REVIEW ARTICLE

Model membrane systems to reconstitute immune cell signaling

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Introduction

Cells are composed of complex machineries working together to keep the cellular homeostasis. Maintenance of global homeostasis requires both an adaptive interaction of cells with their surrounding environment via their plasma membrane (PM) and the coordination of tailored cellular responses requiring signal transduction across different organelles. Thus, cellular membranes are essential structures endowing living organisms with an array of biological functions required for adaptation, survival, and proliferation. To understand these

Abbreviations

APC, antigen-presenting cell; BSLBs, bead supported lipid bilayers; cSMAC, central supramolecular activation clusters; CTL, cytotoxic T lymphocytes; DCs, dendritic cells; DOPC, 1,2-dioleoyl-sn-glycero-3-PC; DPPC, 1,2-dipalmitoyl-sn-glycero-3-PC; dSMAC, distal supramolecular activation clusters; EVs, extracellular vesicles; GUVs, giant unilamellar vesicles; IS, immune synapse; ITAMs, immunoreceptor tyrosine-based activation motifs; LB, lipid bilayer; NK cells, natural killer cells; PIP2, phosphatidylinositol 4,5-bisphosphate; PM, plasma membrane; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-PC; PS, phosphatidylserine; pSMAC, peripheral supramolecular activation clusters; PTM, post-translational modifications; SE, synaptic ectosomes; SLBs, supported lipid bilayers; SM, sphingomyelin; SMAC, supramolecular activation clusters; SUVs, small unilamellar vesicles; TCR, T-cell receptor; TIRFM, total internal reflection microscopy; TM, transmembrane.

Understanding the broad variety of functions encoded in cellular membranes requires experimental systems mimicking both their biochemical composition and biophysical properties. Here, we review the interplay between membrane components and the physical properties of the plasma membrane worth considering for biomimetic studies. Later, we discuss the main advantages and caveats of different model membrane systems. We further expand on how the use of model systems has contributed to the understanding of immune cell signaling, with a specific focus on the immunological synapse. We discuss the similarities of immune synapses observed for innate and adaptive immune cells and focus on the physical principles underlying these similarities.
cellular events, however, we need to solve the molecular details of these complex machineries and the grand principles behind their function.

From the structural point of view, biological membranes are formed by lipid bilayers (LBs) arranged as asymmetric, semipermeable, and dynamic structures. These features of the LB are defined in part by its comprising phospholipids, which interact with the environment through their hydrophilic, polar head groups while maintaining a hydrophobic core of acyl chains. The high heterogeneity of phospholipid species leads to their asymmetric distribution both laterally and between the inner and outer leaflet of the bilayer [1–3]. This spatial and compositional asymmetry helps define the physical and mechanical properties of the membrane and correlates with the specific functions of a given organelle and cell type [4,5].

The rich molecular composition of biological membranes also defines key physical properties, such as fluidity, phase transition, electrostatic forces, tension, and curvature, which adaptively change to evoke functional responses driven by surface receptors and other interacting macromolecules. Thus, in order to study the minimal forces and components conferring membrane function requires reductionist approaches mimicking both the physical properties and the molecular composition essential to replicate the process under study. In this review, we will first briefly discuss the physical properties of membranes—a critical consideration for reconstitution experiments—and then provide a general overview of the most common modeling systems. We will also discuss how these models of cellular membranes have been used for structural, biophysical, and biochemical studies, and discuss their main advantages and caveats. We will continue by expanding our discussion on how synthetic LBs have promoted understanding of the function of highly dynamic membrane-associated structures, such as the immunological synapse, with a focus on how immune cell signaling links to membrane reorganization.

**Mimicking biological membranes based on their molecular anatomy and physiology**

The paramount goal in exploiting model membranes is to reach an anatomical (e.g., structure/composition) and physiological (e.g., functional) understanding of how LBs work as platforms coordinating the assembly of higher-order molecular structures and interconnecting the cytoskeleton, membrane lipids, proteins, glyocalyx, and the extracellular matrix (Fig. 1). As reductionist mimics of cellular membranes, model LBs have provided useful tools to understand cellular processes in an isolated and controllable environment. Mimicking key components of the PM has in fact showed how few components can evoke function and collaborate in membrane-associated processes, such as immune cell signaling.

Anatomically, the central elements of the PM are structurally diverse lipids that comprise hydrophobic acyl chains and hydrophilic head groups. The structural variations (head groups, acyl chain length, saturation, ether vs ester bonds, etc.) result in hundreds of lipid species in a single cell membrane. Acyl chains in the inner hydrophobic plane intertwine with sterols and protein transmembrane (TM) domains which collaborate in the dynamic organization of the PM. Between 25% and 30% of proteins encoded in the human genome contain hydrophobic TM domains, including receptors, scaffolding proteins, transporters, ion channels, adhesion molecules, and enzymes (catalytic receptors)—all taking part in different membrane functions [6–8]. Furthermore, aiding homeostasis is the glyocalyx, a dense layer formed by glycolipids, glycoproteins, and proteoglycans in the PM and projecting to the exoplasmic space to interact with the environment (reviewed in more detail in Ref. [9]). Thus, membranes coordinate the cell’s adaptability to its environment by bridging events sensed on the extracellular matrix with intracellular signaling cascades and the cytoskeleton (Fig. 1).

Importantly, to offer good mimetics of the biological process under investigation, reconstitution systems should reasonably match both the biochemical and biophysical properties of the modeled biological membrane. To achieve near-physiological conditions, current methods exploit different lipid species, bilayer geometries, and protein anchor systems to study membrane-associated processes. Briefly, we will discuss the most important biophysical parameters of native and reconstituted membranes, and how these are influenced by either LB components or their physical support.

