma membranes. These fundamentally important molecular interactions have been inaccessible to biochemical analysis until recent methods using fluorescence imaging and mechanical approaches. Recent years have seen the beginning of a golden age, or red and green age to borrow the most popular colors of fluorescence microscopy, in which it is feasible to follow multiple individual receptor types and even single molecules in cell-cell synapses. This review focuses on the new information about interactions at membranes and how this relates to cell-cell communication as a whole and the immune system specifically. We begin with a general discussion of T cell activation and then dissect out the steps of this process in terms of its two-dimensional chemistry and biophysics, while keeping our fingers on the biological pulse running through this discussion. We end with examples from the recent literature that point to a general molecular hardware for formation of informational synapses between cells. We define an informational synapse as a specialized cell-cell junction that mediates chemical or electrical cell-cell communication and displays a specific supramolecular structure that subserves information transfer across the gap between two cells (after Purves & Lichtman 1985). The term synapse is derived from a Greek word meaning to grasp, and it is the chemical basis of this molecular embrace that calls our attention. A non-inclusive list of these structures includes the interneuronal and neuromuscular synapses and the more recently described immunological synapse.

MULTIPLE INFORMATIONAL SYNAPSES FOR THE IMMUNE RESPONSE

T cell activation is a highly developed form of self-discrimination rife with formation of informational synapses. These may be referred to as immunological synapses, a long-standing concept that has taken on new life (Paul & Seder 1994). The immunological synapse is the site of T cell receptor (TCR) interaction with major histocompatibility complex (MHC)-peptide complexes within an adhesive nest. The peptides can be from self-proteins that should be tolerated by failing to produce a response in mature T cells, whereas foreign peptides have the potential to produce a strong response in mature T cells. For example, a typical antiviral response involves at least five immunological synapses (Figure 1). Influenza virus infection triggers growth and differentiation of two precursor T cell populations. Helper T cell precursors are activated through interaction with antigen-presenting cells (APC) that have pinocytosed material from virally infected cells or may harbor the virus themselves (synapse 1). Helper T cells express the coreceptor CD4 and use MHC-peptide complexes with MHC class II molecules on the APC. This synapse triggers differentiation of the helper T cell precursor into an effector cell over a period of 1 to 2 days. There is reason to believe that this immunological synapse, or a series of synapses, is sustained for this period (Gunzer et al. 2000). The effector CD4 T cell can form a new immunological synapse with B cells to help antibody production (synapse 2) or with a macrophage to help with intracellular destruction of phagocytosed particles (synapse 3). Synapse 1 can induce changes in the APC through specific signaling pathways involving CD40 in a process that has been referred to as licensing the APC (Ridge et al. 1998). Cytotoxic T lymphocyte (CTL) precursors then interact with the licensed APC to trigger massive growth (cell division) and differentiation into effector CTL (synapse 4). CTLs express the CD8 coreceptor and use MHC-peptide complexes with MHC class I molecules. The effector CTLs migrate to the site of infection and kill virally infected cells through a contact-dependent mechanism that requires only a few minutes to induced target apoptosis (synapse 5). Thus the CTL-target synapses may last less than an hour. This entire process takes several days during which the infection may be controlled by a variety of innate mechanisms that are more rapid than the TCR-mediated response but frequently cannot eliminate the virus. Before first contact with the virus, the immature T cell or thymocyte forms synapses with cells in the thymus including thymic epithelial cells and dendritic cells (synapse 0). If this synapse does not form, the thymocyte dies. If the TCR interaction with self MHC-peptide complexes is too strong, then the thymocyte dies by apoptosis. Only cells with a narrow range of interactions with self MHC-peptide complexes develop into mature T cells. The central molecules in T cell synapses are adhesion molecules, costimulatory molecules, and antigen receptor/ligand complexes. We define each of these in detail subsequently. The interaction of the TCR with MHC-peptide complex is at the heart of the six immunological synapses described above. The most intensively studied immunological synapse

is between the T cell and B cell or between T cells and surrogate APCs composed of supported planar phospholipid membranes decorated with purified adhesion molecules and MHC-peptide complexes. The planar membranes are passive and reveal the cell autonomous response of the T cell. With all of this action focused on molecular interactions between apposed membranes it is clearly important to understand the basic two-dimensional chemistry behind the formation of synapses. Key issues regarding the immunological synapse include the mechanism of discrimination between self and non-self, sensitivity to very small numbers of non-self MHC-peptide complexes, the mechanisms of sustained signaling, and the mechanisms used to tune thresholds to provide different outcomes.

Two-Dimensional Chemistry: Fluorescence Methods

Not only is binding between two apposing surfaces biologically more relevant to cell-cell recognition than binding of fluid phase molecules (three-dimensional chemistry), but these two kinds of interactions are also physically distinct. Indeed, the two-dimensional on-rate k_{on} (in $\mu\text{m}^2 \text{s}^{-1}$) and binding affinity K_a (in μm^{-2}) have dimensions different from their three-dimensional counterparts (in $\text{M}^{-1} \text{s}^{-1}$ and M^{-1} , respectively). Before discussing reasons underlying these differences, it is useful to review the existing two-dimensional binding parameters and methods for their measurement, which are based on either fluorescent or mechanical means to measure the two-dimensional interactions.

The original use of fluorescence to define receptor redistribution in a contact was the classical study of McClosky & Poo (1986). They used fluorescent IgE to form a bridge between the receptor for the constant fragment of IgE (Fc ϵ RI) and cells coated with a ligand for the variable fragment of the same IgE molecules. These bridges accumulated at the interface between the two cells, and the relative number of bridges correlated with adhesive strength. In order to measure two-dimensional affinity, receptor-expressing cells are placed on a glass-supported bilayer reconstituted with lipid-anchored, fluorescently labeled ligands (Dustin et al. 1996, 1997). Receptor-ligand interactions locally deplete free ligands, creating a density gradient that drives net diffusion of free ligands into the contact area (since there are fewer free ligands to diffuse out). This further pushes the process toward formation of more interactions and diffusion of more free ligands into the contact area, leading to ligand accumulation within the contact area, as revealed by the higher fluorescence intensity inside than outside the contact region. Just like solution binding, the density of interactions at equilibrium initially increases with the density of free ligands in the bilayer but tends to saturate beyond a certain point. Results from fluorescence photobleaching recovery experiments demonstrate that these interactions are highly dynamic with complete turnover in 5–10 min for the fast dissociating CD2/CD58 interaction (Dustin 1997). At physiological receptor and ligand densities, hundreds or thousands of dynamic interactions per cell are typically observed. These properties enable the derivation of equations based on the law of mass action to determine binding affinities for interactions in the contact

area. One such derivation specific for the cell adhesion to planar bilayer situation has been described (Dustin et al. 1997). Thus far this method has been used to measure CD2 expressed on Jurkat cells interacting with CD58 and with CD48, yielding two-dimensional K_d of $1.1 \mu\text{m}^{-2}$ for the former and $47 \mu\text{m}^{-2}$ for the latter (Dustin et al. 1996, 1997). Additional data points have been added recently including the interaction of CD28 and CD80 and the constant fragment of IgG and one of its low-affinity receptors (CD16). This set of four measurements is internally consistent with respect to the relationship that an interaction with three-dimensional K_d on the order of $1 \mu\text{M}$ has with two-dimensional K_d on the order of $1 \mu\text{m}^{-2}$ (Figure 2).

