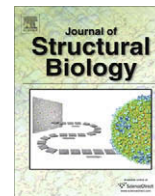




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Review

Supported bilayers at the vanguard of immune cell activation studies

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ABSTRACT

Biological adhesion between cells is critical for development of multicellular organisms and for the function of the adaptive immune system of vertebrates. A gap in understanding of adhesion systems arises from the difficulty of collecting quantitative data on the molecular interactions underlying adhesion, which is typically studied by population statistics such as percent adhesion in the presence of empirically defined forces to separate less adherent cells. Supported lipid bilayers formed on glass surfaces offer a useful model system in which to explore some basic features of molecular interactions in adhesive contacts. We have exploited that lateral mobility of molecules in the supported planar bilayers and fluorescence microscopy to develop a system for measurement of two-dimensional affinities and kinetic rates in contact areas. Affinity measurements are based on a modified Scatchard analysis. Measurements of kinetic rates are based on fluorescence photobleaching after recovery at the level of the entire contact area. This has been coupled to a reaction-diffusion equation that allows calculation of on- and off-rates. We have found that mixtures of ligands in supported planar bilayers can effectively activate T lymphocytes and simultaneously allow monitoring of the immunological synapse. Recent studies in planar bilayers have provided additional insights into organization principles of cell-cell interfaces. Perennial problems in understanding cell-cell communication are yielding to quantitative measurements based on planar bilayers in areas of ligand driven receptor clustering and the role of the actin cytoskeleton in immune cell activation. A major goal for the field is determining quantitative rules involved in signaling complex formation.

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1. Introduction – cells with a purpose

Multicellular organisms depend upon cell–cell adhesion to provide mechanical integrity and to compartmentalize the extracellular space for various physiological activities. The ubiquitous and highly mobile cells of the immune system use a large array of highly evolved adhesion mechanisms for navigation in the body and identification of infected cells. The vertebrate immune system is based on relatively recently evolved adaptive immune system that is built onto an evolutionarily ancient innate immune system. The central cells of the innate immune system are various types of phagocytes that ingest particulates and tissue fluids and have some “innate” ability to destroy invaders based on highly conserved structural motifs associated with viruses (e.g. double stranded RNA), bacteria (e.g. lipopolysaccharide, flagellin) and parasites (e.g. chitin). A subset of these cells, dendritic cells, have become specialized not to destroy, but to process and present pieces of proteins or lipids to cells of the adaptive immune system along with cell surface and soluble factors that convey information about the context in which these structures were encountered. T lymphocytes of the adaptive immune system utilize highly diverse T

cell antigen receptors (TCR) that are generated by somatic recombination within the genome to recognize the protein and lipid fragments presented by the dendritic cells. This fundamental cell–cell recognition process is supported by an array of adhesion systems that operate in parallel in the interface. A historical advantage of working with immune cells is the functional activities could be clearly modeled in vitro – none better than T lymphocyte mediated killing of cells in the context of viral infection, intracellular bacterial infection and transplantation. These clear and robust in vitro assays enabled early pioneers to generate antibodies that blocked these functions and were thus clearly recognizing important targets (Sanchez-Madrid et al., 1982). These approaches led to the first identification of adhesion receptors and immune cells still provide excellent model systems for studying basic principles of adhesion. In parallel with these early efforts at identification of adhesion receptors of the immune system, supported bilayer technology has been a key tool at the vanguard of immune cell recognition research for almost 30 years.

2. Immune cell triggering by dimensionally constrained monovalent ligands

Studies in the late 1970’s established that antibodies bound to phospholipid anchored haptens (small molecules that are bound

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by antibodies) in large vesicles were non-clustered and freely mobile, yet could trigger activation of immune cells that interacted directly with the large vesicles. This ability of effectively monovalent ligands to trigger immune cell activation conflicted with the observation the soluble monovalent haptens were unable to activate cells and that only dimeric or oligomeric haptens were able to trigger immune cell functions by cross-linking the receptors (Metzger, 1992; Schlessinger et al., 1976). Signaling activity in microaggregates generated by antibody cross-linker has been directly visualized (Stauffer and Meyer, 1997). It had been argued by the proponents of the cross-linking model that the curvature of the vesicles would effectively cluster the immune cell antibody receptors by focusing them at a point defined by the point of minimal separation between the cell membrane and the spherical vesicle. In order to test this McConnell and colleagues incorporated the same phospholipid hapten into a supported planar monolayer deposited on a flat alkylated glass surface (Hafeman et al., 1981). The antibody-hapten complexes were mobile in this monolayer as in the vesicle membranes and they still triggered activation of an oxidative burst and secretion of the lysosomal protein cathepsin B onto the surface. McConnell visualized the accumulation of the fluorescently tagged antibodies in the contact area, but encountered problems with accelerated photobleaching in contact areas with the activated immune cells so could not document the pattern of receptor engagement that led to activation. Transformed mast cells that could bound to supported bilayers presenting laterally mobile IgE evolved small patches of IgE that were interpreted as microaggregates of ~100 receptors per $0.1 \mu\text{m}^2$ spot (Weis et al., 1982). This increased density of receptors in a small area was proposed to arise due to topological constraints of small membrane projections such as microvilli. This mechanism of clustering has not been confirmed nor is it clear that complexes assembled by membrane curvature driven aggregation would have sufficient integrity to initiate signaling (McKeithan, 1995). Nonetheless, these experiments framed an enduring enigma of how apparently monovalent ligands in a fluid phospholipid bilayer can achieve receptor clusters and activation without an apparent mechanism for cross-linking (Figs. 1 and 2).