One of the most critical properties to take into consideration for modeling key biological membranes, such as the PM, is membrane fluidity. Diffusivity of the molecules residing in the PM of eukaryotic cells is key for their function [10,11]. From yeast to mammals, sterols and acyl chain unsaturation are key factors of cellular membranes modulating membrane fluidity. As such, building synthetic LBs using phospholipids composed of fatty acids of varying lengths and saturation, as well as of varying sterol percentages, allows a fine tailoring of lipid packing and diffusion coefficients [12–14]. Beyond phospholipid composition, cholesterol concentration plays a dominant role in determining lipid packing and bilayer fluidity, with increasing
concentrations leading to substantial packing and reduction in the diffusion coefficient of synthetic LBs [14]. A related concept is liquid–liquid phase separation in the membrane. When saturated and unsaturated lipids and cholesterol are mixed with certain proportions, they show liquid-ordered (lo) and liquid-disordered (ld) coexistence (reviewed in more detail in Ref. [15]). A fundamental consequence of higher ordering is slower diffusion which is crucial for effective ligand–receptor interaction and signaling. Hence, lipid ordering and domain formation are still attractive questions in membrane biophysics and in biochemistry of protein–protein and lipid–protein interactions. This phenomenon is very useful to elucidate which lipid environments certain lipids and proteins partition [16,17] or which environments certain molecules (e.g., viruses) bind to [18]. An additional factor influencing fluidity is the presence of a support, which can be considered as a mimic of the cytoskeleton [19–25]. Even at equal phospholipid compositions, supports significantly influence lipid lateral mobility by imposing hindrances dependent among others on the substrate roughness, curvature, and nanoscale topography [14,26–29].

Compositional and biophysical asymmetry of the PM is another key aspect for its function [3]. In the cytoplasmic leaflet of the PM, electrostatic forces play a critical role in the organization of functional interactions, particularly between acidic lipids and basic proteins. Acidic, negatively charged lipids, such as phosphoinositide and phosphatidylserine (PS), coordinate the targeting of polybasic/polycationic proteins to the inner leaflet of the membrane-forming transient nucleation domains that dissociate upon Ca^{2+} influx [30,31]. Phosphoinositides, including phosphatidylinositol 4,5-biphosphate (PI[4,5]P_2 or PIP_2) and phosphatidylinositol 3,4,5-triphosphate (PI[3,4,5]P_3 or PIP_3), are minor (~1%) but pivotal components of

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Fig. 1. Complexity of the PM and the central role of LBs. Model membranes aim to dissect the complexity of the PM and ‘dial in’ complexity as needed. As the field evolves, new model systems are exploiting near-physiological conditions to unveil the contribution of each one of these components in the collaborative task of membrane homeostasis.
the PM acting cooperatively in the recruitment of membrane-associated proteins including enzymes relevant for cell migration and endo/exocytosis (reviewed in more detail in Ref. [30]). Although PS contains a smaller density of negative charges compared to phosphoinositides, it also plays a key role in coordinating the delivery of cationic proteins to the inner leaflet of the PM, the endosomal compartment and lysosomes where PS is considerably more abundant [30,32].

Collaborating with ordering and electrostatic forces, membrane tension and curvature are other related parameters that define the adaptive functionality of the PM [33]. Certain lipids impose tension to the membrane which can be compensated by curving the membrane, a vital process for endo- and exocytosis [34]. For instance, the enzymatic conversion of sphingomyelin (SM) to ceramide instigates local membrane tension and curvature, a feature required for the budding of extracellular vesicles (EVs) from the PM [35]. The key role of curvature-modulating ceramides is further supported by the observation that addition of sphingomyelinase to synthetic vesicles containing SM is sufficient to generate local membrane enrichment and tension leading to membrane invaginations [36]. Phospholipid scramblases and flippases are critical in the regulation of local PM electrostatic forces and curvature by transporting lipids between the ectoplasmic and cytoplasmic leaflets of the PM [37,38]. Asymmetric distribution of lipids in model membranes in the absence of these enzymatic activities has been a challenge, yet there have been several recent methods to achieve this with controlled flipping of the lipids [39].

Aforementioned points highlight the importance of lipid selection in the reconstitution of bilayers for functional studies. As such, the choice of phospholipids with varying charges, saturation, and fatty acyl chains must be taken into account for the fine calibration of LB fluidity, ordering, and electrostatic interactions. Current methodologies are constantly being improved to mimic more closely the composition, asymmetry, and biophysical properties of cellular membranes. For instance, lipid matrixes comprising the neutral phosphocholine (PC) can be further tailored by varying the ratios of species differing in their acyl chain saturation; two [1,2-dioleoyl-sn-glycero-3-PC (DOPC)], one [1-palmitoyl-2-oleoyl-sn-glycero-3-PC (POPC)], or no unsaturation [1,2-dipalmitoyl-sn-glycero-3-PC (DPPC)] lead to diffusion coefficients in the order DOPC > POPC ≫ DPPC [14]. Moreover, cholesterol concentration can be tuned to further control the fluidity of the cell membrane. Thus, the selection of each model requires the consideration of how each component influences the biophysical properties of the system. Next, we will discuss model synthetic LBs and their methodological advantages and shortcomings.

### Giant unilamellar vesicles

With sizes up to 100 μm, giant unilamellar vesicles (GUVs) are synthetic vesicles formed by free-standing bilayers enclosing an isotonic solution [40] (Fig. 2). GUVs are commonly generated by simple rehydration of supported LBs (SLBs) formed and dried on electrodes [41]. Rehydration of dried LB under an electric field and using isotonic solutions results in high yields of giant vesicles of tailorable sizes [41]. Modifying the voltage and frequency of the alternating current (AC) electric field permits adjustment of vesicle size, but initial GUV diameters range from 10 to 100 μm and average 20 μm [42]. Due to their free-standing nature, soft exterior, geometry (curvature), and size, GUVs are good models to study membrane processes occurring within 3D cell–cell interfaces [43]. Indeed, much information on cell membrane dynamics has come from studying GUVs, most famously the phenomena of phase separation and its characterization [44]. GUV geometry makes them an attractive model especially as they are highly accessible through optical microscopy. Furthermore, bilayer behaviors such as clustering or liquid–liquid phase separation, as well as vesicle-to-cell interactions, are easy to reproduce [41,44].

