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Review

Microdomains in the membrane landscape shape antigen-presenting cell function

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ABSTRACT

The plasma membrane of immune cells is a highly organized cell structure that is key to the initiation and regulation of innate and adaptive immune responses. It is well-established that immunoreceptors embedded in the plasma membrane have a nonrandom spatial distribution that is important for coupling to components of intracellular signaling cascades. In the last two decades, specialized membrane microdomains, including lipid rafts and TEMs, have been identified. These domains are preformed structures ("physical entities") that compartmentalize proteins, lipids, and signaling molecules into multimolecular assemblies. In APCs, different microdomains containing immunoreceptors (MHC proteins, PRRs, integrins, among others) have been reported that are imperative for efficient pathogen recognition, the formation of the immunological synapse, and subsequent T cell activation. In addition, recent work has demonstrated that tetraspanin microdomains and lipid rafts are involved in BCR signaling and B cell activation. Research into the molecular mechanisms underlying membrane domain formation is fundamental to a comprehensive understanding of membrane-proximal signaling and APC function. This review will also discuss the advances in the microscopy field for the visualization of the plasma membrane, as well as the recent progress in targeting microdomains as novel, therapeutic approach for infectious and malignant diseases. *J. Leukoc. Biol.* 95: 251–263; 2014.

MEMBRANE MICRODOMAINS

The plasma membrane is essential for cell function as a result of its unique role in the communication between the inside and outside of the cell. Immune cells rely on the activation and collaboration of multiple different receptors in the plasma membrane that are central to leukocyte function, including antigen recognition and presentation, cell adhesion, and cytokine production. A contemporary goal of researchers has been to uncover how immune cells physically organize and compartmentalize receptors and signaling molecules into efficient, regulated membrane-proximal signaling complexes. Whereas many studies have been performed on investigating the plasma membrane of T lymphocytes, this review focuses on new insights into the immunological relevance of microdomains in the membrane of APCs.

In 1972, Singer and Nicolson [1] presented their classical view of membrane structures, in which monomeric and amphiphilic membrane proteins diffuse in a two-dimensional fluid lipid bilayer. The identification of specialized membrane domains, including lipid rafts, TEMs, and caveolae, has been a major breakthrough in cell biology and has changed the classical fluid mosaic model. Although different in size and composition, we define them as membrane microdomains throughout this review. Lipid rafts are microdomains with an estimated size of 10–200 nm, present in the plasma membrane of all eukaryotic cells [2]. The outer leaflet of lipid rafts consists of cholesterol that binds to glycosphingolipids and promotes formation of a liquid-ordered phase within the disordered glycerophospholipid bilayer of the plasma membrane (**Fig. 1**). The inner leaflet of lipid rafts is composed of saturated phospholipids [3, 4]. Inside of the cell, the concentration of cholesterol is increased from the ER to the Golgi, which may underlie the important role of cholesterol in the trafficking of transmembrane proteins to the plasma membrane [5]. Typical raft constituents within the outer leaflet are GPI-anchored proteins [6], which partition to the lipid rafts in virtue of their glycolipid anchor. Different models have been proposed that underlie plasma membrane compartmentalization. Lingwood and Simons [7] refer to lipid rafts as a membrane-organizing principle, in which rafts allow lateral segregation of proteins in the plasma membrane and recruitment of signaling molecules

Abbreviations: CLL=chronic lymphocytic leukemia, CLR=C-type lectin receptor, DC-SIGN=DC-specific ICAM-3-grabbing nonintegrin, DRM=detergent-resistant membrane, FLIM=fluorescence lifetime microscopy, FRET=Förster resonance energy transfer, GM1=ganglioside monosialic acid antibody, HCV=hepatitis C virus, MCD=methyl-β-cyclodextrin, MIIC=MHC II-enriched compartment, NSOM=near-field scanning optical microscopy, NWO=Netherlands Organisation for Scientific Research, SHP1=Src homology-2-containing tyrosine phosphatase 1, SMIP=small modular immunopharmaceutical, SPT=single-particle tracking, STED=stimulated emission depletion, TEM=tetraspanin-enriched microdomain