3. T lymphocyte activation by purified MHC–peptide complexes

The ligands that mediate activation of T lymphocytes are MHC–peptide complexes that are highly asymmetric type I transmembrane proteins with large extracellular domains and relatively small cytoplasmic domains. The monolayers used in the above study were deposited on the alkylated glass from an air–water interface, a method requiring organic solvents that are not compatible with membrane proteins. McConnell's lab overcame this problem by making the observation that supported bilayers can be formed simply by incubating aqueous solutions of unilamellar liposome solutions with the clean glass coverslips or beads (Babbitt et al., 1985; Brian and McConnell, 1984; Gay et al., 1986; Watts and McConnell, 1986). Transmembrane proteins like MHC class I and class II antigens are readily incorporated into unilamellar liposomes by detergent dialysis. After fusion of these proteoliposomes to glass surfaces the MHC molecules were laterally immobile. These experiments were performed not to study adhesion per se, but to determine if purified allogeneic or specific antigen bearing MHC molecules could functionally activate T cells since MHC molecules had been shown by genetic studies to control the responses of T cells to foreign antigens. Activation of T cells under these conditions required interaction with 1000 MHC–peptide complexes (Watts and McConnell, 1986), which probably reflects the requirements for MHC–peptide complexes to activate T cells with little or no support for other adhesion ligands found on the surface of dendritic cells. It should be noted that fluid bilayer membranes are not biologically passive and that they will bind fibronectin from serum and support adhesion of immune cells without explicit incorporation of adhesion ligands (Bonte and Juliano, 1986). In later studies we found that even higher levels of pure MHC–peptide complexes were required to mediate adhesion (Dustin et al., 1996b). This suggests that serum attachment factors did contribute to the earlier functional interactions. An interesting study demonstrated that TCR could actually facilitate the interaction of antigenic peptides with empty MHC molecules based on fluorescence resonance energy studies using total internal reflection fluorescence (TIRF) spectroscopy (Watts et al., 1986). It is no

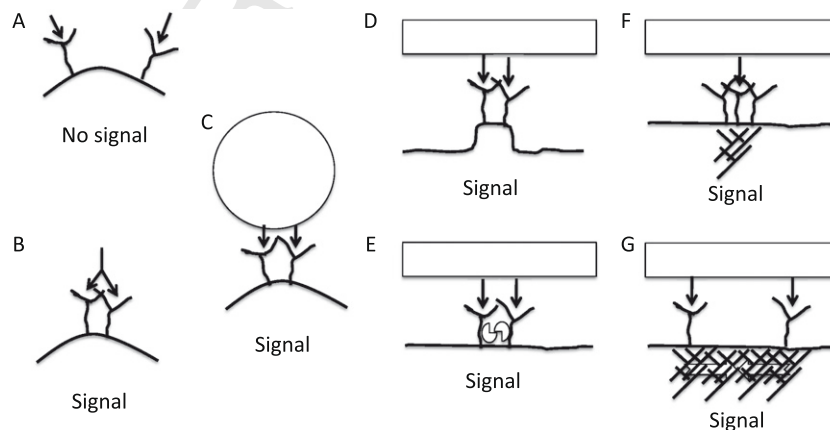


Fig. 1. Models for triggering immunoreceptors. It is unlikely that any “one fits all” model exists for immunoreceptors since they have strikingly different F-actin requirements. The line on the bottom represents the cell membrane that the Y shaped figures are the cell surface receptor. The arrows represent ligands. The sphere is a liposome and the rectangular shapes represent fluid supported bilayers. (A) Monovalent engagement does not activate most immunoreceptors in general. A possible exception has been reported for certain anti-TCR Fab (Gil et al., 2002). (B) Cross-linking with antibodies activates most immunoreceptors, at least transiently. (C) IgG on liposomes activates Fc ϵ receptor. In order to activate a TCR beads need to be larger than 3 μm diameter (Mescher, 1992). (D) The early planar bilayer experiments with Fc receptors reacting to dimensionally constrained monovalent Ig Fc revealed that receptors could form clusters that were capable of signaling. This has been explained by assuming that membrane projections allow concentration of receptors and ligands to support adhesion, but it not clear this would satisfy kinetic proofreading criteria. (E) Trans and cis interactions might cooperate to promote microaggregate formation and signaling (Reich et al., 1997; Tolar et al., 2009). (F) F-actin dependent nucleation of a microaggregate by a single MHC–peptide complex. This is not proven, but implied by titration data. (G) F-actin and myosin IIA dependent force consistent with particle size requirements (Galbraith et al., 2002; Mescher, 1992).