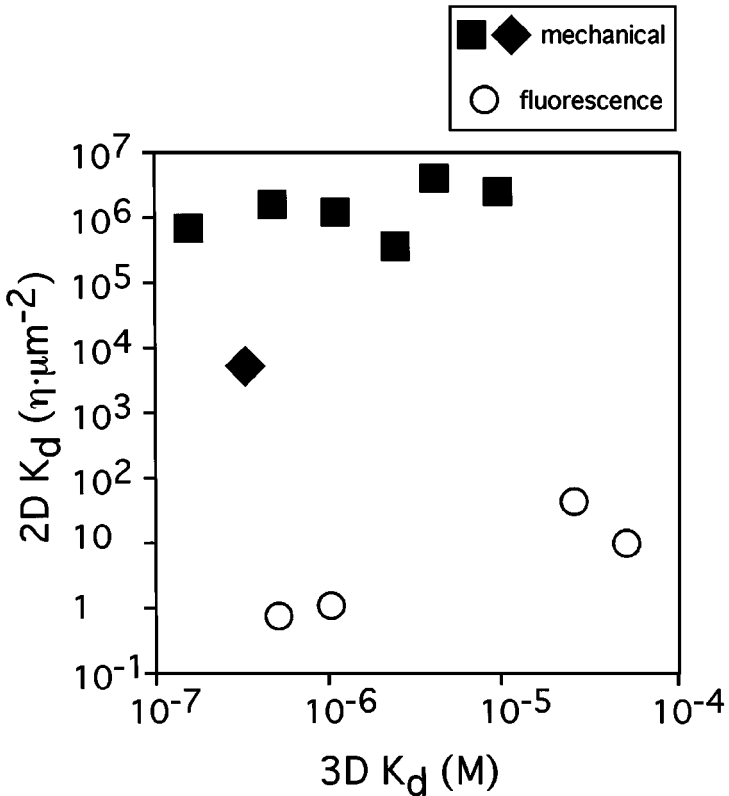


Figure 2 Two-dimensional K_d determined by fluorescence (open circles) and mechanical (filled squares) methods. The diamond symbol is the P-selectin/PSGL-1 interaction measured by the mechanical method. The three-dimensional K_d is in M units. The two-dimensional K_d is reported in $\eta \cdot \mu\text{m}^{-2}$, where η = the ratio of the actual contact area/observed contact area. η is near unity for the fluorescence method, but it is a few percent for the mechanical method. Despite this correction, the two-dimensional K_d measured by the fluorescence and mechanical techniques is still 3–4 logs apart.

The fluorescence method may also be extended to cell-cell interactions. The discovery of green fluorescence protein and its elaboration into useful spectral variants has increased the potential to analyze two-dimensional chemistry in cell-cell interactions (Chalfie et al. 1994; Heim et al. 1994, 1995; Griffin et al. 1998; Tsien & Miyawaki 1998; Miyawaki et al. 1999). The interaction of ICAM-1 with LFA-1 and of HLA-C alleles with KIR receptors has been detected in live cells using this methodology (Wülfiging et al. 1998, Davis et al. 1999). Recent studies focusing on the movements of TCR and CD4 in the immunological synapse between T cells and B cells reveal the complexity of these systems (Krummel et al. 2000). CD4 interacts with an invariant portion of MHC class II molecules that carry self and foreign peptides primarily derived from the endocytic system. CD4 initially colocalizes with clusters of TCR but later is partially excluded from the TCR clusters. These movements may reflect the active transport of molecules by cytoskeletal mechanisms or the passive exclusion of CD4 molecules after release from signaling complexes. Unlike the planar bilayer system, the correlation of fluorescence intensities to molecular densities is much less precise in cell-cell interfaces with current technology, but this will improve in the future. The difficulty arises from the irregular interfaces and unpredictable orientation of the interface to the microscope. Application of mass action law to these interactions is complex because of the heterogeneity of the plasma membrane, undefined cytoskeletal interactions, and the likelihood that interactions may not reach equilibrium (Williams 1991). Despite these limitations, these experiments are essential to test biological hypotheses that depend upon the complex in vivo relationships in true cell-cell synapses.

An alternative to fluorescent proteins for tracking molecules on cells is to use non-inhibitory Fab fragments with fluorescent dyes (Johnson et al. 2000). This approach has been taken to tracking the movement of the TCR and CD45 in the interface between T cells and supported planar bilayers. Both Fab and fluorescent proteins require adding a relatively large (30–50 kDa) tag. Therefore, great caution must be taken to ensure that the function of the tagged molecule can be confirmed in a sensitive functional assay independent of the imaging experiment.

The use of chemical fixation has also been effective in stopping the action to define snap shots of molecular distribution in the immunological synapse. This technique was used to define two supramolecular activation clusters (SMACs) in the immunological synapse (Monks et al. 1998). The power of the fixation approach is that it often achieves a higher resolution than dynamic methods. This approach was used to identify the association of the cytoplasmic-signaling protein, protein kinase C- θ (PKC θ), in the central or cSMAC and the cytoskeletal protein talin in the ring-like peripheral or pSMAC (Monks et al. 1998). A similar pattern of a central cluster of engaged antigen receptors surrounded by a ring of adhesion molecules is also detected in the immunological synapse formed by T cells with supported planar bilayers containing MHC-peptide complexes (TCR ligand) and ICAM-1 (adhesion ligand) (Grakoui et al. 1999) (Figure 1 inset). Thus the fixed cell and the

live cell methods find the same general pattern in model immunological synapses. This consists of a central TCR cluster or cSMAC (green in Figure 1 inset) and an LFA-1 ring or pSMAC (red in Figure 1 inset). Imaging fixed cell-cell interactions and live cell-planar bilayer interactions currently allow similar resolution but complementary information by freezing dynamic molecular patterns and allowing analysis of molecular dynamics, respectively. This resolution can be extended in live or fixed cells using fluorescence resonance energy transfer (FRET) (Siegel et al. 2000; Mahajan et al. 1998, 1999). Fixing cells also allows analysis by immunoelectron microscopy to obtain a complementary view of molecular assemblies at the cell surface (Wilson et al. 2000).