Evidently, GUVs offer unique advantages regarding their 3D features and biophysical properties, positioning them between planar and bead SLBs [14]. However, though GUVs are an attractive model to study the cellular membrane, their soft exterior and fluidity also become their downfall; GUVs rupture in harsh experimental conditions such as extreme pH or high salt concentrations, or upon excessive washing [41]. For these reasons, experimental settings requiring more mechanically stable LBs exploit SLBs formed on a variety of solid substrates of different geometries lending themselves to several investigative techniques such as atomic force microscopy.

### Small and large unilamellar vesicles

Free-standing lipid vesicles of smaller sizes ranging between 50 and 300 nm are commonly referred to as small unilamellar vesicles (SUVs) while large unilamellar vesicles (LUVs) are between 200 and 1 μm. SUVs and LUVs (herein referred to as liposomes) are generated by the breakage of multilamellar liposomes using different physical methods. The mechanical disruption...
of multilamellar liposomes by extrusion is the most efficient method for generating homogeneous preparations of SUVs, followed by sonication/ultrasonication and homogenization [45]. Detergent dialysis from octyl-β-D-glucoside is an additional method to generate liposomes containing TM proteins [46]. Similar to GUVs, liposomes have proven useful in the reconstitution of lipid/protein biological scaffolds but have also allowed reconstitution of EVs with immunostimulant properties. Furthermore, they have aided the study of cellular processes such as ion channels and membrane fusion events during viral infection [47]. SUVs are also used as delivery platforms for drugs and vaccines as they can be coated with sensitive compounds and antigen for a more targeted delivery with improved stability in vivo [48,49]. The use of specific phospholipid species revealed a role of lipid packing in defining the quaternary assembly of different multiprotein complexes, including bacteriorhodopsin and aquaporin-0 [50–55].

**Substrate supported lipid bilayers**

Solid supported membranes were pioneered by McConnell et al. in the early 1980s [56]. Bilayers assemble on the surface in such a way that an ultrathin hydration layer or ‘cushion’ (< 2 nm thick) remains at the interface of the support and polar heads of the inner leaflet, separating them [26,57]. The bilayer remains in proximity only through an energetic minimum that describes electrostatic forces and weak, long range Van der Waals interactions. The hydration cushion is crucial as it preserves lateral movement of lipids, and thus the structural and thermodynamic properties of the bilayer [58]. Yet despite separation, reports of the support’s influence on the inner leaflet—disrupting fluidity and even reacting with embedded proteins—are widespread, and the extent unpredictable [14,28,29]. Design variants include a synthetic cushion comprising polymer networks in lieu of the hydration layer, as well as tethered bilayers comprising spacer...
chains that decouple the bilayer from the substrate but are themselves directly anchored [59,60]. Such tethered or polymer bilayers serve to increase cushion thickness, and therefore reduce the support’s influence on the bilayer.

Supports vary but include oxidized silicon (glass), titanium, aluminum, and mica. Solid supports vastly improve mechanical stability and durability for more thorough investigation of bilayer behavior. Substrate architecture can be finely tuned in numerous ways and SLBs can be divided into planar or spherical designs. While the aforementioned advantages are shared, the obvious geometric differences should not be overlooked (Fig. 2). Indeed, membrane topology is known to influence bilayer behavior. In recent years, Fu et al. showed shallow insertion depths of peripheral proteins into highly curved bilayers compared to planar constructs [61]. Yet, despite such consequential evidence, literature barely compares the two systems. Clarity on their limitations, advantages, and applications are clearly needed.

**Planar supported lipid bilayers**

The self-assembly capabilities of small liposomes on solid supports have promoted the development of planar SLB on a vast array of materials (reviewed in more detail in Ref. [58]). From the methodological standpoint, planar SLBs are assembled onto substrate by one of three means: (a) via vesicle spreading; (b) by sequentially transferring two monolayers (Langmuir–Blodgett method); and (c) by spin-coating substrates of different composition [58,62]. Vesicle spreading perhaps remains the most common method as upon exposure, liposomes simply collapse onto the substrate. The Langmuir–Blodgett dipping method is more complicated as it entails consecutive transfer of monolayers onto the substrate [63]. However, a superior advantage of Langmuir–Blodgett dipping method is the direct control of leaflet asymmetry otherwise not possible with spontaneous fusion of intact liposomes. Importantly, both methods are largely limited by substrate material, pH, and salt concentration. Spin-coating addresses these issues and consistently assembles more uniform bilayers with high reproducibility. Lipids dissolved in chloroform and methanol mixture are pipetted onto substrate which is then immediately spun at ~ 500 g for 30 s [62]. As the solvent evaporates, a thin coat of lipid is distributed comparatively evenly across the substrate which, upon rehydration, form the fluid bilayer.

Utilizing a planar substrate opens doors to surface sensitive analysis. For example, atomic force and total internal reflection microscopy (TIRFM) are routinely used to obtain high-resolution images and topographic information of bilayers and their associated cellular assemblies. Additionally, surface plasmon resonance can reveal the dynamics of real-time binding events at the surface. Yet, all planar SLBs by definition lack three-dimensionality, a significant limiting factor. Although this can be circumvented by the use of substrates encoding 3D features [64], the underlying substrate might restrict TM protein lateral mobility [65]. Also, there lacks any systematic comparison of how different SLB assembly methods impact technical readout, as different SLB preparations might impose hindrances to the lateral mobility of molecules and hence the association of multimolecular assemblies.