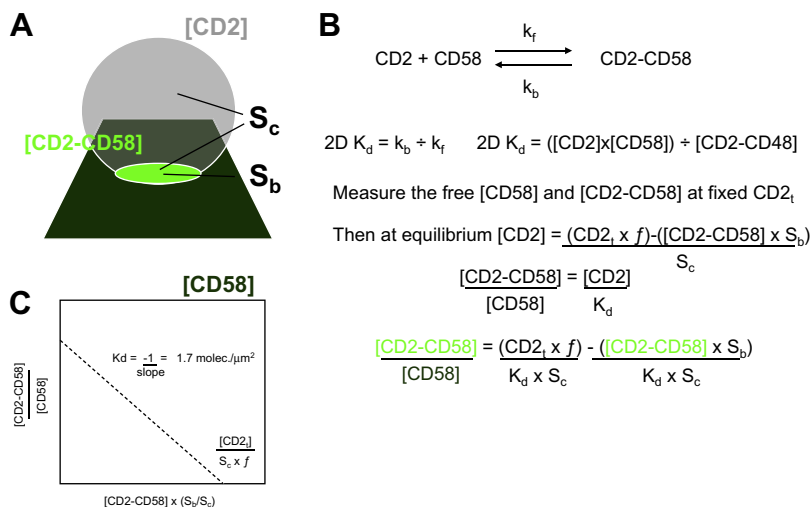


Fig. 2. Model for calculation of 2D dissociation constant. (A) The sphere represents a T cell expressing CD2 that interacts with a plane representing a supported bilayer reconstituted with different densities of purified FITC-CD58. The cell adheres and the system reaches equilibrium in 20–40 min at 24 °C. The bilayer fluorescence and contact area fluorescence were measured. It is also important to use a distinct non-binding, but similar sized protein (such as mouse CD48) labeled with a different dye, like Cy5, to determine the level of exclusion of free CD58 from the contact area, which is usually 20–40%. This is due to steric crowding in the contact area and is used to calculate the free [CD58] in the contact area. Measurements are made on 50–100 cells per data point. Each data point is a different starting CD58 density usually in the range of 10–1000 molecules/ μm^2 . (B) Derivation of the Golan-Zhu plot (Zhu et al., 2007). The S_b is the area of the contact between cell and bilayer measured based on CD58 accumulation and CD48 exclusion. S_c is the area of the entire cell. f is the fractional mobility of CD2 on the surface of the T cell. This is essentially a modified Scatchard plot (Scatchard, 1949) that allows the proportion of the total amount of CD2 ($CD2_t$) to vary based on its lateral mobility and mass-action driven partitioning into the contact area. (C) A schematic of a Golan-Zhu plot. Typically 4–5 densities of CD58 would be tested to provide equally distributed points along the line. The 2D K_d value is based on the best-controlled published data (Dustin et al., 2007b). This paper also describes a model for bridge formation by the CD58-Fc chimeric drug alefacept.

longer thought that TCR facilitates loading of peptides on MHC molecules, although it appeared to do so in these experiments. This study foreshadowed more recent studies that have combined TIRF microscopy with supported planar bilayers to probe the question of how freely diffusing monomeric MHC-peptide complexes activate T cells.

4. Determination of two-dimensional affinity

The generation of monoclonal antibodies that block the function of allogeneic cytotoxic T lymphocytes, which is a type of immune cell important in transplant rejection, identified two adhesion pathways that play a prominent role in human and rodent immune responses. These molecules were initially defined as lymphocyte function associated-1 (LFA-1), LFA-2 and LFA-3 (Sanchez-Madrid et al., 1982). Analysis of blocking of adhesion by pair-wise combinations of these antibodies revealed that LFA-2 (CD2) and LFA-3 (CD58) were in the same pathway, whereas LFA-1 was in a separate pathway (Shaw et al., 1986). A screen for antibodies that were raised to B lymphoblasts (Epstein-Barr virus immortalized cells) from LFA-1 deficient patients and could block LFA-1 dependent aggregation of normal B lymphoblasts identified ICAM-1 as a surface molecule in the same pathway with LFA-1 (Rothlein et al., 1986). Supported planar bilayers were used to provide compelling evidence that CD58 and ICAM-1 were ligands for CD2 and LFA-1, respectively (Dustin and Springer, 1988; Dustin et al., 1987b, 1988; Marlin and Springer, 1987). In parallel studies we found that half of the CD58 on nucleated cells and all of the CD58 on erythrocytes was glycosylphosphatidylinositol (GPI) lipid anchored, with the other half on nucleated cells have a transmembrane domain and short cytoplasmic tail (Dustin et al., 1987a). As noted above, the McConnell lab had found that antibodies bound to lipid-linked haptens were laterally mobile in supported bilayers above the transition temperature. This suggested that a GPI anchored molecule like CD58 should also be laterally mobile. We purified the transmembrane form of CD58 from cells that had a biosynthetic defect in production of GPI anchors and compared this

to CD58 from erythrocytes (Hollander et al., 1988). We found that the mobile GPI anchored CD58 was considerably more active in supported planar bilayers than the immobile transmembrane form (Chan et al., 1991). We speculated that the greater activity of the laterally mobile form of CD58 might be due to its ability to accumulate in the contact area by diffusion and trapping. We tested this by fluorescently labeling the CD58 while protecting the active site using the function-blocking antibody. This fluorescent CD58 was incorporated into a supported planar bilayer and we imaged the interface using a laser scanning microscope system to detect movement of the labeled CD58 after $CD2^+$ T cells were added. We were able to quantify the density binding of CD2 to CD58 in the T cell-bilayer interface (Dustin et al., 1996a). We were then able to perform a Scatchard analysis using this binding data, which was linear and suggested a 2D dissociation constant (K_d) of 21 molecules/ μm^2 . The 2D K_d measurement has subsequently been refined by modeling CD2 diffusion on cells (Dustin et al., 1997; Zhu et al., 2007, 2006) and measuring the exclusion of freely diffusing molecules from the contact area (Bromley et al., 2001; Dustin et al., 2007b). The formation of contracts is dependent upon the 2D K_d more than on the kinetics of the interactions, making the 2D K_d an important parameter (Shao et al., 2005).