Two-Dimensional Chemistry: Mechanical Methods

The idea for measuring chemical kinetics mechanically lies in the fact that the products of the reaction are receptor/ligand tethers that physically link two cells or a cell to a surface. Cell binding can be quantified by several mechanical methods, such as the flow chamber method (e.g., Kaplanski et al. 1993), the micropipette method (e.g., Chesla et al. 1998), and the centrifugation method (e.g., Piper et al. 1998). The interactions of selectins and their ligands are a classical subject for the mechanical method because these interactions typically do not progress beyond formation of a few interactions even when given long periods of time. Adhesive interactions that do develop into stable multimolecular interactions over time can also be studied by the mechanical method, but the contact time must be limited to prevent formation of multimolecular interactions.

Kaplanski et al. (1993) directly measured two-dimensional kinetics using a flow chamber. Here, receptor-expressing cells were driven by a hydrodynamic force to flow over a ligand-derivatized surface. Binding is detected by the abrupt change in the cell's velocity. The k_{on} and k_{off} were estimated from analyzing the probabilities of forming a durable adhesion per time or per length of travel between a cell and the chamber floor and of the duration of these adhesions, respectively. The limitation is that, even after removing the mass action effect of the densities of the receptors and ligands, the binding rate so measured is still an aggregated parameter that lumps k_{on} with the collision frequency, encounter duration, and contact area. The latter three parameters cannot be measured or controlled by the experimenter, making it difficult to dissect k_{on} .

In contrast, the determination for k_{off} is much simpler, as it involves only measurements of the lifetime of durable adhesions mediated by single molecular interactions. Most of the two-dimensional off-rates published to date were measured by the flow cell method (Kaplanski et al. 1993; Pierres et al. 1995, 1996b; Masson-Gadais et al. 1999; Alon et al. 1995, 1997; Chen & Springer 1999, 2001; Smith et al. 1999; Ramachandran et al. 1999). Wherever data are available, these two-dimensional off-rates appear to be comparable to the three-dimensional k_{off} measured using optical biosensors (Pierres et al. 1996b, Nicholson et al. 1998, Mehta et al. 1998), both of which have the same unit of s^{-1} . In the flow chamber, the

adhesion lifetimes are measured under applied forces, yielding k_{off} as a function of force. Force dependence of k_{off} can also be determined using the micropipette (Merkel et al. 1999, Evans et al. 2001) or microcantilever (Tees et al. 2001) techniques by measuring rupture forces in a range of rates of force application. These measurements indicate that selectin/carbohydrate ligand bonds dissociate a few times per second at zero force. Force results in an exponential increase in k_{off} , with a 10-fold increase every 200 pN increase in force for selectin-ligand interaction, but only 10 pN for antibody-antigen interaction. For the case of biotin-avidin (and -streptavidin) interactions, there seem to be three separate regimes where the forces required to cause a 10-fold increase in k_{off} are in the order of several tens, ten, and one piconewton, respectively. The importance of these measurements is that selectin-ligand tethers are brief but undergo small changes in off-rate under physiologically relevant forces. In contrast, the antibody-antigen and biotin-avidin interactions, although having much higher affinities, undergo dramatic increases in k_{off} under similar forces. The properties of selectins are optimal for initial tethering of cells from shear flow, but this type of interaction is not optimal for formation of a stable synapse. The implications of these measurements for biologists include a better understanding of how adhesion molecules work when they are subjected to a mechanical stresses such as that encountered in cell migration, and remodeling the extracellular environment.

Two-dimensional k_{on} has also been measured by a micropipette method in which the experimenter stages the cell-cell contact by micromanipulation (Chesla et al. 1998). The likelihood of adhesion is estimated from the binding frequency in a series of controlled touches. Just as with the concentration of bonds in solution binding, the adhesion frequency increases with contact time initially and approaches a steady state. The initial slope of the curve is proportional to k_{on} , the steady-state binding is a measure of K_a , and the time required to reach half of the steady-state binding is inversely proportional to k_{off} . This idea of measuring two-dimensional kinetics from quantifying the dependence of adhesion probability on contact time has also been implemented using other methods such as optical tweezers (Thoumine et al. 2000). Most of the two-dimensional k_{on} s published to date have been measured by variations on this method. The effective on-rate measured, $A_c k_{\text{on}}$, where A_c is the contact area, is 10^{-6} – $10^{-8} \mu\text{m}^4 \text{s}^{-1}$ for low-affinity Fc γ receptors (CD16 and CD32) expressed on nucleated cells interacting with IgG coated on human red blood cells (Chesla et al. 1998, 2000; Williams et al. 2000a,b, 2001), $\sim 10^{-5} \mu\text{m}^4 \text{s}^{-1}$ for antibody-antigen interaction (Levin et al. 2001), and $\sim 10^{-3} \mu\text{m}^4 \text{s}^{-1}$ for selectin-carbohydrate ligand interactions (M. Long & C. Zhu, unpublished data).

The cost of increased experimental control with the micropipette method is time, as only one interaction between a single pair of cells may be tested at a time. Assaying a large number of cells in a single test by centrifugation circumvents this limitation (Piper et al. 1998). Here the adhesion probability is estimated from the fraction of adherent cells. In contrast to the micropipette method that detects adhesions formed in the absence of tensile forces, the centrifugation method enables one

to determine how K_a depends on force because a controlled force can be applied to alter the adherent fraction. This method is applicable only to systems where multimolecular interactions are not observed in the time frame of the experiment (several minutes). The selectin-ligand interactions are ideal for this type of analysis because they do not form multimolecular interactions at physiological ligand densities.

With a few exceptions, two-dimensional binding parameters determined by different mechanical methods using the same biological materials are consistent. For instance, the zero force k_{off} of E-selectin/ligand interactions measured by micropipetting (M. Long & C. Zhu, unpublished data) agrees well with the zero-force extrapolation of k_{off} determined by the flow chamber (Kaplanski et al. 1993; Alon et al. 1995, 1997; Smith et al. 1999; Ramachandran et al. 1999; Chen & Springer 1999, 2001) and by the microcantilever method (Tees et al. 2001). The effective binding affinity $A_c K_a$ of CD16a-RbIgG interaction measured by micropipette (Chesla et al. 2000) agrees with the zero-force extrapolation of $A_c K_a$ determined by centrifugation (Li et al. 1999). However, for receptors of comparable three-dimensional K_a , the two-dimensional K_a s measured with the mechanical method are orders of magnitude smaller than those measured by the fluorescence method (Figure 2). Careful examination of this apparent discrepancy revealed issues fundamental not only to whether (and if so, how) two-dimensional and three-dimensional kinetic rates and affinities can be related, but also to how the cell uses receptors to effectively adhere to surface-bound ligands.