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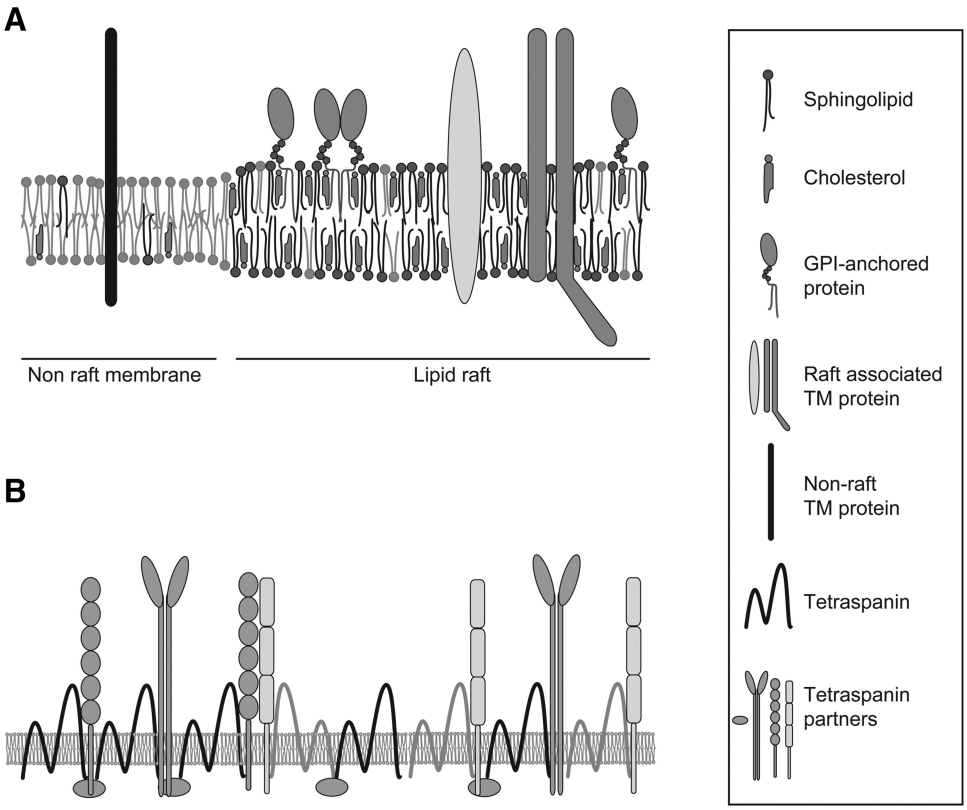

Figure 1. Schematic representation of a lipid raft and a TEM. (A) Lipid raft. Lipid rafts are enriched in sphingolipids, cholesterol, and GPI-anchored proteins. Certain transmembrane (TM) proteins are specifically associating with lipid rafts, whereas other proteins are excluded. (B) TEM. Tetraspanin proteins specifically recruit one or more partner molecules, including (immuno-)receptors and signaling molecules, whereby they induce the formation of multimolecular complexes in the membrane. These protein–protein interactions can be direct or indirect (via other members of the tetraspanin family), forming so-called TEMs or the "tetraspanin web" (see text for details). Molecules depicted in this schematic representation of membrane microdomains are not drawn to scale.

to compartmentalize cellular events, including signal transduction, membrane traffic, and endocytosis. Upon cross-linking of raft-associated receptors, two or more rafts may coalesce into larger domains, where more sustained signaling can occur, and anchoring to the actin cytoskeleton can take place (reviewed in ref. [7]). Kusumi et al. [8] propose a major role for the actin cytoskeleton in their "picket-fence" model that is described by a hierarchical, three-tiered, mesoscale-domain architecture, present in the plasma membrane. Mesoscale refers to a size greater than a nanometer and smaller than a micron. The first hierarchy includes membrane compartments of 40–300 nm, formed by a fence of an actin-based membrane cytoskeleton and pickets of transmembrane proteins anchored to this fence. The second tier includes raft domains of 2–20 nm, consisting of cholesterol, glycosphingolipids, and GPI-anchored proteins. The third tier includes dynamic protein complexes of 3–10 nm, containing membrane-associated and integral membrane proteins. In this picket-fence model, transmembrane proteins and phospholipids can undergo hop diffusion among membrane compartments, whereas they can move freely within a compartment, emphasizing the important role of the actin cytoskeleton [8] versus the more classical model of lipid-raft compartmentalization described by Simons and Sampaio [5]. The nanoscale size of rafts is also consistent with the "lipid shell" model, which proposes that each protein is surrounded by a ring of laterally organized lipids [9].

Sixty years ago, small (60–80 nm) microdomains, called caveolae, present on specific cell types, were first identified using electron microscopy [10, 11]. Caveolae are cholesterol-enriched membrane invaginations consisting of caveolin proteins and cavin proteins. These proteins interact with each other to regulate signal transduction, endocytosis, and transport of free cholesterol (reviewed in refs. [12, 13]). Like lipid rafts, caveolae can cluster together and form extensive networks [14]. In the immune system, they are found in certain myeloid [15] and lymphoid [13] cell lineages, depending on the activation and maturation state of the cell. Studies on murine macrophages revealed that caveolae structural proteins are important regulators of macrophage number and phenotype in the lung [16], and caveolin proteins may promote differentiation of monocytes into macrophages [17]. The role of caveolae in other APCs is not well established and will therefore not be the focus in the remainder of this review.