5. Kinetic rates of interactions in contact area

While contact formation is largely dependent upon the 2D affinity, signaling reactions are dependent upon the kinetic rates of the receptor-ligand interactions (McKeithan, 1995). We noted early in our studies of the CD2-CD58 interaction in the planar bilayer system that when CD58 was photobleached by a focused laser beam that fluorescence recovered rapidly even when a large proportion of fluorescence in the contact area was destroyed (Dustin et al., 1996a). This observation suggests that the bleached CD58 is rapidly dissociating from and being replaced by bright CD58 molecules that diffuse in from outside the contact area. We later designed a system with a laser beam that could be defocused to bleach CD58 in the entire contact area (Dustin, 1997). In this case

fluorescence can only recover due to dissociation of interactions followed by new binding with a recovery process governed by the single reaction rate constant $k_f(\text{CD2} + \text{CD58}) + k_b$, where k_f and k_b are the forward and reverse rates, respectively. Cheng Zhu's lab determined the diffusion reaction equations needed to calculate k_f and k_b from the fluorescence photobleaching after recovery time course (Tolentino et al., 2008; Wu et al., 2008). Interestingly, the fitting of the recovery data revealed a k_b of 0.074 s^{-1} , which if at least 100-fold slower than the solution k_b of 7.8 s^{-1} (van der Merwe et al., 1994). The apparent k_f was $0.015 \mu\text{m}^2 \text{ s}^{-1}$. It seems very unlikely that conditions in the contact area would result in a 100-fold stabilization of the CD2–CD58 bond, so the most likely explanation is that the dissociated CD58 tend to rebind the same CD2 many times prior to actually diffusing away and allowing for exchange only after a half-time of about 10 s. This duration of interaction suggests that CD2–CD58 interactions have a sufficient effective duration to contribute to TCR signaling processes and may also account for recent evidence for direct signaling through CD2–CD58 interactions (Kaizuka et al., 2009). Extensive measurements have not been made for the TCR–MHC–peptide interaction, but data from a single MHC–peptide density allowed an estimate of the 2D Kd for the 2B4 TCR interaction with I-E^k with moth cytochrome C peptide 88–103 of 10 molecules/ μm^2 , in the same range as the CD2–CD58 interaction (Grakoui et al., 1999). This convergence is significant since the 3D interaction of the TCR has a similar affinity, but a slower on and off rate compared to CD2–CD58 (Grakoui et al., 1999; van der Merwe et al., 1994).

6. Segregation of adhesion molecules by size

Adhesion systems involved in immune cell activation normally operate in combinations rather than one at a time. Springer speculated that adhesion molecule size would be important for the organization of contact areas by forcing segregation of adhesion receptors like CD2–CD58, which span about 15 nm, and larger adhesion receptors like LFA-1–ICAM-1, which appeared capable of spanning greater than 30 nm. It is been confirmed that contacts established by the CD2–CD58 adhesion mechanism generate uniform intermembrane spacing of 13 nm (Choudhuri et al., 2005; Milstein et al., 2008). The spacing generated by the integrin LFA-1 is a more complex issue. Integrins are large, non-covalent heterodimers with a globular domain connected to the membrane by two legs (Luo et al., 2007). LFA-1 activity is regulated by TCR signaling (Dustin and Springer, 1989) and has three affinity states with Kd of 1 mM, 10 μM and 100 nM (Shimaoka et al., 2003). The complete crystal structure of the platelet fibrinogen receptor $\alpha\text{IIb}\beta\text{3}$ demonstrated that integrins have a “genu” or knee like structure that allows the legs to bend such that the binding site can exist anywhere from 5 to 25 nm from the membrane (Zhu et al., 2008). Electron microscopy studies demonstrate that higher affinity conformations correlate with more extended genus (Luo et al., 2007), it remains possible that affinity and bending/extension may be independent. This would allow variable intermembrane spacing, which is in fact observed in the contact areas between T cells and planar bilayers containing LFA-1 (Shaw and Dustin, 1997). When CD58 and ICAM-1 are incorporated into the same planar bilayer activated T cells form contact in which the accumulation of CD58 and ICAM-1 appear to be completely segregated with interaction domains on a readily resolved micron scale (Dustin et al., 1998). More recently, we have performed experiments with engineered versions of CD48 that change the intermembrane spacing for different ligands binding to the same receptor, mouse CD2. CD2, CD58 and CD48 are all members of the immunoglobulin superfamily that are built from tandem arrays of small β -sandwich domains that are about 4 nm long. Van der Merwe and colleagues generated ex-