Two-Dimensional Chemistry: Learning from Differences

It is useful to discuss the striking discrepancies between the two-dimensional affinities measured by the fluorescence and mechanical methods with the existing theory for the relationships between the three-dimensional and two-dimensional binding parameters. Assuming that the binding process can be decomposed into a transport and a reaction step, the overall affinity can be expressed as a product of the affinities of the two steps (Bell 1978, Lauffenburger & Linderman 1993). The affinity of the transport step can be interpreted as the space where an encounter complex is formed, which is a volume in three-dimensions but an area in two-dimensions. The enthalpic contributions to binding are thought to be very similar for molecules in solution or tethered to membranes. While this has not been directly tested, it is very likely. On the other hand, the entropic loss on binding is different for two-dimensional and three-dimensional interactions because more degrees of freedom are lost on binding of soluble molecules than membrane-anchored molecules. The assumption that enthalpies are constant and entropies are correctable allows one to relate the kinetic rates for the reaction step in the three-dimensional and two-dimensional cases. Thus the ratio of three-dimensional to two-dimensional affinities is a ratio of volume to area because the three-dimensional and two-dimensional affinities of the reaction step are canceled in the ratio after correcting the entropic contributions to the free-energy changes. This ratio has the dimension of length and is referred to as the confinement length (Bell et al. 1984). Initially, it was thought

that this length is characteristic of the size of the interacting molecules or the gap distance between two apposing membranes (Lauffenburger & Linderman 1993). Its first (indirect) determination by the flow chamber, however, found values in the tens of microns (Kuo & Lauffenburger 1993). In contrast, the confinement length has values in nanometer range when the two-dimensional affinities are measured by the fluorescence method, but in the range of tens to hundreds of microns when the two-dimensional affinities are measured by the micropipette method. Molecules are usually randomly coated onto surfaces employed in the mechanical assays as opposed to uniformly reconstituted in lipid bilayers employed in the fluorescence method. However, only about a 10-fold difference can be accounted for by the effect of molecular orientation (S.E. Chesla & C. Zhu, unpublished data). What is the basis of the remaining multi-log difference between confinement lengths measured by these methods?

A major difference between two-dimensional and three-dimensional transport that was not discussed explicitly in the above binding parameter conversion theory relates to how molecules approach each other. Instead of approaching each other by free diffusion, as in the three-dimensional case, molecules in two-dimensional interactions are brought together (and apart) by cells to which they are anchored. Obviously, surfaces that are separated by a distance greater than the molecular size would physically prevent any binding, effectively making the confinement length of infinite length. Therefore, two-dimensional binding constants strongly depend on how two apposing surfaces are positioned with respect to bringing the receptor and ligand together in space. This line of reasoning suggests that the confinement length reflects the size of the search space where molecules find their binding partners. It can also be thought of as a measure of order in the contact area, with a more ordered contact area having a smaller value. Using this concept, it may be said that the micropipette method quantifies binding that takes place in a disordered contact where the molecules have to search a huge effective space for their binding partners. In contrast, the fluorescence method measures interaction that occurs between aligned membranes where the search space is very small because binding partners are readily accessible. The biological correlates of these measurements are different, although a process of synapse formation will have stages corresponding to each measurement.

Why would the search spaces be so different in the fluorescence and mechanical methods? The answer may lie in the different regimes in which the two methods are designed to work. The direct measurement of the micropipette method is the likelihood of adhesion, not the number of bonds. As such, this approach requires infrequent adhesions in order to use small number statistics to predict from the measured adhesion frequency the average number of bonds in the adherent contacts, which must be low and hence unable to reorganize the surface micro-topology, which is rough. Thus the micropipette method probes the formation of a few interactions in a subminute time scale across rough cell membranes, with extensive microvilli protruding hundreds of nanometers. Adhesive receptors, requiring tens of nanometers of close proximity to ligand-presenting surfaces owing to their small

size, thus have a limited effectiveness, which manifests as requiring a large search space.

In contrast, adhesions assayed by the fluorescence method take place over a much longer time scale of tens of minutes. During this period, the initially discontinuous and spatially separated point contacts become connected (see the Supplemental Material link at www.annualreviews.org). This irons out the wrinkles in the cell membrane and aligns it with the lipid bilayer to form an ordered structure, which greatly facilitates ligand binding. Depletion-driven diffusion allows the freely mobile ligands to be recruited from outside into the contact area, which further tilts the equilibrium toward bond formation by the law of mass action, resulting in thousands of interactions. Thus the search space is on the order of the expected fluctuations in intermembrane distance. In an established adhesive site where the apposed membranes are aligned by a large number of interactions, the fluctuations from thermal- and cell-active motions are of a magnitude of 10 nm. This conclusion is further supported by interference reflection microscopy measurements that demonstrate small fluctuations in established contacts areas (Simson et al. 1998).

The role of surface roughness is reflected by the fact that the contact area A_c and the two-dimensional affinity K_a always appear as a product, not isolated individuals, in equations used to analyze the two-dimensional binding data, regardless of whether they are measured using the micropipette or fluorescence methods. This product, referred to as effective affinity, accounts for both the affinity for the receptor-ligand to bind and the effectiveness of their presentation by the host membranes. In the fluorescence method, the molecular presentation is optimal in aligned membranes. The contact area is separately measured by interference reflection microscopy to allow K_a to be dissected from the effective affinity (Izzard & Lochner 1976). In the micropipette method, although the apparent contact area A_c^* is monitored and controlled by direct microscopic observation, the true, or functional A_c is difficult to access and likely contains a small fraction of A_c^* . For example, the contact area may appear to be $3 \mu\text{m}^2$, but only the tips of microvilli may actually touch thus generating an effective contact of only $0.06 \mu\text{m}^2$. Therefore, in Figure 2 the two-dimensional K_d s are presented in units of $\eta \cdot \mu\text{m}^{-2}$, where $\eta = A_c/A_c^*$ is the fractional effective contact area. It may be close to unity for the fluorescence method but was estimated to be only a few percent (2% in the example above) for the micropipette method (Chesla et al. 1998). This hypothesis is supported by a recent study in which binding parameters of the same molecule expressed on cells with smooth and rough surfaces were compared (Williams et al. 2001). For the same apparent contact area A_c^* , a 50-fold increase in $A_c K_a$ was seen for receptors on smooth red blood cells over rough nucleated cells, while the k_{off} s were similar. No enhancement was seen in solution binding. This suggests that the increase in the effective affinity is from the more ordered contacts produced by the smooth red cells. Compared with the 10,000-fold difference between the two-dimensional K_d measured by the fluorescence and mechanical methods, this effect is only 50-fold because the number of interactions remains low even in