During the 1990s, a novel type of membrane microdomain was identified: the TEM [18]. Tetraspanins are a family of four transmembrane proteins present on the plasma membrane and on intracellular vesicles of virtually all mammalian cell types. Their structure is characterized by four transmembrane domains: a small and a large extracellular loop, and two short cytoplasmic tails [19]. The size of a TEM varies among cell types, and diameter sizes between 100 and 300 nm have been reported (**Table 1**). The assembly of TEMs is dependent on tetraspanin–tetraspanin interactions and tetraspanin interactions with transmembrane receptors, enzymes, adhesion molecules (integrins and others), and signaling molecules (reviewed in refs. [23, 24]). These interactions can be divided into three levels [21, 25]. Level 1 refers to very robust and direct interactions that are stable in strong detergents (i.e., Tri-

TABLE 1. Characteristics of TEMs and Lipid Rafts

	TEM	Lipid raft
Estimated diameter size	100–300 nm [20]	10–200 nm [2]
Content/characterization	Enriched in tetraspanins, tetraspanin-partner proteins, cholesterol	Enriched in cholesterol, sphingomyelin, lipids with saturated acyl chains, raftophilic proteins (GPI-anchored, caveolin, other)
Biochemical features	Partition into low-density fractions of sucrose gradients; resistant to mild detergents (Brij 97) at 37°C[a]; partially dependent on cholesterol[b]	Partition into low-density fractions of sucrose gradients; resistant to nonionic detergents (Tx-100) at 4°C; dependent on cholesterol[b]
Signaling molecules	PKC, PI4K, phosphatases, Rac, other	Src kinases, H-Ras, PI3K/Akt, BCR- and TCR-associated signaling effectors

Although TEMs and conventional lipid rafts display some similarities (both enriched in cholesterol and partition into low-density fractions of sucrose gradients), there are a number of critical differences that distinguish these two microdomains. [a]Tetraspanin–protein interactions have been classified into three categories, based on their stringency in different detergents (see text for more details). This concept is also relevant for lipid rafts, as the protein content of DRMs differs, depending on the detergent used. [b]Although direct interactions between tetraspanins and their partners are resistant to cholesterol depletion [21], signal transduction downstream of TEMs is affected by MCD treatment [22].

ton X-100). Level 2 interactions are less robust and are disrupted by strong detergents but are stable in hydrophobic detergents (i.e., Brij 96 or Brij 97). Level 3 consists of weaker, indirect interactions, only detected in mild, less hydrophobic detergents {i.e., Brij 99 or 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate}. These different interaction levels reveal the concept of a tetraspanin web, in which tetraspanins are organizers of multimolecular complexes on the plasma membrane. Each tetraspanin molecule recruits specifically one or more partner molecules, following interaction with another tetraspanin molecule to form larger complexes [25] (Fig. 1). The composition of TEMs is highly dependent on the specific cell type studied, and it has been shown that different TEMs exist within one particular cell type [20]. In the Golgi complex, tetraspanin proteins can be palmitoylated, which allows dynamic homo- or heterodimerization with another tetraspanin molecule or with associated proteins in the plasma membrane. Palmitoylation is the covalent attachment of fatty acids, such as palmitic acid, to cysteine residues of membrane proteins, which promotes protein–protein interactions in the lipid environment. This post-translational modification was shown to be important for the assembly of TEMs, as described previously for tetraspanins CD9 [26] and CD151 [27]. These protein–protein interactions in the plasma membrane have an important role in intercellular (adhesion, migration, synapse formation) and intracellular (organizers of membrane-signaling complexes) interactions, as well as intracellular protein transport and endo- and exocytosis. TEMs are not static entities; instead, there is now prevailing evidence from studies in living cells that their localization and composition are dynamic [26, 28]. There is constant diffusion of individual tetraspanins and partner proteins among specific microdomains, which may induce clustering of two or more TEMs to enhance the strength of a response. Furthermore, the diffusion dynamics of the different molecules present in the microdomain can be influenced by ligation of a receptor within the TEM.

Despite the different biochemical principles regulating rafts and TEMs, these microdomains are dynamic in time and space, and their molecular constituents may exchange between different types of microdomains [21, 26, 29]. In the remainder of this review, we will focus on the characterization and function of lipid rafts and TEMs in APCs.

APPROACHES TO CHARACTERIZE MICRODOMAINS

The formation of membrane microdomains depends on lipid–lipid, lipid–protein, and protein–protein interactions, indicating the existence of a variety of biochemical principles that allows these interactions to occur at the molecular level. Strong interactions between cholesterol and sphingolipids promote their cosegregation in raft domains, which in turn, can sequester specific signaling proteins, allowing the formation of large signaling complexes. In the past, the nanoscale size and dynamic nature of lipid rafts have posed technical challenges for their identification and the study of their composition. A solution to this problem came with the discovery that segregation of certain proteins into lipid rafts was determined by their capacity to reside in a specific fraction of membrane-derived material that appeared to resist detergent treatments [30, 31]. These so-called DRMs were isolated initially after cell lysis in the presence of the detergent Triton X-100 and subsequent ultracentrifugation on sucrose gradient, where the floating fraction was found to contain DRMs (for a detailed description of the most widely used protocols, see ref. [32]). Another frequently used technique to study lipid rafts is MCD treatment, which depletes cholesterol, an essential constituent of lipid rafts, from the plasma membrane.