tended forms of CD48 by adding Ig domains from CD2 or CD22, with the natural binding sites of these spacer domains mutated (Wild et al., 1999). The extended forms of CD48 inhibit antigen recognition by T cell hybridomas and primary T cells (Milstein et al., 2008; Wild et al., 1999). Addition of Ig-like domains might be expected to increase intermembrane spacing by up to 4 nm per domain, but van der Merwe and we observed that spacing was increased by 1 nm per domain (Choudhuri et al., 2005; Milstein et al., 2008). We observed that a 1 nm difference in size did not force segregation, but a 2 or 3 nm spacing difference led to complete segregation in a narrow range of ligand concentrations in which the two systems coexisted (Milstein et al., 2008). Outside this narrow range the more functionally abundant ligand occupied the entire contact area. Therefore, robust lateral segregation over a wide range of ligand densities, as observed for CD58 and ICAM-1 containing bilayers likely reflects both the physical process in the extracellular space, and active membrane/cytoskeletal processes that allow adhesion systems to establish competitive niches within the contact area.

7. Formation of the immunological synapse

The immunological synapse started as a model that cytotoxic T cell function could be equated to that of a neural synapse stabilized by LFA-1 and with TCR acting as a ligand gated Ca^{2+} channel to trigger delivery of secretory vesicles to the target cells upon engagement with antigen (Norcross, 1984). The TCR was soon shown to operate based on a tyrosine kinase cascade to activate phospholipase C- γ as a pathway to cytoplasmic Ca^{2+} elevation (Samelson et al., 1986) and this specific model was largely set aside. Imaging studies 10 years later revealed a specific organization of TCR in a central micron scale structure and LFA-1 as forming an annular disc surrounding the TCR cluster in the interface between antigen specific T cells and antigenic MHC–peptide complex bearing B cells (Monks et al., 1998). Kupfer described these patterns of terms of supramolecular activation clusters (SMACc) since they appeared to involve thousands of non-covalently associated molecules and were correlated with T cell activation (Monks et al., 1998). Paul Allen, Andrey Shaw and I proposed that these SMACs together constituted an immunological synapse (Dustin and Shaw, 1999; Dustin et al., 1998). We went onto use the supported planar bilayer containing ICAM-1 and a range of biological active MHC–peptide complexes to reveal the pathway to formation of the immunological synapse. Surprisingly, this was based on centripetal transport of TCR engaged MHC–peptide complexes that were initially engaged in the periphery of the expanding contact area in what appeared at a coarse-grained view as pattern inversion (Grakoui et al., 1999). Unlike prior studies with antigen receptor triggering and bilayers, this system explicitly addressed the position of essential adhesion and specificity controlling elements to reveal a carefully orchestrated process. A surprising finding was that the TCR–MHC–peptide interactions in the central SMAC appeared to be stable over hours, which suggested an extreme version of the rebinding process that was revealed in the contact-FRAP studies above. We will address this further below. These dynamic images led to a number of models for the role of F-actin and the potential of the synapse to mediate processes like directed secretion and asymmetric cell division (Dustin and Cooper, 2000; Dustin and Chan, 2000).

8. Microclusters drive activation – back to basics

The fundamental signaling machinery of the immunological synapse was not apparent in early coarse-grained views. Kupfer found the protein kinase C- θ and the Src family kinase Lck were accumulated in or near the central SMAC (Monks et al., 1998),

but subsequent studies revealed a rapid loss of TCR proximal signaling in this region and persistence of signaling elements in the periphery where LFA-1 appeared to dominate (Freiberg et al., 2002; Krummel et al., 2000; Lee et al., 2002). Models were developed to accommodate a flexible role of the central SMAC in signal termination when signaling was strong and signal enhancement when signaling is weak (Lee et al., 2003). A break-through in understanding signaling dynamics in the immunological synapse was made by returning to the basic technological platform introduced by McConnell in 1982 – TIRF microscopy on supported bilayers. In key observations first made by Varma, microclusters of TCR were observed to continually form in the periphery of the immunological synapse and stream inwards to the formed central SMAC (Varma et al., 2006). TIRF was essential to reduce background to a level where the small aggregates, with as few as 10 receptors each, were visible (Varma et al., 2006). In an important test of the significance of these structures, we showed that anti-MHC-peptide complex antibody immediately blocked the formation of the microclusters and signaling was fully extinguished as the last microclusters reach the central SMAC after about 2 min. Thus, the thousands of TCR accumulated in the cSMAC were insufficient to sustain Ca^{2+} signals (Varma et al., 2006). This basic finding was backed by studies that demonstrated the presence of active signaling molecules only in the microclusters and not in the central SMAC (Campi et al., 2005; Yokosuka et al., 2005, 2008). Earlier studies had demonstrated TCR signaling molecules associated with anti-CD3 induced microaggregates (Bunnell et al., 2002), but the advantage of the supported bilayers system over the immobile solid support is that the signaling and inactivation is a spatiotemporal process that allows deconvolution of the significant signaling and non-signaling structures in the TCR “life-cycle”. An interesting hybrid system has been developed by the Grove’s lab based on using electron beam lithography to general nanoscale metallic lines on the substrate that partition of bilayer into various geometric patterns. The compartments defined by the metallic lines allow the formation of microclusters, but the prevent the transport of these clusters across the lines. A $1 \times 1 \mu\text{m}$ grid allows the formation of many small microclusters that cannot be transported centrally to form a central SMAC and this manipulation actually measurably increased signaling (Mossman et al., 2005). The planar bilayer system can also be adapted to use natural MHC-peptide ligands or anti-CD3 antibodies for studies on polyclonal T cells or cells with TCR of unknown specificity (Kaizuka et al., 2007). The mobility of molecules in the planar bilayers also allow for sorting within the plasma membrane interface. Saito and colleagues discovered that CD28-CD80 interactions are initially co-localized with TCR-MHC-peptide interactions in microclusters, but are sorted into a distinct compartment at the boundary between the central and peripheral SMACs (Yokosuka et al., 2008). This mode of rearrangement would not be possible with immobile ligands. While the smallest TCR microcluster that can be tracked have greater than 10 TCR, a single MHC-peptide may be sufficient to trigger a microcluster based on titration data (Varma et al., 2006). However, direct observations are needed to test this possibility. These studies have advanced our understanding of TCR signaling processes in the synapse, but also have left us with the same fundamental question that faced McConnell in 1982 – how do freely diffusing monovalent ligands trigger formation of clusters. It is possible that the solution to this problem will be different for different receptor systems.