**Spherical supported lipid bilayers**

While surface analysis is more limited with spherical constructs, their geometry prominently positions them as a valuable biomimetic model. Spherical supported models describe a bilayer wrapped around a spherical inorganic material, typically silica. Assembly can be achieved by spontaneous fusion of liposomes with micron-sized beads forming BSLBs [66]. By design, this allows precise control of membrane size to best mimic native microcurvature, or even templating surface architecture [67]. Such control also negates the issue of polydispersity previously described for GUV populations. In fact, as nanoparticles, BSLBs are inherently colloidal—a behavior that extends their applicability significantly: Their robustness makes them amenable to optical tweezers for finessed spatial manipulation and individual assessment [68]; and as dispersions, their concentrations can be precisely adjusted to maximize signal-to-noise ratios in spectral analysis [69]. Alternatively, solution compositions can be tailored for instance to better mimic physiological environments or to contain protein for easy membrane coating. Finally, and more recently, microarrays comprising wells designed to hold individual BSLBs have been utilized for high-throughput screening (~ 100 000 per mm²) of molecular interactions, but could also refine and improve design parameters through a library of constructs [69].

Bead supported lipid bilayer topology imparts a lateral tension across the bilayer and does not always align with native characteristics. Phase transitions occur at lower temperatures with highly curved SLBs, some 2–3 °C below that of free-standing vesicles [57]. Due to the electrostatic forces that hold the bilayer in-place, conventional BSLBs are generally restricted to only cationic or neutral compositions, whereas native
PMs comprise > 20% anionic lipids. The reconstitution of SLBs with anionic lipids has to be studied carefully as others have reported the asymmetric distribution of negatively charged lipids which are repelled to the outer leaflet by silicon oxide substrates [70]. The ectoplasmic localization of negatively charged lipids such as phosphoinositides and PS might help reconstitute events on the PM’s cytoplasmic face. However, such localization might also limit the use of SLBs in reconstitution of ectoplasmic surfaces with proteins requiring the interaction of TM domain and anionic phospholipids.

One of the key factors influencing the efficiency by which cells either rely or sense activating signals is ligand mobility [71]. Yet side-by-side comparisons of diffusion times between BSLB and other model membranes are rarely presented and correlated with a functional response in interacting cells. Our recent research emphasizes the limitations of each model and how substrate nature influences bilayer lateral mobility [14]. Previous conclusions on cellular processes have been drawn from all three models, and so clarifying discrepancies is crucial to confirm and facilitate decisions on which model is most suited for the investigation at hand. In the following sections, we will expand on how these experimental systems have advanced our understanding of the machinery regulating immune cell responses.

Studying immune cell signaling with model membranes

Model membranes have enriched our experimental toolkit for the dissection and modeling of communication between immune and target cells, especially that of the structured contact formed at their cell–cell junction termed ‘the immunological synapse’. The synergy between the advances in membrane mimetics and microscopy has provided high-resolution evidence of the link between the structural organization of cell surface receptors, their signaling cytosolic domains, and the signal transduction occurring in response to receptor triggering. Among them, TIRF microscopy has been widely adopted to study events occurring at the interfaces of cells and glass functionalized with SLBs of diverse phospholipid composition [72–75]. The inclusion of lipid species possessing polar heads of different affinity linkages facilitated the tailoring of SLBs with increasing complexity. The use of biotin (lipid)– avidin (linker)–biotin (protein) bridges, DGS-NTA (Ni):His-tagged proteins and glycosylphosphatidylinositol-modified proteins [76–78], favored the reconstitution of a number of cell membrane proteins and the modeling of cell–cell interfaces [79–81]. Furthermore, the adaptability of synthetic SLBs to different experimental platforms has been instrumental in reconstituting both the exoplasmic and cytoplasmic face of different immune cells, aiding understanding of the physicochemical complexity of cell activation at the immune synapse (IS).

Shedding light on the immunological synapse with model membranes

Microscopy studies looking at contacts formed between immune cells and compositionally diverse model membranes unraveled the link between protein reorganization at the interface and immune cell response. Synthetic LBs were first used by Weis et al. [82] in combination with TIRFM to study the interfaces between rat basophils and membranes containing lipid hapten, which permitted a coating of immunoglobulin E (IgE) antibodies exposing their Fc portion. Later that decade, Brian and McConnell demonstrated activation of mouse cytotoxic T cells to planar SLB loaded with antigen [major histocompatibility complex (MHC) class I allomorph H-2Kk], validating their use for the functional study of immune cell response to mimetics of the antigen-presenting cell (APC) PM [83]. Further to these initial uses of SLBs, was the description and functional characterization of the immunological synapse (IS). The pillar work of Grakoui et al demonstrated that T lymphocyte activation at the IS derives from dynamic receptor:ligand interactions resulting in the temporal evolution of supramolecular activation clusters (SMAC) previously defined as end-points in fixed cell–cell conjugates by Kupfer et al. (see below) [84,85] (Fig. 3A). More specifically, the molecular rearrangements required for full signaling include the following: (a) the initial ligation of surface antigen receptors; (b) the maintenance of membrane proximity by integrins; (c) the progressive gain in binding valency and physiological avidity of receptors with an intrinsic low ligand affinity; (d) the exclusion of large phosphatases which altogether promotes signaling by forming receptor microclusters [86]; and (e) the physical association of signaling microclusters with the actin cytoskeleton—accelerating the central clustering but also the association of these clusters with the machinery of endosomal sorting complexes required for transport (ESCRT) for their inclusion on budding vesicles [87,88] (Fig. 3A,B).