It should be noted that DRM extraction has been used primarily to determine the association of membrane proteins with lipid rafts. In fact, although localization of TEMs in DRMs has been documented [33, 34], seminal work from the Hemler laboratory [21, 35] has demonstrated that TEMs are discrete units that are distinct from lipid rafts but can occasionally interact with them (Table 1). In addition, proteomics approaches have demonstrated that the composition of TEMs is specifically distinct from that of lipid rafts, as different sets of proteins were detected in lipid rafts and TEMs (reviewed in

ref. [36]). Part of the controversy was caused by the sensitivity of DRMs and TEMs to cholesterol depletion by MCD, although for the integrity of TEMs, the effects of cholesterol depletion were reported to be milder [22]. MCD has been widely—and sometimes without including basic controls of cell viability or residual cholesterol levels—used to determine the association of membrane proteins with lipid rafts. Lack of receptor functionality upon MCD treatment has often been attributed to impaired colocalization with lipid rafts. However, one should consider that MCD has been shown to exert pleiotropic effects, which include destruction of clathrin-coated pits [37, 38] and rearrangement of the cytoskeleton [29, 39].

Despite the slightly artifactual nature of DRMs and MCD treatment, one should acknowledge that these approaches have been instrumental to the original identification of the potential raftophilic/raftophobic nature of a large variety of proteins. However, it is increasingly recognized that protein–protein and protein–lipid interactions that mediate the formation of specific membrane compartments or scaffolds are often transient and highly dynamic. Therefore, although DRMs give a broad view of domain composition, they represent a bulk snapshot of a specific situation and cannot provide information on the spatiotemporal variations of the membrane domains. Moreover, whereas the use of the detergent Triton has been shown to induce formation of DRMs [40], different detergents have a different ability to solubilize membrane proteins selectively or enrich glycosphingolipids and cholesterol [41], emphasizing the notion that DRMs might not be fully representative of lipid rafts. Fast developments in fluorescence microscopy techniques have now made it possible to measure protein aggregation state, dynamics, and interactions in living cells, facilitating in situ measurements of biochemical parameters and revealing novel aspects of membrane microdomain organization and function.

Although a detailed overview of all of the state-of-the-art microscopy techniques that can image membrane domains falls outside of the scope of this review, it is worth highlighting the increasing application of novel imaging techniques that are perfectly suited to visualize the nanoscale organization of the plasma membrane, including lipid rafts and TEMs. Images of membrane domains were obtained initially by using immunogold labeling and transmission electron microcopy on membrane sheets or intact cells [42–45]. With the advent of super-resolution microscopy techniques in the past decade, techniques, such as NSOM, STED, and localization microscopy, were shown to be capable of directly mapping out the nanoscale landscape of the cell surface [46–48] (**Fig. 2**). Nanoscale proximity of raft components and transmembrane adhesion receptors has been revealed by NSOM imaging of the cell surface of human monocytes [49, 50], whereas pioneering measurements of STED, combined with fluorescence correlation spectroscopy, have been able to determine fast diffusion of phospholipids and sphingolipids in living cells [51, 52]. More recently, photoactivated localization microscopy, in combina-