9. B lymphocytes acquire antigen by force

Whereas T lymphocytes bind antigens only when they are presented on the surface of cells with MHC-peptide ligands, B lymphocytes bind intact antigens many of which are soluble. Batista

considered the possibility that physiological acquisition of antigens by the B cell antigen receptor (BCR)¹ in vivo would involve binding of surface presented complexes through an immunological synapse and developed cellular and supported bilayer based tools to study this process (Batista et al., 2001; Carrasco et al., 2004). In order to present a wide variety of purified proteins to B cells, Batista and colleagues developed methods to monobiotinylate proteins and then to capture these to the planar bilayer using biotinylated lipids and streptavidin, which can be fluorescently labeled for visualization. This has been a generally useful method for attaching many proteins to bilayers, particularly antibodies. The only caveat with this approach is that the exact valency of the system is not defined. Streptavidin likely binds to the bilayer through biotin binding sites and then can probably capture up to two monobiotinylated antibodies. B lymphocytes will form a similarly organized immunological synapse with microclusters converging on a central SMAC, but the biological imperative for the B lymphocyte is to pull the antigen off the surface such that contraction and force dependent extraction are directly related to the B lymphocytes subsequent ability to present antigens to T cells (Fleire et al., 2006). Interestingly, signaling by primary B cells in response to dimensionally constrained monovalent ligands on a planar bilayer requires CD19 (Depoil et al., 2008). Perhaps CD19 is a factor needed for aggregation of BCR by monovalent ligands. While the Batista lab works with mature primary B lymphocytes, the Pierce lab has focused on a complementary model based on a plasmacytoma cell line lacking BCR subunits (Tolar et al., 2005). They have reconstituted this cell line with fluorescent protein tagged BCR subunits and have used this to monitor changes in the intramolecular spacing of the cytoplasmic domains of the receptor complexes. The advantage of using the cell line is that all BCR subunits are fluorescently tagged and the disadvantage is that signaling requirements may differ due to the maturity of the cell line and potential differences related to transformation. The results were nonetheless interesting in that the authors were able to observe increased spacing of the cytoplasmic domains of the BCR by fluorescence resonance energy transfer (Tolar et al., 2005). These findings are consistent with recent work showing that key tyrosine phosphorylation sites are buried in the plasma membrane and dissociate upon activation (Xu et al., 2008). More recently, they have offered a novel solution to the old problem of receptor triggering by monovalent ligands, in this case short peptides with hexahistidine at the C-termini bound to Ni chelating lipids. This is a useful strategy for attaching any recombinant proteins that can be generated with a hexahistidine to decahistidine tag in a monovalent form. They have demonstrated that the extra Ig-like domain that is spliced into the heavy chain of cell surface BCR undergoes a weak, lateral homophilic interaction that facilitates aggregation of engaged BCR (Tolar et al., 2009). This is conceptually similar to earlier models for TCR aggregation (Reich et al., 1997). A model based on a ligand dependent release of an aggregation domain from an inhibitory interaction, which then can propagate to neighboring unligated receptors could account for the ability of one or a few ligands to nucleate larger receptor clusters. It remains unclear how F-actin would facilitate this process. One notion would be that the conformational change involved in exposure of this oligomerization domain requires a force generated by the actin cytoskeleton.