As a general model, activation of immune cells occurs when the integration of phosphorylation events across multiple receptor-associated tyrosine kinases surpasses a minimal threshold for activation—a high
steady-state phosphatase activity in the cell maintains this threshold [89]. Also, due to the inherent low affinity of the triggered receptors, cell activation requires a high valency of binding (also referred to as avidity) and an optimal interaction dwell-time of bound molecules [90–93]. Multiple different receptor-ligand pairs may contribute to this high valence by coming together spatially to form a single microcluster or larger microdomain. At this level, the formation of distal, peripheral, and central SMACs (cSMAC) facilitates high binding valency by promoting the formation of higher-order receptor assemblies in the PM [94]. These higher-order clusters increase the spatial proximity between receptor cytoplasmic tails and Src-family of protein tyrosine kinases. This subsequently leads to downstream signaling in an environment protected from the antagonizing action of phosphatases [94]. In T cells, the Src-family of protein kinase member Lck mediates the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) present in CD3 subunits within T-cell receptor (TCR) complexes. Since Lck associates with the cytoskeletal tail of the TCR coreceptors CD4 and CD8, which also bind the MHC, the binding of TCR and antigenic peptide MHC (pMHC) leads to the spatial proximity of Lck and the CD3 subunits δε and γε heterodimers and the CD247 ζ homodimer [95–97]. Phosphorylated CD3 ITAMs further amplify signaling by recruiting Syk-family of kinases, ZAP-70 among them (reviewed in more detail in Ref. [98]). The formation of higher-order cytoplasmic structures between Src-family of kinases Lck and Fyn, and Syk-family of kinases ZAP-70 and Syk is key for the recruitment of adaptor proteins including linker for activation of T cells (LAT), SLP-76, Crk, and Cas-L and the resulting formation of migratory signalosomes [99]. Adhesion molecules like CD2-CD58 and costimulatory molecules like CD28-CD80/86 may contribute to the valency to allow activation with fewer pMHC. The high contrast of TIRFM with SLBs allowed the detailed characterization of these migratory signalosomes, commonly referred to as TCR microclusters, which rapidly form at the interacting pole of the T cell and in response to even scarce quantities of antigenic pMHC on the synthetic SLB [84,100].

The initial imaging of CD4+ T cells interacting with glass-SLB mimicking APCs demonstrated that TCR microclusters immediately form to prolong initial signaling, and as the synapse progresses over time these microclusters centripetally migrate and coalesce in a stepwise process into bigger SMACs. LFA-1 and TCR microclusters form in the distal SMAC...
(dSMAC) and migrate centripetally to form two separated SMACs over minutes of interaction (Fig. 3C). The dSMAC forms a radial lamellipodia which allows cytoskeleton reorganization while facilitating receptor–ligand interactions as well as mechanosensing [101,102]. Bound ICAM-1:LFA-1 microclusters accumulate and arrange peripherally forming a symmetrical ring surrounding the synaptic cleft formed at the center of the contact [84]. This peripheral ring of bound ICAM-1:LFA-1 pairs was termed as the peripheral SMAC (pSMAC) by Kupfer [85,103,104] (Fig. 3C). At the borders of the nascent synaptic cleft, the migrating TCR microclusters then dissociate from F-actin and form a TCR-enriched domain termed as cSMAC [99,104,105]. Although initially the cSMAC was considered a signal termination domain, we recently showed that it also has a more specialized function as a synaptic ectosome budding domain critical for the release of effectors in a vesicular form [85,103]. The application of direct stochastic optical reconstruction microscopy (dSTORM) to the imaging of SLBs further demonstrated that microclusters containing either CD40L or TCR, which are initially formed in the periphery and distal area of the contact, are sorted into the cSMAC and in a later step incorporated into synaptic ectosomes (SE) and kept as discrete structures. Importantly, it is also shown that EPN-1, which binds PIP2 leading to membrane curvature and the formation of clathrin invaginations, also forms ring-like structures in the immediate periphery of the cSMAC [47]. This suggests that the cSMAC might comprise different domains participating in the compartmentalization of endocytosis, exocytosis, and EV budding at the IS. A similar partition of the cSMAC was proposed by Griffiths et al. for CD8+ cytotoxic T lymphocytes (CTL), in which a signaling domain surrounds an inner secretory region specialized in the delivery of cytotoxic signals from intracellular stores [106]. Because TCR+ EVs are mostly originated from the PM and delivered in a polarized fashion at the cSMAC—as evidenced by both correlative fluorescence-electron microscopy and dSTORM on glass-SLBs—we have coined the more appropriate term SE to refer to these effector EVs [47,87]. As we have recently shown using a combination of both planar SLB and BSLB methods, PM-derived SEs incorporate a number of surface-bound ligands, such as ICOS and CD40L, which are centripetally recruited in microclusters to the synaptic cleft [47,107]. In a comparable way to CTLs, the synaptic release of EVs might also include vesicles stored in multivesicular bodies and containing effectors, such as CD40L and FasL [108–112].

The synapses of different immune cell types

This SMAC organization is not specific to SLBs, and similar SMAC structure was also observed in GUVs functionalized with target proteins [113,114]. These experiments were illuminating as they also revealed the molecular determinants of SMAC formation. When GUVs were coated with pMHC, T cells could recognize the GUVs and formed the synapse where pMHC was enriched in the contact area while the phosphatase CD45 was significantly reduced (Fig. 4A). This contact was sufficient for downstream signaling and recruitment of Zap70 to the membrane of T cells (Fig. 4B). Moreover, GUV systems showed that any tight adhesion, such as that between CD2 and CD58, is sufficient to displace CD45 from the contact area (Fig. 4C). The latter phenomenon mimics CD45 exclusion triggered by TCR binding; this further supports the kinetic segregation model that describes productive signaling as a consequence of local enrichment of kinases—a behavior ensured by their ectodomains exerting steric hindrance on phosphatases [113,115]. The GUV system is quite versatile and has also been used to show phosphatase exclusion and synapse formation in other cells such as in B cells or mast cells (Fig. 4D) [114,116]. The requirements of receptor aggregation and phosphatase repulsion have been extensively shown to be a critical step for signaling. Rather than mandatory, phosphatase exclusion contributes by lowering the threshold for T-cell activation, thus increasing the sensitivity for antigen [117]. Recent analysis of the rearrangement of FcεRI in mast cells stimulated on SLB loaded with either Fce or trinitrophenyl-BSA-immobilized IgE has demonstrated that both monovalent IgE and immunocomplexes are equally competent in excluding CD45 and drive cell activation by recruiting Syk [116]. The ability of monovalent IgE to form multivalent clusters in SLB was first observed by McConnell almost 40 years ago [82] and is assumed to be a product of membrane topology and receptor diffusion. However, similar observations are now present for T cells as well, pointing toward a potential cross-linking-independent mechanism [118,119].