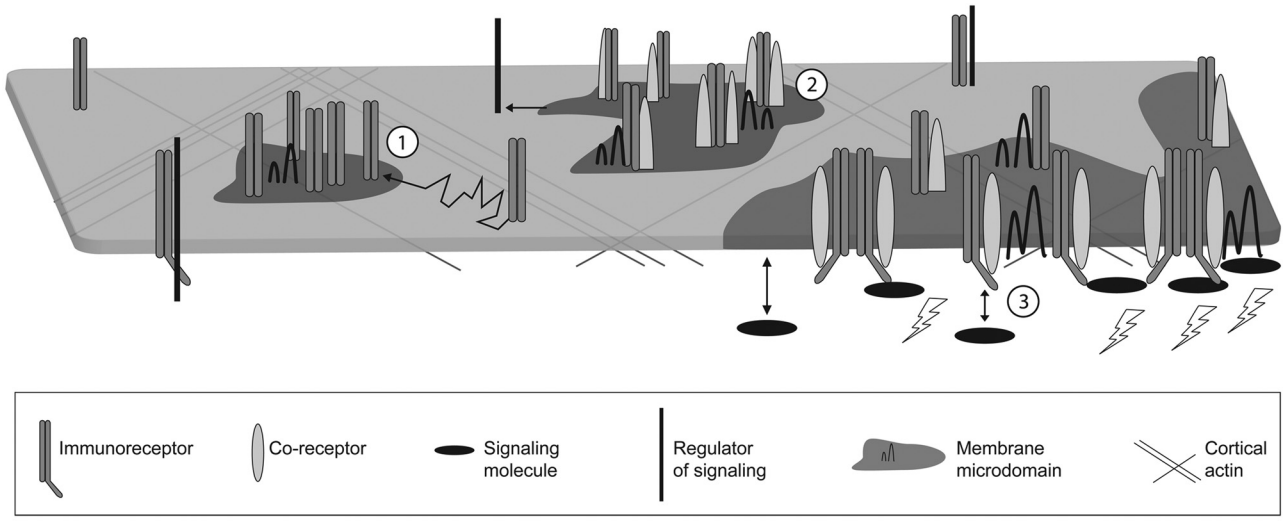

Figure 2. Model illustrating microdomains in the plasma membrane that can facilitate immunoreceptor clustering, cross-talk, and immune cell signaling. (1) Clustering. Immunoreceptors can be clustered within membrane microdomains, leading to an increase of receptor avidity. (2) Cross-talk. Membrane microdomains provide a local environment that facilitates cross-talk between different immunoreceptors and their coreceptors, resulting in enhancement (or dampening) of their function. In addition, regulators of signaling can be excluded. (3) Signaling. Signaling molecules are recruited specifically toward membrane domains, where stable signaling complexes are created. It should be noted that the plasma membrane is much more crowded with proteins than depicted in this model, adding an extra level of complexity. An example of visualization of membrane microdomains is given in the lower-left corner: confocal and super-resolution microscopy (STED) of tetraspanin microdomains in the plasma membrane of human B cells, demonstrating the need for advanced imaging techniques to investigate single-membrane microdomains and their constituents. The diameter size of a TEM in B cells is estimated to be ~100 nm. Original scale bars, 2 μm.

tion with pair-correlation analysis, has been shown to be a promising tool to analyze complex organization patterns of membrane proteins, enabling quantification of protein cluster size, density, and abundance in the cell membrane [53].

Single-molecule imaging, such as FRET-FLIM and multicolor SPT, can provide a view of protein dynamic behavior at the molecular level [54]. Elegant FRET-FLIM studies from the Sanchez-Madrid group [28] have demonstrated the existence of specialized, preformed, tetraspanin-enriched adhesive platforms at the plasma membrane of endothelial cells containing VCAM-1 or ICAM-1, ligands for the leukocyte integrins VLA-4 ($\alpha4\beta1$) and LFA-1, as well as the tetraspanins CD151 and CD9, respectively. This study also shows that the spatial organization of membrane receptors in nonimmune cells affects immune cell function during cell–cell interactions, such as adhesion and transmigration. Despite the numerous interactions discovered between tetraspanins and immune receptors, only few similar studies have been performed on immune cells [55].

Whereas FRET-FLIM generally provides averaged values of ensemble measurements, SPT offers the opportunity to distinguish fractions of proteins exhibiting different dynamic behavior. An excellent example was provided by Jaqaman and colleagues [56], who used SPT to investigate the dynamics and signaling of the scavenger receptor CD36 at the plasma membrane of macrophages that was controlled by the cortical cytoskeleton. Recent work from our laboratory also demonstrated the power of SPT to study immune receptor nanoscale organization and function. On monocyte-derived DCs, we followed single molecules of the PRR DC-SIGN and demonstrated a direct relationship between spatial nanopatterning and lateral diffusion to provide DC-SIGN with the exquisite ability to bind many different viruses [57]. On the cell surface of human monocytes, where the integrin LFA-1 is organized in nanometer-sized domains [45, 49], we unraveled the intricate coupling between conformation and lateral diffusion of LFA-1 microdomains. Domain mobility was crucial for the formation of stable and large clusters that initiate LFA-1-mediated leukocyte adhesion [58]. In addition, these novel imaging techniques have now provided compelling evidence that tetraspanins regulate the mobility of integrin molecules in the plasma membrane. The physical association of the laminin-binding $\alpha6$ integrin with tetraspanin CD151 was demonstrated to promote random-confined diffusion of the integrin in mammary cells, which may regulate its interaction with the cytoskeletal protein talin [59]. In B lymphocytes, we have shown that the tetraspanin CD37 is required for the mobility and clustering of $\alpha4\beta1$ integrin molecules in the plasma membrane, which is important for plasma cell survival and antibody production [60]. These studies provide novel, mechanistic insights into the contribution of TEMs to integrin lateral motility and adhesive properties. Still, studies addressing the mechanisms underlying TEM dynamics and interactions in the plasma membrane are scarce, in particular, in immune cells. The elegant study on the mobility of single molecules of tetraspanin CD9 in prostate carcinoma cells revealed that CD9 mostly exhibits Brownian diffusion at the plasma membrane but is transiently confined to platforms that are enriched in CD9, and its interaction partners [26] again highlight the importance of investigating TEMs in living cells. Although no single technique is able to unravel the concept of membrane receptor compartmentalization and function, we believe that the best approach toward understanding the principles of membrane microdomains is to compare and contrast results obtained with the existent methods, as well as exploring new techniques as they are developed and improved. The advantages and limitations of the discussed microscopy techniques that are able to image all types of microdomains are reviewed nicely elsewhere [61]. A major current challenge in the field is to increase insight into the molecular mechanisms underlying the regulation of membrane-proximal signaling by membrane microdomains in immune cells.