the smooth-surfaced red cell case. Further increases in initial alignment may be achieved by titrating the repulsive effect of the surface oligosaccharides and glycoproteins that would enforce an optimal distance between membranes at a given level of force holding the membranes together. At the appropriate level of force and glycocalyx components, the two-dimensional K_d in the micropipette experiment might be further enhanced, but to approach the value measured in ordered contact areas would probably require a large number of interactions to restrict the membrane fluctuations.

PRE-CLUSTERING AS AN EVOLVED STRATEGY TO IMPROVE k_{on}

There are cases in biology where the initial interaction in the disordered contact still has to happen quickly. As pointed out above, very-high-affinity interactions, such as those of antibodies and antigens, can have 2-log faster k_{on} than the low-affinity interactions typical of adhesion molecules. However, these unregulated high-affinity interactions are rare or non-existent among adhesion molecules. The only interaction examined that has low three-dimensional but high two-dimensional affinity is that of P-selectin and its ligand PSGL-1. In fact, the two-dimensional K_d of this interaction, measured by the mechanical method, is intermediate between the other mechanical measurements and the fluorescence measurements (Figure 2, diamond symbol). Here, the much higher two-dimensional affinity results from the much faster two-dimensional k_{on} , as the k_{off} is also fast ($\sim 1 \text{ s}^{-1}$). Such rapid two-dimensional kinetics are a functional requirement of the selectin/ligand interactions, which is to capture flowing leukocytes on endothelium during brief encounters when they collide with the vessel wall. To mediate cell rolling, these interactions must also be transient, of low number, and hence unable to align cell membranes to form ordered contacts. How does the selectin-ligand interaction shave 2-logs off of the gap between ordered and disordered interactions? The answer may be that selectins and PSGL-1 are clustered on the ends of microvilli, a form of lateral order that can be established prior to contact. This clustering may have two major effects. One is that the local clustering increases the effective concentration. A second factor is that the positioning on the ends of microvilli may result in increased access of the ligand to the small points of initial contact between the cells. Pre-clustering has been widely invoked in discussion of regulated adhesion including selectins, integrins, cadherins, and Ig superfamily members (Berlin et al. 1995, von Andrian et al. 1995, van Kooyk et al. 1994, Yap et al. 1997). However, pre-clustering implies a low lateral mobility to hold molecules in the pre-clustered distribution. Low mobility may in part account for the poor ability of selectins to mediate stable adhesion (Finger et al. 1996). Thus when pre-clustering is used as a strategy to increase k_{on} , it will reduce the potential for stable adhesion unless the molecules can be mobilized rapidly after initial capture, or the molecule has immobile clustered and mobile subpopulations that can participate in sequential capture

and firm adhesion, respectively. An integrin, such as VLA-4, that mediates both capture and firm adhesion is likely to operate in this manner (Berlin et al. 1995). Assembly of antigen receptors in pre-assembled clusters and MHC molecules in lipid rafts may be a process to facilitate signaling and to increase k_{on} , while still allowing rearrangements required for immunological synapse formation (Schamel & Reth 2000, Anderson et al. 2000).

IMPLICATIONS OF TWO-DIMENSIONAL CHEMISTRY FOR SYNAPSE FORMATION

A major function of the immunological synapse seems to be to interpret chemical interactions of the TCR and a wide range of MHC-peptide complexes to determine the likelihood that a few of these peptides are foreign. This task can be divided into two problems—discrimination and sensitivity. The foregoing discussion of two-dimensional chemistry provides insights into each problem. As in solution, we have broken the chemistry of receptor-ligand interactions into kinetic rates and an affinity that is the ratio of these rates. In two-dimensional chemistry, the k_{on} is highly sensitive to the organization of the contact area. Thus this parameter varies over 4 logs depending upon the organization of the contact area. In contrast, k_{off} is very similar when measured in three-dimensional or two-dimensional systems. Thus the k_{off} is a robust chemical parameter that reflects the fit between the receptor and ligand-binding surfaces but varies only a small amount with environmental factors. Forces exerted on the molecules in the contact area may increase the k_{off} , but the organization of synapses with hundreds or thousands of interactions is likely to insulate individual interactions from forces greater than ~ 10 pN. In T cell recognition, the response of T cells to MHC-peptide complexes can be changed by mutating key residues in the peptide that make contact with the TCR (Evavold et al. 1993). These “altered peptide ligands” generate a range of biological outcomes from activation of mature T cells (agonist MHC-peptide complexes) to positive selection of T cells during development (self MHC-peptide complexes) (Evavold et al. 1993). When these responses are related to the TCR/MHC-peptide interaction in solution, the best correlation was found with k_{off} (Matsui et al. 1994, GJ Kersh et al. 1998), which is also the most robust parameter in the two-dimensional system. This k_{off} -based discrimination is evident in the earliest signaling events in the nascent immunological synapse, including the pattern of tyrosine phosphorylation in the cytoplasmic domains of the TCR, which is established in minutes (E.N. Kersh et al. 1998). Furthermore, the formation of the central cluster of TCR and MHC-peptide complexes in the immunological synapse at 5 min to 1 h correlated with k_{off} but not with K_a (Grakoui et al. 1999). Thus the formation of structure of the immunological synapse is linked to chemical kinetics through early signaling. There is also evidence that the interaction of the TCR with MHC-peptide complexes involves significant rearrangements in the binding interface or induced fit. This is consistent with a general scanning mechanism for MHC-peptide recognition

that is chemically related to the manner in which sequence-specific DNA binding proteins locate their target sequence within a sea of related, but irrelevant structures (Willcox et al. 1999, Boniface et al. 1999). The mechanism by which the kinetic differences are converted to signals in the T cell is not known, but it is suspected that signaling complexes that must be assembled from multiple components require time (McKeithan 1995). For example, the CD4 surface molecule recruits Src family tyrosine kinases to the TCR complex *in cis* (in the same membrane) (Shaw et al. 1989, Turner et al. 1990). The CD4 interaction has a lateral k_{on} for interaction with the TCR, and this lateral interaction may be stabilized by the presence of the bound MHC (König et al. 1992). The critical time that the TCR/MHC-peptide complex needs to stay together (a few seconds) may be based on the time required for recruitment and action of CD4 and *lck* to start the signaling cascade.