Overall, ISs of different functionality were promptly characterized in natural killer (NK) cells, mast cells, CTLs, regulatory T cells (Treg), and B cells [106,120–124] (Fig. 5A,B). These also included synapses hijacked by viral pathogenic factors which resulted in suboptimal signaling and aberrant T-cell–SLB interfaces shaped by the ligation of viral proteins [125–129] (Fig. 5C). Moreover, IS-like structures have also been described for macrophages in which segregation of
phagocytic receptors follows the same central clustering pattern to antigen receptors in lymphocytes leading to the formation of phagocytic cups [130,131]. More importantly, all of these synapses share the same fundamental membrane reorganization events observed for the activation and functional response of T cells. As such, initial receptor binding leads to phosphatase exclusion and concurrent recruitment of Src-family kinases, followed by Syk-family kinases, the recruitment of adaptors, and the remodeling of the cytoskeleton.

**Studying the effector phase of the IS**

Model membranes allowed us to also dissect the events following cell activation induced by a minimal target cell. Following the productive signaling of receptors is the initiation of secondary mechanisms driving the effector response through release of soluble signals from the activated immune cell into the synaptic cleft [47,87]. These mechanisms entail the fusion of intracellular vesicles within preformed domains at the PM for the targeted release of cellular cargo, such as preformed EVs (called exosomes), and the de novo formation and shedding of SEs from the PM [47,87]. In NK cells and CTLs, effector cargo includes small soluble signals, such as regulatory cytokines and proapoptotic/cytotoxic factors, as well as EVs stored in multivesicular bodies that might partially share the composition of SEs. Perforin and granzymes are common cytotoxic molecules released by NK cells, CTLs, and Tregs [132,133]. Secretion of content from multivesicular bodies or intracellular vesicles depends on membrane fusion events directing cargo release into specific domains at the PM. SUVs have provided additional tools to understand the sequence of events required for vesicular and PM fusion and the key proteins involved. For instance, SNARE proteins reconstituted in SUVs are sufficient (i.e., the minimal requirement) to mediate the fusion of apposing membranes at physiological temperatures [134]. However, in a cellular context the process is far more complex requiring the functional organization of multiprotein complexes including Rab-GTPases primarily interacting with the target membrane (with high curvature) and bringing SNAREpins proteins alongside their aiding factors to form the pivotal machinery mediating fusion of cytoplasmic vesicles with the PM [135]. Vesicle fusion mechanisms participate in a vast array of biological processes including the release of neurotransmitters in the neurological synapse. Remarkably, several molecular reorganizations occurring in the IS are also present and share a high degree of structural identity with neurological synapses. Of note, many features of neurological synapses are in parallel with IS, including the following: cell polarity; the participation

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**Fig. 4.** The study of cis and trans molecular reorganization occurring between GUV and immune cells. (A) T cells interacting with GUVs decorated with pMHC and CD45. The size-dependent segregation of proteins within the immunological synapse can be observed for CD45 and pMHC in trans (i.e., exclusion in the opposing membrane). In this case, both CD45 (green) and pMHC (magenta) are on the surface of GUVs mimicking a professional APC. Binding of pMHC by TCR on the T cell leads to the central enrichment of pMHC, which results in the exclusion of CD45 from the cell-GUV contact area. (B) T cells expressing Zap70-mNeonGreen interacting with GUVs decorated with pMHC. After the contact formation, Zap70 is recruited to the T cell pole. (C) T cells interacting with GUVs decorated with CD58. Compared to (A) in this example, CD45 is present on the T-cell surface (in cis, green) and is excluded from the contact area where CD58 (magenta) is enriched. (D) Rat basophilic leukemia (RBL-2H3) cells expressing Syk-Citrine and FcεR-SNAP interacting with GUVs decorated with IgE-bound antigen. After the contact formation, Syk is recruited to the membrane where the receptor is enriched. Scale bar = 20 µm. See refs 116 and 118 for details.
of adhesion molecules providing stability; and the formation of a synaptic cleft for targeted delivery of neurotransmitters by stimulated neurons [136]. Importantly, a minimal diameter of 0.5 µm has been established for functional signalosomes comprising either antigen receptors (like TCR microclusters), engaged phagocytic receptors on immature dendritic cells (DCs; which require target particles with a minimal size of half a micron), or neurological contacts [136]. The latter suggests that to achieve a signaling threshold, T cells, phagocytic cells, and neurons require the formation of a minimal structural assembly driving the exclusion of phosphatases from immunological and neurological synapses [136].

The striking functional arrangement of bound receptors among different immune cells undergoing synaptic communications suggests a common evolutionary origin and the selection of a mechanism for efficient signaling in the face of spatiotemporal restrictions. For instance, the IS of B cells adopts a molecular organization resembling that observed in T cells and NK cells, and combines the process of activation with the uptake of antigens from the surface of cells, such as macrophages and DCs [121,122]. As such, F-actin polymerization resulting from initial B-cell receptor (BCR) triggering leads to membrane deformation and the formation of a phagocytic interface essential for the uptake of antigens, both events observed in macrophages and DCs [130] (Fig. 6A,B).