SIGNAL TRANSDUCTION REGULATED BY PLASMA MEMBRANE MICRODOMAINS

It is well-established that signaling transduction efficiency is dependent on the selective concentration of signaling proteins into discrete clusters in the plasma membrane (reviewed in refs. [62, 63]), including their recruitment into specialized microdomains, such as rafts and TEMs [64, 65]. Whereas some proteins constitutively reside in rafts, like most Src family kinases, other proteins become only transiently associated with rafts, for example, upon activation. Similarly, PKC recruitment to TEMs and subsequent activation are inducible processes that depend on integrin activation [66]. Thus, microdomains can serve as "scaffolds" to localize and transiently concentrate specific signaling molecules [67]. The time that a protein resides within a microdomain can be altered upon oligomerization of the protein after, for example, multivalent ligand binding. In addition, activated receptors can induce coalescence of rafts with a different composition, resulting in large and stable structures on the plasma membrane [64, 68], again underscoring the dynamic nature of the signaling capacity of membrane microdomains.

Within the microdomain environment, receptors can be modified by local kinases, leading to further downstream signaling. Indeed proteomics data demonstrate an enrichment of specific signaling molecules in raft fractions compared with total membrane fractions [69] (Table 1). Although the exact mechanisms underlying this enrichment need to be identified, it is apparent that special domains or motifs in the molecular structure of signaling proteins determine their localization to the plasma membrane [62]. This concept is illustrated nicely by members of the ubiquitously expressed proteins of the Ras family of GTPases. The importance of Ras for APC biology is illustrated by R-Ras-deficient murine DCs that show impaired maturation and T lymphocyte priming, which are linked to defective LPS signaling [70]. H-Ras and K-Ras are highly homologous proteins; still, they generate very distinct signaling outputs as a result of their different lipid anchors interacting with different regions of the plasma membrane. H-Ras, which is palmitoylated, has transient interactions with lipid rafts, whereas K-Ras clusters in nonraft areas of the plasma membrane [42]. Palmitoylation, by itself, is not a prerequisite for raft localization, as this modification has also been shown essential for tetraspanin–tetraspanin interactions, the stability of

TEMs, and its signaling capacity [71, 72]. Several tetraspanins have been reported to interact with G protein subunits, PI4K, and activated PKC isoforms in many different cell types, including APCs (reviewed in refs. [24, 65, 73]). Whether these signaling proteins interact directly to tetraspanins is unclear, but the intracellular or the transmembrane regions of tetraspanins have been implicated in their interaction with PKC [66, 74, 75]. Interestingly, some tetraspanins contain a PDZ domain binding motif in their C-terminal tail, which facilitates the anchoring of transmembrane proteins to the cytoskeleton, and hold together signaling complexes, thus providing a direct way to sequester components of a signaling cascade to TEMs [24, 76]. Furthermore, several studies have demonstrated that TEMs are implicated in regulation of the JNK and Akt signaling pathways [60, 77–79]. In B cells, we have recently shown the requirement of tetraspanin CD37 for proper $\alpha 4\beta 1$ integrin mobility and clustering, which is essential for Akt signaling and survival of IgG1-producing plasma cells [60]. Many studies support the concept that tetraspanins act as linker molecules to recruit kinases in the proximity of integrins [66, 72, 80]. Strikingly, it was shown that tetraspanin CD37 is able to signal by itself, as it contains predicted ITAM- and ITIM-like motifs in its two intracellular tails [81]. Antibody cross-linking of CD37 on B cells resulted in recruitment of Lyn and SHP1 via its ITIM-like domain, leading to cell death, whereas its ITAM-like domain recruited PI3K and Akt, leading to cell survival. Thus, upon antibody ligation of CD37, two opposing stimuli act simultaneously on the Akt signaling pathway, although the involvement of FcRs cannot be excluded completely in this study. Different groups have demonstrated that TEMs can also inhibit signal transduction pathways by the recruitment of phosphatases [82, 83] or alternatively, by the sequestering of kinases away from their site of action [84, 85]. Recently, the C-terminal cytoplasmic domain of tetraspanin CD81 was shown to interact directly with the GTPase Rac and to inhibit Rac activation [86].

Taken together, microdomains are implicated in enhancing or dampening signaling from immune receptors by serving as platforms for the dynamic assembly of signaling complexes. Considering the complexity of TEMs and lipid rafts, it not surprising that they regulate many different signaling pathways by their capacity not only to include but also to exclude proteins from microdomains. For instance, phosphatase CD45, a negative regulator of receptor signaling, is well-known to be excluded from lipid rafts [87]. Another example is the interaction of tetraspanins (CD81, CD82) with CD4 and CD8 coreceptors that can sequester these proteins away from the TCR signaling complex in the plasma membrane [84]. Next to regulating the recruitment of signaling molecules and activation of immune receptors on the plasma membrane, microdomains can also promote cross-talk among different receptors (Fig. 2). These fascinating processes will be discussed below, taking PRRs, MHC class II, and the BCR as classical examples of immune receptors regulated by membrane microdomains during the course of an immune response.