The sensitivity of T cells to agonist MHC-peptide complexes is very high with as few as 100 MHC-peptide complexes or less per APC being sufficient to activate a mature T cell (Harding & Unanue 1990, Peterson et al. 1999). This is equivalent to 0.2 molecules/ μm^2 . Because the k_{off} is relatively constant (as discussed above), immunological synapse formation must work to improve the k_{on} . This can be achieved by ordering the contact and decreasing the confinement length. There appear to be at least two specific mechanisms through which the immunological synapse optimizes k_{on} . The clearest view of contact topology is offered by the interaction of T cells with supported planar bilayers. Here, interference reflection microscopy provides a distance map of the contact area. T cells use large adhesion molecules to grip the surface and then actively generate areas of close contact where smaller molecules such as TCR and MHC-peptide complexes can interact (Grakoui et al. 1999). This mechanism initially seeks to minimize the confinement length before any TCR are engaged. As mentioned above, this will require the cell to apply just enough force through actin cytoskeletal mechanisms to compress the glycocalyx to a spacing of around 15 nm between membranes. How does the cell know how much force to apply given that glycocalyx composition will vary from cell to cell? It is interesting to consider that application of cytoskeletal force might be adjusted by a feedback mechanism that links force generation to receptor engagement. In this way an empirical process determines the optimal spacing over a range of glycocalyx properties. A different mechanism for minimizing the confinement length is to use an abundant adhesion molecule interaction that is of the same size as the TCR/MHC-peptide interaction to pre-assemble the contact at the optimal spacing. One adhesion mechanism that may fill this role is the CD2/CD48 interaction (van der Merwe et al. 2000). Co-crystallization of CD2 and its human ligand CD58 shows that this interaction would span a gap of 14–15 nm between cells (Wang et al. 1999). When the size of the mouse CD2 ligand CD48 was increased by adding one or three Ig-like domains, the extended CD48 molecules acted as dominant-negative inhibitors of TCR engagement (Wild et al. 1999). The natural form of CD48 significantly increased T cell activation. Therefore, CD2/CD48 interaction appears to augment T cell activation by generating an organized contact area that is isometric with the TCR/MHC-peptide interaction and has a small confinement length. These two mechanisms—active close contacts

and isometric adhesion mechanisms—are likely to operate *in vivo* to enhance T cell sensitivity. Both would act to convert regions of the immunological synapse into attoliter (10^{-18} L) reaction spaces in which one foreign MHC-peptide complex has an effective concentration in the micromolar range.

A MOLECULAR MACHINE TO READ A KINETIC CODE

In order to be fully activated, the T cell must sustain signaling for many hours. The reasons for this prolonged requirement for TCR-mediated signals may relate to passing certain cell-cycle checkpoints and possibly also to the time required for chromatin remodeling to activate cytokine gene expression (Avni & Rao 2000). There are two alternative strategies to achieve sustained signaling that lead to T cell proliferation. One is the formation of a stable immunological synapse in which the T cell remains with one APC for the duration of primary stimulation. The other is the formation of a series of transient synapses with multiple APCs over the same time period. We consider the molecular mechanisms involved in each type of interaction and how these different modes of stimulation can each result in T cell proliferation.

The immunological synapse is a highly polarized structure that allows coordination of T cell migration with the antigen recognition process, sustained signaling, and bidirectional communication with the APC. It is characterized by a bull's eye pattern with a central cluster of TCR surrounded by a ring of LFA-1, a major integrin adhesion molecule involved in T cell-APC interactions. Secretory granules and the Golgi apparatus are centered on the central cluster to maintain a focused cytokine-mediated communication with the APC (Griffiths 1995, Paul & Seder 1994). This type of organization may even be mapped onto the classical polarized systems, the epithelial cell. In this analogy, the adhesion ring corresponds to the basolateral surface and the central cluster corresponds to the apical surface. There is no direct correlate of the tight junction complex, but the demarcation between the central cluster and the ring is very sharp and probably involves molecular specialization to prevent mixing of these compartments. The adhesion ring is itself surrounded by a very active ruffling membrane. The ruffling membrane suggests ongoing activation of rac family G proteins that interact with downstream molecules to stimulate membrane ruffling (Nobes & Hall 1995). The generation of this pattern involves initial formation of TCR/MHC-peptide interactions in the periphery of the contact that are then transported to the central region. This radial inward movement also reflects the polarization of the T cell toward the APC. Immersing the T cell-APC interaction in a collagen gel breaks the stable polarization between the T cell and APC, in which case the T cell forms more transient interactions with a series of APC over time (Gunzer et al. 2000). These sequential interactions are likely to result in similar compartmentalization of signaling as the stable immunological synapse and can also lead to proliferation but the coordination of migration and antigen recognition is lost. Interestingly, collagen is sequestered from T cells in secondary lymphoid tissues

(Ebnet et al. 1996). For this reason we favor the idea that primary T cell activation in lymph nodes operates through the formation of a stable synapse (Dustin et al. 2001). In contrast, effector interactions in collagen-rich tissues may operate in a serial mode much like the classical model for serial killing of targets by CTL (Martz 1987).

The immunological synapse coordinates adhesion and signaling with the cytoskeleton. It requires an intact actin cytoskeleton, and it has been suggested that this is a classical case of tension generation by myosin II contraction of an actin filament network (Rozdzial et al. 1995, Dustin & Cooper 2000). The trigger for this process is signaling through the TCR and LFA-1, with cross-talk between these spatially distinct signaling regions in the synapse (Dustin & Chan 2000). It is well known that TCR signaling rapidly activates adhesion through changes in LFA-1 activity (Dustin & Springer 1989, van Kooyk et al. 1989). The mechanism of this process is not known with certainty but candidates include the branches of the mitogen-activated protein kinase pathway and the regulation of actin cytoskeleton through Syk family tyrosine kinases and the Vav exchange factor (Miranti et al. 1998) (Figure 3). While the details of this signaling pathway are not known, it may also involve activation of the small G protein Rap1 (Katagiri et al. 2000, Reedquist et al. 2000, Suga et al. 2001). Physiological TCR engagement and activation of adhesion pathways are likely to be totally interdependent processes that cannot be separated. Nonetheless, signaling processes appear to be compartmentalized