Supported lipid bilayers have also allowed the characterization of the IS in more specialized subsets of T cells, such as helper follicular T cells (TFH) which release chromogranin B alongside CD40L in response to antigen, CD40 and ICOSL [107]. Importantly, the release of chromogranin B at the cSMAC suggests that TFH may promote the generation of long-lived plasma cells by releasing dopamine at the IS. Similarly, imaging of glass-SLB further demonstrated similar molecular organizations in the lytic synapses formed by NK cells and CTLs, with the specialized release of cytotoxic molecules in a sealed contact termed the ‘kiss of death’ (reviewed in more detail in Ref. [137]). Lytic synapses link recognition of agonistic signals in tumor cells or infected cells with the release of cytolytic granules; yet although the rearrangement of SMACs is similar, evidence suggests that cytotoxic T cells can kill target cells without the need of stable contacts [138]. The maintenance of self-tolerance depends in part on Treg forming synapses with activated immune cells. Treg synapses might be cytotoxic via FasL-dependent triggering of proapoptotic receptors, such as CD95 (Fas), or antagonistic by depleting activating molecules, such as CD80 and CD86, from the surface of DCs using a CTLA-4-dependent trogocytosis/transendocytosis [139–141]. Macrophages also form organized membrane domains during phagocytosis and display a striking similarity in the rearrangement of bound surface receptors participating in the uptake of foreign particles, such as FcR, Dectin-1, and others [130]. The terms phagocytic cups and phagocytic synapse were coined by Goodridge et al. [130] to illustrate this molecular organization which, among others,
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containing proteins sorted into the cSMAC (P. F. Cifuentes, S. Valvo, E. Sezgin, Y. Peng, T. Dong, M. Maj, B. Peacock, D. Aubert, M. L. Dustin, manuscript in preparation). The physical resistance of BSLBs to shear forces and different media compositions made their analysis possible by multiparametric flow cytometry and mass spectrometry allowing the simultaneous comparison of protein transfer dynamics at the IS. BSLBs have aided the discerning of how different ligands tailored the release of effectors by T cells, and how the degree of TCR triggering and density of costimulatory ligands influenced the sorting of these T-cell molecules into SEs. Importantly and more specifically, selectivity on the sorting of bound molecules such as pHLA bound TCRαβ, CD40 bound CD40L, and ICOSL bound ICOS was not observed for adhesion molecules, including LFA-1 bound ICAM-1 [47].

In addition, BSLBs provided a robust quantification of the amount of effector molecules transferred as a result of SE release. This quantification alongside the use of recombinant proteins and free-standing SUVs allowed us to investigate the relationship between CD40L clustering, sorting, and binding valency on the agonist effect of shed T-cell SEs on DCs. Using SUVs with an average size of 86 nm, we developed a synthetic version of CD40L+ SEs. For this, we used absolute quantifications of CD40L transfer from CD4+ T helper cells (TH) into antigen+ and CD40+ BSLBs. Using SUVs bearing physiological densities of the T-cell effector molecule CD40L, we showed that the high valency of binding provided by vesicular CD40L, but not its concentration in the media, efficiently activated CD40+ DCs [47]. This observation shed light on the biological function of SE release at the synapse, suggesting high-order clustering of effectors is needed to promote APC activation not normally triggered by densities present on the steady-state PM of T cells. These are some examples of how synthetic vesicular elements have been used to unravel the multimolecular

The use of model membranes for systematically interrogating a number of ISs demonstrated how spatial reorganization of bound receptor–ligand pairs is a mandatory process for not only achieving complete receptor signaling, but also activation of the engaged immune cell and delivery of effectors at the cell–cell interface. TIRFM was unmatched at improving the contrast of events occurring in the intimacy of the LB and the cell membrane. However, limitations in the applicability of TIRFM to determine quantitative differences in molecules transferred from cells to glass-SLB are an important constraint. In part, this might relate to differences in 3D distribution of SE deposits on planar SLBs, which might in turn influence the penetrance and photon excitation provided by the evanescent light. Light distribution across the TIRFM field of view and the access points of labeling antibodies within tight contacts might also limit the quantification of ectosome molecules. Recently, the easiness of SLB assembly on SiO2 (silica) beads has favored the development of BSLBs with tailored protein composition to dissect the mechanisms responsible for effector molecule release at the IS. Most specifically, we dissected the machinery responsible for SE release by combining imaging of glass-SLBs with integrated proteomic analysis of BSLBs [47]. BSLBs proved to be good surrogates of both glass-SLBs and APCs, mimicking the lateral mobility of the PM [25], cell curvature, and efficiently capturing budded vesicles containing proteins sorted into the cSMAC (P. F. Cespedes, E. Kurz, D. G. Saliba, A. Kvalvaag, L. B.

![Fig. 6](image-url). Lateral distribution of proteins in the longitudinal plane of the immunological synapse of (A) B cells and (B) the phagocytic synapse formed by Dectin-1-mediated engulfment of yeast cells. LFA-1 bound ICAM-1 pairs segregate to the periphery of the contact, and antigen bound BCR pairs cluster centripetally in the cSMAC where internalization occurs. Similarly, Dectin-1 centripetally accumulates in the phagocytic cups favoring the exclusion of phosphatases CD45 and CD148 from Dectin-1 clusters and promoting the engulfment of the recognized yeast cell. F-actin polymerization in the periphery of these synapses is essential for the capture and processing of antigens.

seggregates the phosphatases CD45 and CD148 from foci of bound Dectin-1 where phosphorylated Syk accumulates (Fig. 6B). As we discussed above, phosphatase segregation is essential in providing functional stability to nascent signalosomes, which mediates signal integration essential for conveying a biological response in the limited dwell-time of receptor–ligand interactions happening on the PM.

Fig. 6. Lateral distribution of proteins in the longitudinal plane of the immunological synapse of (A) B cells and (B) the phagocytic synapse formed by Dectin-1-mediated engulfment of yeast cells. LFA-1 bound ICAM-1 pairs segregate to the periphery of the contact, and antigen bound BCR pairs cluster centripetally in the cSMAC where internalization occurs. Similarly, Dectin-1 centripetally accumulates in the phagocytic cups favoring the exclusion of phosphatases CD45 and CD148 from Dectin-1 clusters and promoting the engulfment of the recognized yeast cell. F-actin polymerization in the periphery of these synapses is essential for the capture and processing of antigens.
interactions, the architecture, and the forces involved in the formation of working immunological networks.