PRRs

APCs are equipped with a broad panel of specific PRRs that are essential for the recognition and uptake of pathogens. APCs bind pathogens by means of complex multimolecular interactions among different PRRs and a wide variety of microbial structures called PAMPs. Efficient pathogen recognition and uptake are dependent on a high level of organization of PRRs in the plasma membrane of APCs. There is now increasing evidence that specialized microdomains can serve as organizing platforms that facilitate PRR multimerization and clustering, cross-talk between different PRRs, and integration of downstream signal transduction pathways. The two main PRR subclasses that are expressed on the plasma membrane of APCs include CLRs and TLRs.

Receptor multimerization is a well-defined mechanism that increases receptor avidity and ligand engagement. A classical example is the CLR DC-SIGN, which exists as tetramers in the plasma membrane and is clustered into microdomains during DC differentiation [44, 88]. A direct structural relationship between tetramer stability and nanoclustering formation, sustained by the neck region of DC-SIGN, has been reported recently [57]. This provides DC-SIGN with the ability to bind nanoscale pathogens (viruses) of different sizes, highlighting a physiological role for nanoclustering. Another CLR, Dectin-1, has been found to cluster in a "phagocytic synapse" (a structure reminiscent of the immunological synapse formed between APCs and T cells) in myeloid cells, which is crucial for triggering phagocytosis and full antifungal activity [89]. Different studies have shown that Dectin-1 interacts with tetraspanin proteins (CD63 and CD37) on the cell surface of APCs, which may facilitate Dectin-1 clustering [90, 91]. CD37 deficiency leads to impaired stabilization of Dectin-1 molecules in the membrane of APCs as a result of increased internalization. The Dectin-1-CD37 interaction also has functional consequences, as Dectin-1-mediated IL-6 production by APCs is inhibited by CD37.

TLRs have also been reported to dimerize into homodimers and/or heterodimers, which facilitates recruitment of intracellular signaling adapters and kinases. Dimer formation of TLRs occurs at the extracellular leucin-rich repeat domain and the cytosolic Toll/IL-1R domain. TLR localization into microdomains may induce receptor clustering further. TLR2 and TLR4-CD14 recruitment to lipid rafts has been described [92–94], although other studies documented TLR4 association with tetraspanin microdomains in macrophages [95]. It is not unlikely that different pools of TLRs exist in the membrane that are localized to different microdomains. Regulation of these receptor pools may be linked to ligand-specific cellular responses and/or depend on the activation status of the cell. This hypothesis may be resolved by investigating TLR clustering and localization in living APCs using high-resolution microscopy (such as multicolor SPT). Recently, the scavenger receptor CD36 has been found in multimolecular complexes composed of tetraspanin CD9, CD81, integrins, and Syk kinase [96]. Although not a classical PRR, CD36 can recognize *Plasmodium falciparum* and bacterial diacylglycerides. Similarly, the scavenger receptor CD5 that recognizes β-glucan in the fungal cell wall was reported to interact with the tetraspanin CD9, although functional implications of this interaction have not been reported [97]. Thus, assembly of PRRs in membrane microdomains may underlie the mechanism of receptor multim-

erization (reviewed in refs. [98–100]). It is important to note that besides the presence of PRRs in preassembled clusters, pathogen binding itself will induce PRR clustering further, possibly altering PRR-induced signaling intensity and duration.

Receptor cross-talk refers to the collaborative induction of a response by different receptors that is distinct from the response elicited by individual receptors. Microdomains are believed to provide a platform for efficient receptor cross-talk in the plasma membrane. For example, mutual signaling from TLR2 and Dectin-1 is documented to be required for proinflammatory cytokine production by APCs upon β-glucan binding [101]. Besides synergistic responses, the outcome of PRR cross-talk may also dampen unwarranted immune responses when receptors act antagonistically. For example, there is now evidence of existing inhibitory interactions between different TLRs in the membrane of human PBMCs [102]. A consequence of such receptor cross-talk is the integration of different signal transduction pathways that eventually determine the outcome of the immune response aimed to eradicate the pathogen (reviewed in ref. [100]). However, less well-known is the existence of cross-talk among different signaling molecules, already at the level of the plasma membrane [62]. In this context, signaling molecules that contain specific protein or lipid-binding specificity can dock at the plasma membrane, which often coincides directly with their activation. For example, the kinase Syk gets recruited to the phosphorylated ITAM-like motif in the intracellular tail of Dectin-1 upon yeast binding [103]. Although the precise role of lipid rafts and TEMs in PRR cross-talk needs to be established, evidence is accumulating that membrane microdomains are important for the formation of multimolecular signaling complexes by providing a local environment that allows for efficient physical interactions between signaling molecules and PRRs [98].