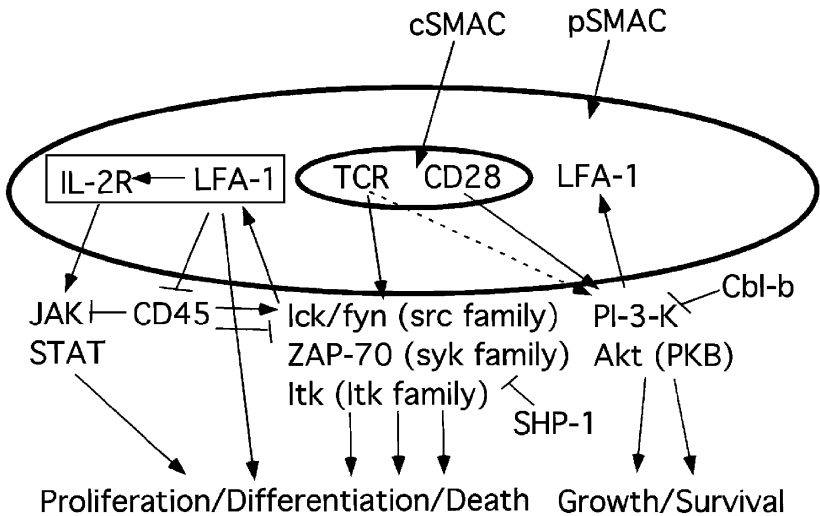


Figure 3 Compartmentalization of the signaling in the immunological synapse. Simplified summary of positive and negative signaling in the mature immunological synapse with a central cluster of TCR and CD28 (cSMAC) and a ring of LFA-1 (pSMAC). Arrows are positive effects and perpendicular lines are negative effects. Signaling from both the cSMAC and pSMAC is required for full T cell activation.

(Figure 3). The TCR signaling pathway controls the activation of three tyrosine kinase families, *src*, *syk*, and *itk*, and a lipid kinase phosphatidylinositol-3'-kinase (PI-3-K). These kinases are assembled together around a tyrosine phosphorylated scaffold formed by the TCR-associated CD3 ϵ , γ , δ , and ζ subunits and the linker of activated T cells (LAT). Critical downstream pathways include activation of the mitogen-activated protein kinase and Jun kinase pathways and NF κ B and NFAT transcription factors that are required for full T cell activation. The ability of the T cell to suppress TCR-mediated death signaling is carried out by co-stimulatory receptors such as CD28 and ICOS (Noel et al. 1996, Genot et al. 2000, Tafuri et al. 2001). These survival receptors augment activation of PI-3-K, and the generation of phosphatidylinositol-3,4,5-trisphosphate augments recruitment and activation of the pleckstrin homology (PH) domain-containing kinases such as *Itk* and *Akt* (also known as protein kinase B) (Burgering & Coffey 1995). *Akt*, a serine/threonine kinase, activates or inhibits downstream molecules to promote T cell survival (Scanga et al. 2000). CD28 looks like an adhesion molecule that might activate PI-3-K in response to T cell contact with any ligand-expressing cell, but this may not be the case in vivo. CD28 engagement is tightly controlled by TCR engagement to an extent that unlike adhesion molecules LFA-1 and CD2, CD28 does not appear to augment TCR engagement (Viola & Lanzavecchia 1996). Thus it is appropriate to classify CD28 in a separate category from LFA-1 and CD2. It remains to be seen whether ICOS is similarly regulated.

Signaling from the adhesion ring is also likely to play a key role in cell cycle progression. The best-characterized biochemical pathway is based on activation of the Jun/Fos transcriptional co-activator JAB1 by LFA-1 (Bianchi et al. 2000). Sustained LFA-1 engagement in conjunction with IL-2 receptor engagement also downregulates the cell cycle inhibitor p27kip, thus enabling movement from G1 to S phases of the cell cycle (Geginat et al. 1999). Another interesting property of the LFA-1 ring is that it is relatively depleted of the transmembrane tyrosine phosphatase CD45 (Johnson et al. 2000). CD45 has recently been shown to act as a Janus kinase phosphatase and thus a negative regulator of cytokine signaling pathways (Irie-Sasaki et al. 2001). In order to enter the cell cycle, the T cell must integrate TCR, survival, and cytokine signals. It is attractive to speculate that the LFA-1 ring is a site of active cytokine signaling owing to the depletion of CD45 and by analogy to focal adhesion complexes that also have a well-known role in sustaining growth factor signals (Miyamoto et al. 1995).

The immunological synapse is also laced with inhibitory signaling mechanisms that contribute to setting signaling thresholds at appropriate levels. Signaling for activation of immature T cells may operate near the theoretical limit of single-molecule sensitivity. In order to prevent auto-immunity, the sensitivity of mature T cells is less than the sensitivity of thymocytes. This amounts to a biochemical margin of safety, where mature T cell activation requires 50–100 MHC-peptide complexes to achieve full activation (Peterson et al. 1999). There are several biochemical mechanisms that contribute to the attenuated sensitivity of mature T cells. One mechanism is the recruitment of tyrosine phosphatases such as SHP-1. Strong recruitment of SHP-1 can extinguish signaling (Binstadt et al. 1996), whereas

moderate recruitment is likely to be involved in setting thresholds in both T cells and thymocytes (Johnson et al. 1999). Another axis of regulation is based on ubiquitin addition by ring finger family member E3 ubiquitin ligases such as c-cbl and cbl-b (Levkowitz et al. 1999). One distinct regulatory network is based on cbl-b inhibition of PI-3-K (Fang et al. 2000). In the absence of cbl-b, the TCR-mediated recruitment of PI-3-K is sufficient to fully activate mature T cells without a requirement for CD28 engagement. In the presence of cbl-b, the addition of ubiquitin to the p85 subunit inhibits PI-3-K and imposes a requirement for additional PI-3-K activation through CD28 to activate effectors such as Akt to achieve full T cell activation and survival. Ubiquitin addition may inhibit a pathway by proteasome-mediated degradation and/or by steric inhibition in the absence of degradation (Fang et al. 2000). Negative regulators of inositol lipid phosphorylation also impinge on this pathway. PTEN is a phosphatidylinositol-3'-phosphatase that counteracts PI-3-K and regulates signaling thresholds (Wang et al. 2000). For example, the T cell line Jurkat lacks PTEN, a fact that may account for Jurkat's general high sensitivity to activating signals (Shan et al. 2000). PTEN also has a role in controlling the sensitivity of T cells to FAS-mediated apoptosis, a major mediator of antigen-induced cell death (Di Cristofano et al. 1999). SHIP is phosphatidylinositol-5'-phosphatase that generates PI-3,4-P₂. Akt has a PH domain that preferentially binds PI-3,4,5-P₃, and action of SHIP reduces Akt recruitment and activation, but generates new binding sites for negative regulators that have PH domains interacting preferentially with PI-3,4-P₂. Thus the formation of the immunological synapse may provide a mechanism to balance positive and negative biochemical pathways with TCR and CD28 engagement to achieve activation at an appropriate level of TCR engagement. The activation of T cells through serial engagement in a collagen gel may place less emphasis on sustaining this balance. In this situation, T cells may carry a finite biochemical memory of earlier encounters with APC. This memory may be encoded in slowly reversible processes such as degradation of cell cycle inhibitors and in chromatin remodeling.