10. The F-actin machine behind the synapse

A striking contrast between Fc receptor systems studied by Metzger and McConnell and the TCR system that we have focused

¹ Abbreviations used: BCR, B cell antigen receptor; LFA, lymphocyte function associated; PKC, protein kinase C; SMAC, supramolecular activation cluster; TCR, T cell antigen receptor; TIRF, total internal reflection fluorescence.

on is that immunological synapse formation, TCR signaling and microcluster formation are all F-actin dependent (Bunnell et al., 2002; Campi et al., 2005; Grakoui et al., 1999; Varma et al., 2006), whereas signaling through the Fc ϵ receptor is enhanced when F-actin is depolymerized (Torigoe et al., 2004). In fact, the acquisition of F-actin independence by TCR microclusters is a property associated with inactivation of signaling in the central SMAC (Varma et al., 2006). How is F-actin involved in the immunological synapse? The TCR is directly linked to F-actin polymerizing machinery via Vav, which activates both Rac and CDC42 (Fischer et al., 1998). Integrins like LFA-1 may also contribute to this process. Rac activates the Wave2 complex, which activates both Arp2/3 and Formin mediated actin polymerization in lamellipodia (Gomez et al., 2007; Nolz et al., 2006). CDC42 activates WASp, which is another activator of the Arp2/3 complex involved in formation of filopodia and podosomes (Carman et al., 2007). TCR microclusters appear to form in lamellipodium like structures on planar bilayers containing ICAM-1 and MHC-peptide complexes (Kaizuka et al., 2007; Sims et al., 2007). Therefore the F-actin structures that are associated with microcluster formation and transport are dynamic, branched F-actin networked formed by Arp2/3. However, when WAVE2 is knocked down, early TCR signaling proceeds normally suggesting that alternative pathways, perhaps WASp based, are fully functional (Nolz et al., 2006). T cells express myosin IIA, but its function in synapse formation and signaling is controversial (Jacobelli et al., 2004; Wülfing and Davis, 1998). A requirement for force generation in T cell activation is consistent with a distinct size threshold for T cell activation by MHC-peptide coated particles (Mescher, 1992). Sheetz found that bead size thresholds for focal adhesion formation are attributable to force dependence of this process (Galbraith et al., 2002). Myosin IIA knock down by siRNA and treatment with the myosin II specific inhibitor blebbistatin both block the translocation of TCR microclusters and impair signaling at a step between Src family kinase activation and phosphorylation of ZAP-70 and LAT (Ilani et al., 2009). TCR and integrin microclusters appear to form normally in the absence of myosin IIA. The immunological synapse is radially symmetric, like a spreading fibroblast with a radial lamellipodium (Sims et al., 2007). The symmetry of this system is promoted by WASp and suppressed by PKC θ to control the stability of the immunological synapse (Sims et al., 2007). The role of PKC θ in immunological synapse stability was surprising, but the result, originally obtained with naïve CD4⁺ helper T lymphocytes (a type of immune cell that orchestrates many cells in the adaptive immune response), has been extended to cytotoxic T lymphocytes (Beal et al., 2008). The radially symmetric lamellipodium is characterized by a 3–5 μ m wide annular region of centripetal F-actin flow, which dissipates in the central region occupied by the central SMAC (Kaizuka et al., 2007). TCR and LFA-1 microclusters are transported toward the center. The coupling to centripetally moving actin will exert forces on the integrin that are sufficient, in simulations, to induce conformational changes that increase integrin affinity (Zhu et al., 2008). Short-lived LFA-1 microclusters tend to dissociation in the F-actin depleted center and thus accumulate in the peripheral SMAC, perhaps because of the force dependence of maintaining high affinity LFA-1 (Zhu et al., 2008). TCR microclusters that achieve actin independence are transported to the central SMAC. Shorter-lived TCR microclusters may disappear at the boundary between the central and peripheral SMACs. The coupling between TCR microclusters and F-actin flow is not perfect and based on speed the TCR microclusters appear to be linked to the F-actin ~40% of the time. The generation of chrome barriers in planar bilayers using e-beam lithography has been extended to fine analysis of microcluster movement (DeMond et al., 2008). It was found that microclusters navigate around barriers as long as the barrier was angled toward the center of the synapse. This con-

firmed the notion that there is a molecular “clutch” between the TCR and integrin clusters and the F-actin flow that allows the microclusters to display a mixture of diffusive and directed movement. This clutch appears to operate in both directions since immobile integrin clusters appear to be able to slow the centripetal F-actin flow with consequences for stability of signaling complexes (Nguyen et al., 2008). These observations point to important missing information in our understanding of lateral mobility of free and engaged ligands in the plasma membranes of different types of antigen presenting cells. Synaptic patterns differ between B cells and dendritic cells as APC, with B cells resembling structures formed with supported planar bilayers (Grakoui et al., 1999; Monks et al., 1998) and dendritic cells displaying more disrupted patterns (Brossard et al., 2005; Tseng et al., 2008). This suggests limited mobility or a high degree of compartmentalization, which is consistent with the demonstrated role of the actin cytoskeleton on antigen presenting function of dendritic cells (Al-Alwan et al., 2001a, 2001b). Understanding how both sides of the immunological synapse influence each other is an important area for future research in which planar bilayers will play an important role; for example, by reconstituting the dynamics of live antigen presenting cell molecules to T cell surface receptors reconstituted in supported bilayers.