In the next section, we discuss some of the observations made possible with the reconstitution of the cytoplasmic face of the PM and the physicochemical events coordinating signal transduction and reorganization of the cytoskeleton.

**Reconstituting intracellular organization and signaling events**

Supported lipid bilayers have been also used successfully for the reconstitution and modeling of events occurring at the inner leaflet of the PM (Fig. 7A,B). Using high-resolution TIRF microscopy, Su et al. explored the relationship between phosphorylation and the physical properties of T-cell antigen receptor (TCR)-associated signaling complexes. This *in vitro* reconstitution required peptide sequences representing CD3ζ, LAT, and Lck anchored to the SLB and ZAP-70, Grb2, Sos, or SLP-76 in solution, which were all phosphorylated upon ATP addition [142]. The resulting condensates excluded the CD45 phosphatase domain, which was surprising as this is normally thought to require the extracellular domain. However, very early studies on reconstitution of CD45 function with constructs containing only the cytoplasmic domain suggest that this phase separation process may contribute to physiological CD45 function [143]. Importantly, the addition of actin was sufficient to reconstitute filaments enriched in LAT, supporting SLBs as tools for functional dissection of highly complex signaling cascades that organize cytoplasmic adaptors—such as those present in TCR microclusters.

An alternative membrane-based reconstitution system using suspensions of LUVs and FRET to detect complex formation was applied to analysis of checkpoint using suspensions of LUVs and FRET to detect condensates that nucleate F-actin [149] (Fig. 7C). This study was a predecessor to the work on the TCR-LAT-driven condensates. Interestingly, the SH3 and polyproline domain adapter protein CD2AP plays an important role in maintaining the IS and the slit diaphragm [150,151].

**Concluding remarks and future perspectives**

A considerable body of evidence derived from the study of model membranes demonstrates the pivotal role of membranes as structures orchestrating complex biological processes depending on the combined, additive, or antagonistic effects of lipids, proteins, and sugars. Advanced imaging, spectroscopy, and numerous other complementary techniques have highlighted the dynamics of the PM; frequently, pivotal functions arise from membrane components rearranging to form higher-ordered structures that connect to the internal compartment of cells. The feasibility of reconstituting cellular membranes into reductionist systems has promoted the systematic evaluation of biophysical and biochemical factors governing LB functionality. From all the examples discussed, we can highlight that the common paradigm explaining the functioning of LBs is their intrinsic disequilibrium and high dynamism (for instance between activating and inhibitory elements), which is locally lost upon triggering of responsive elements within its structure. The beauty of these systems has been unmatched for elucidating how interaction of a few molecules suffices to promote a biological response of interest. However, advances in the field are likely requiring the study of membranes of increasing complexity to mimic near-physiological conditions and more complex cell behaviors. For instance, the exquisite high sensitivity of CD4+ and CD8+ T cells has been replicated only partially by using model LB. Two recent works showed that triggering of Ca2+ influx depends on both the number of agonistic pMHC included within single TCR microclusters and the spatiotemporal coincidence of their bound elements, rather than by the total number of pMHC encountered by the
Manz et al. [93] showed a minimum of four pMHC per TCR microclusters is required to instigate signaling, which is substantially higher to the previously reported range of 1–50 total pMHC triggering T-cell activation in other experimental systems [153–156]. The latter suggests that other factors need to be considered to reproduce such high sensitivity to antigen, including the tailoring of SLB with costimulatory ligands for CD28 and CD2 to reduce the T-cell activation threshold [93,157,158]. Another factor that should be considered is the adaptive response of the opposing APC membrane, which also clusters MHC in response to its engagement [159]. This suggests that improvements in the self-ordering capabilities of the synthetic LB might also be exploited to reduce T-cell activation thresholds. Fully reconstituting the APC PM might require circumventing current limitations, including the lack of methods for the proper partitioning and orientation of protein TM domains on SLB. Protein TM domains might define new functions by interacting with elements of the LB hydrophobic core, such as tetraspanins, sterol, and a variety of acyl chains.

Other limitations derive from the reconstitution of the glycocalyx of model cells by tethering glycolipids and glycoproteins to synthetic LBs. One recent interesting observation was the finding that recombinant proteins per se could strongly influence the shape of synthetic membranes due to the interactions of their glycosylated ectodomains [160]. Protein extracellular domain extension and volume, which is partially influenced by post-translational modification (PTM), such as O- and N-linked glycosylations, could also determine the diffusion properties of proteins and the biological processes they mediate as part of the glycocalyx. To tackle these future needs, the study of extracted native membranes will allow us to evaluate how our current understanding applies in more complex biological situations, such as different metabolic states. Metabolism might directly influence the functional properties of cellular membranes by modulating glycosyltransferase activity and fatty acid saturation in the PM [161–163]. Other PTM modifications affecting receptor half-lives and their surface expression levels might also collaborate in defining the biophysical and biochemical complexity of native
membranes. Elucidating how these factors influence the behavior of highly dynamic cellular events will open new avenues for the design of therapeutics modulating membrane-associated processes, such as the high sensitivity of T cells for antigen, and the physical interaction of receptor–ligand pairs driving immunity and tolerance. Future studies will certainly focus on these paramount questions and more, and the combined used of reductionist and native systems might provide new insights on membrane biology and how we can tailor it for translational applications. Without doubt, having such valuable tools will allow us to study the effect of checkpoint inhibitors and immune stimulators at a physiological level previously unmatched by synthetic systems alone. For this purpose, the exploitation of native membrane systems such as giant PM vesicles [164] and their adaptation into supported PM bilayers [165] is a mandatory step to unravel the fundamental dynamics of membrane physiology for different phenotypical states.

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Conflict of interest

The authors declare no conflict of interest.

Authors contribution

All authors contributed to the discussion, planning, and writing of the manuscript.

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