A novel mode of inhibition of immunological synapse formation has been suggested by studies of N-linked glycosylation. A deficiency in the enzymes that regulate production of galectin-1 binding sites on T cells suggests that the interaction of the bivalent lectin galectin-1 has an inhibitory effect on T cell activation through a retarding effect on immunological synapse formation (Demetriou et al. 2001). Restriction of lateral mobility has been proposed as a mechanism for regulating a number of adhesive processes including LFA-1 regulation (Kucik et al. 1996). The report of Demetriou et al. is novel in that the molecular mechanism of restricted mobility is not a cytoskeletal interaction but an extracellular lectin associated with the thymic and lymph node microenvironment.

COMMON TOOLS FOR INFORMATIONAL SYNAPSES

One of the attractive aspects of considering a variety of informational synapses together as related structures is the idea that they may share a common machinery and some common mechanisms for maintenance and plasticity. It is clear that

some of the basic machinery will be shared, for example, actin cytoskeleton and proteins involved in directed secretion. Of greater interest is the recent observation that editing of synapses in the retina requires expression of MHC class I molecules and a signaling component of the TCR complex known as CD3 ζ (Huh et al. 2000). MHC class I molecules show a greatly variegated expression pattern in the retinal projections. This pattern is surprising because these molecules are typically expressed on all nucleated cells. The CD3 ζ chain is part of the signal transduction unit in TCR signaling that recruits syk family tyrosine kinases after phosphorylation by src family tyrosine kinases. The model proposed by Shatz and colleagues (Huh et al. 2000) is that a primordial TCR-like molecule may associate with CD3 ζ in visual system synapses. Signaling through this molecule and certain MHC class I molecules appears to be involved in culling inappropriate self-connections in what may be a neural version of T cell negative selection (Figure 1). Thus there is emerging evidence that immunological and neural synapses may share more specific mechanisms than might have been suspected based on the clear differences in the appearance and functions of these systems. These parallels call our attention back to the fundamental similarity of the self/non-self discrimination problems from which immunological and neural cell-cell recognition systems evolved. Informational synapses are likely to share a number of properties beyond polarity and ultrastructural and molecular specialization. Important issues include how the environment shapes synaptic connections and how signals from multiple sources, including soluble cytokines and chemokines, are integrated with the two-dimensional chemical interactions that characterize the synapse. We can anticipate that the chemical discrimination in the neural synapse may share basic properties with the immunological synapse that follow directly from the chemistry of two-dimensional interactions.

The foregoing discussion of two-dimensional chemistry brings some clarity to the dominant role of kinetics, and particularly k_{off} , in governing signaling in the immunological synapse. The important point is that the initial binding reactions can take place over a huge range of effective k_{on} depending upon the organization of the contact area. A generally more robust parameter is the k_{off} of the chemical interaction that can then determine the assembly of downstream signaling complexes. The study of two-dimensional chemical reactions in synapses is a very new area opened up by advances in imaging and biomechanical and molecular genetic techniques. New insights into the operation of informational synapses are likely to reveal more common elements and surprising specialization in colorful red, green, and gold.

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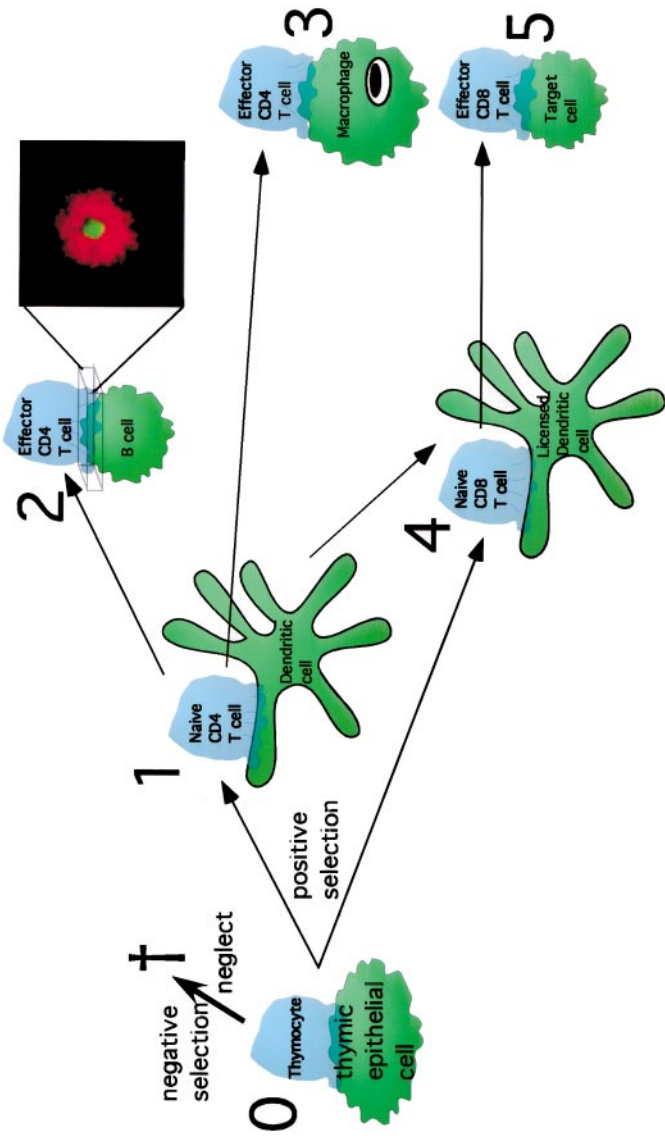
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Thymus Lymph node Tissues

Figure 1 Immunological synapses. 0, thymocyte/thymic epithelial cell; 1, CD4 (helper) T cell/dendritic cell; 2, CD4 T cell/B cell; 3, CD4 T cell/macrophage (with intracellular bacteria); 4, CD8 (cytotoxic) T cell/licensed dendritic cell; 5, effector CD8 T cell/target cell.