The physical processes that control the size of microclusters are not clear. It is interesting that TCR microclusters exclude CD45 from regions about 0.5 μ m across by TIRFM (Varma et al., 2006). Similarly, the segregated patterns formed in mixed contacts with two receptor–ligand systems differing in intermembrane spacing differing by 2–3 nm and protein enriched domains formed when two planar bilayers form intermembrane junctions are all on the scale of 0.5–1 μ m (Milstein et al., 2008; Parthasarathy and Groves, 2006; Varma et al., 2006). This suggests that the length scale of membrane fluctuations may govern the size of microclusters. Modeling that take into account membrane bending parameters support this model (Weikl and Lipowsky, 2004). A major gap in our current understanding is how dynamic F-actin contributes with membrane properties to TCR microcluster formation. One idea may come from studies of integrin microcluster formation, which is also F-actin dependent. High-speed imaging two-color TIRFM has demonstrates that α -actinin cross-linked F-actin serves as a scaffold for integrin cluster formation, rather than as a reaction to initial integrin engagement -based adhesion (Choi et al., 2008). For integrins, this process is initially myosin IIA independent, but nascent clusters rapidly undergo myosin IIA dependent maturation, which appears also to be the case for TCR clusters (Ilani et al., 2009). A preformed, actin dependent scaffold maybe critical to account for the high sensitivity of TCR to a small number of ligands.

11. What is the function of the immunological synapse?

The immunological synapse pattern has captured our imagination and led to a great deal of speculation, but deriving hard evidence for specific functions has been challenging. This is because manipulations that control the dynamic synaptic patterns often have direct biochemical effects on the cell that confound interpretation. Furthermore, since the SMACs are interdependent, its difficult to manipulate them independently. In many cases the functional ideas about the synapse reflect working models with varying degrees of experimental support. Here are three functions for which there is experimental support:

- (1) Stop signal – the symmetry of the immunological synapse results in an arrest of T cell mobility in 2D in vitro systems (Sims et al., 2007). Synapse symmetry in vitro and T-DC interaction time in vivo are well correlated in genetic exper-

iments (Sims et al., 2007). Stop signals increase dwell time with APC and may be important in signal integration during afferent and efferent phases of immune responses (Skokos et al., 2007), but this remains a working model since in vivo swarming may be able to achieve the same advantages in signal integration.

(2) Directed secretion – the cSMAC is an actin depleted zone at the center of the immunological synapse and thus appears to be an ideal location for directed secretion (Stinchcombe et al., 2006). This process can be observed directly using supported planar bilayers (Beal et al., 2008; Liu et al., in press). The gain in efficiency of killing in going from a partial to complete pSMAC ring is about 3-fold (Beal et al., 2008). While the pSMAC does not appear to act as a tight gasket, it may dynamically contain large complexes through a continual inward transport of adhesion molecules that generate partial barriers (Kaizuka et al., 2007).

(3) Asymmetric cell division – this is a fundamental principle in biology for generation of differentiated cells from stem cells. The termination of the synapse by asymmetric cell division might give rise to unequal daughter cells fated for memory (stem cell like) and effector cells (terminally differentiated) (Dustin and Chan, 2000). T cells appear to undergo LFA-1-ICAM-1 dependent early asymmetric divisions that give rise to memory cell precursors (Chang et al., 2007). This idea has been challenged by others studies that suggest that memory cells arise from equivalent effector cells, rather than being set aside early in responses (Wherry and Ahmed, 2004). However, while there are many common elements in these models and it is agreed that early events in the time frame of the first synapse formed by a naïve T cell set the stage for generation of memory T cells.

12. Conclusions

Supported bilayer technology has provided important insights into immune cell function including the identification of the ligands for the TCR, CD2 and LFA-1, the chemistry and physics of adhesion and the formation and function of the immunological synapse. There are still many questions to be addressed and the planar bilayer technology will continue to be useful due to its ability to simulate key aspects of membrane presentation of ligands. Those interested in more technical information on this planar bilayer formation and flexible methods for ligand deposition are referred to a recent protocol (Dustin et al., 2007a) and video describing some of these methods (<http://www.jove.com/index/details.stp?ID=947>). One of the most valuable aspects of the supported planar bilayer technology has been the ability to collect highly quantitative data in an area where this have been challenging: the cell–cell interface. This quantitative data should bridge the gap between physics based theories of adhesion and what happens in functional interfaces. An area of supported planar bilayer technology that has been under utilized in studies of immune cell adhesion are polymer supported bilayers (Floyd et al., 2008). This technology allows a 100 nm hydrated layer to be generated under the bilayer, which may accommodate some transmembrane proteins in a laterally mobile form. These systems appear to be sufficient physical stability for adhesion studies, but also add an important degree of freedom to the bilayer surface in terms of thermal fluctuations. This technology might be particularly useful for reconstitution of intact integrins to study the topology of these interactions. Planar bilayers may also facilitate ultrastructural studies with electron tomography to examine the structure of microclusters formed by the T cell receptor or integrins. This approach has been successful with desmosomes (Al-Amoudi et al.,

2007; He et al., 2003), which are highly enriched in one molecule. Labeling technologies will probably be needed to highlight molecules of interest in more heterogeneous T cell synapses. Studies with supported planar bilayers are powerful in terms of resolution and sensitivity, but its always important to test the predictions of these model systems in in vitro cell–cell or in vivo systems. We have taken steps in this direction by using in vitro cell–cell models and in vivo imaging to T cell-dendritic cell interactions (Milstein et al., 2008; Sims et al., 2007; Skokos et al., 2007; Tseng et al., 2008). Supported bilayer technologies are positioned to continue to occupy a key niche where biology and state of the art optical microscopy meet to address many open questions.

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