MHC CLASS II

APCs use MHC class II molecules to present nonself peptides to $CD4^+$ T cells. As the abundance of relevant MHC class II-peptide complexes is rather low, it is necessary to organize and cluster MHC on the APC surface for efficient antigen-specific T cell activation. Early crystallography studies of MHC class II molecules revealed a structure described as a dimer of heterodimers [104] that supported a model, in which superdimers of MHC II molecules may facilitate the activation of T cells by cross-linking TCRs [105]. However, this could not explain how sufficient numbers of agonistic MHC-peptide complexes from all over the cell surface could be concentrated in the center of a synapse to activate T cells. It is now widely accepted that MHC molecules cluster in the plasma membrane of APCs [106]. MHC class II-peptide complexes in mature DCs are collectively transported to the plasma membrane, where they remain preclustered at the cell surface [107, 108]. This has led to the concept that MHC-peptide complexes cluster into specialized microdomains in APCs prior to the formation of the immunological synapse [109]. This concept has been supported by different studies that provide evidence for a functional association of MHC class II molecules with lipid rafts. The disruption of raft integrity on B cells indeed results in inhibition of MHC class II antigen presentation at low antigen concentration. As the surface expression of MHC class II and the peptide binding to MHC class II were unaltered after raft disruption, a model was postulated in which lipid rafts locally concentrate MHC class II-peptide complexes, crucial for efficient antigen presentation at low doses of antigen [110]. However, there is also compelling evidence that MHC class II molecules interact with tetraspanins (CD9, CD63, CD37, CD53, CD81, CD82), demonstrated by immunoprecipitation studies [18, 111], FRET microscopy [55], and electron microscopy [112, 113]. These studies revealed that tetraspanins are enriched on the internal membranes of MIICs in B cells, where they can interact with MHC class II, HLA-DM, and HLA-DO [112, 113]. In immature DCs however, CD63 was found exclusively in MIICs, whereas CD9, CD53, and CD81 mainly interact with MHC class II at the plasma membrane [114], indicating that individual tetraspanins can interact selectively with MHC class II in different compartments. Selective enrichment of tetraspanin molecules in the central supramolecular activation complex, formed between APCs and T cells, has also been demonstrated [115].

In addition, coclustering of murine MHC class II molecules I-A and I-E has been reported to depend on TEMs. In CD9-deficient DCs, MHC I-E failed to cocap with I-A molecules, and exogenous expression of CD9 in CD9-negative B cells enhanced I-A/I-E interaction [116]. This model was challenged in a study in which cholesterol depletion, but not absence of CD9, did affect I-A/I-E interaction [117]. This controversy may be explained by sensitivity of DRMs and TEMs to cholesterol depletion by MCD, favoring a model where both microdomains are involved in MHC clustering. The mobility of MHC class II molecules in the plasma membrane is also dependent on cholesterol [118]. Furthermore, single-molecule tracking revealed that MHC class II molecules undergo hop diffusion among membrane compartments [119], providing a possible explanation for the molecular exchange among microdomains.

Interestingly, some studies reported that tetraspanins associate predominately with peptide-loaded MHC class II complexes [114, 120]. Tetraspanins were found in complexes with multimerized MHC class II molecules, as shown by immunoprecipitations with the antibody CDw78 [121]. These domains were enriched with the CD86 costimulatory molecule and the peptide editor HLA-DM and carried a restricted peptide repertoire. In contrast, the HLA-DR molecules, found in rafts, displayed a highly diverse peptide repertoire [120]. However, these studies must be interpreted with caution, as the specificity of the CDw78 antibody has been questioned [122]. Silencing of tetraspanins CD9, CD63, and CD81 (but not CD82) resulted in increased MHC class II expression but did not affect MHC class II peptide loading [123]. Still, the importance of tetraspanins in antigen presentation and T cell activation has been validated by studies with APCs from tetraspanin-deficient mice. DCs lacking CD37 or CD151 are hyperstimulatory to T cells by different mechanisms. Whereas CD151 regulates costimulation, CD37 is implicated in inhibiting antigen presentation via MHC class I and class II molecules [124]. Thus, individual tetraspanins on APCs have specific functions that can

even be opposing; for example, tetraspanins CD9 and CD82 may promote MHC clustering, whereas others, such as CD37, may dysregulate MHC clustering at the plasma membrane. In addition, cooperation among tetraspanins has also been demonstrated; CD37xTssc6 double-knockout DCs were significantly more hyperstimulatory than DCs isolated from either single-knockout mouse [125]. Taken together, TEMs have been implicated in the assembly (clustering), stabilization, and/or trafficking of MHC class II-peptide complexes. The complexity of the composition of the tetraspanin web and their localization within the cell warrant further studies to clarify the precise function of TEMs in the MHC class II lifecycle.

BCR

The BCR complex is composed of a membrane-bound Ig, which mediates binding to antigen and the Igα/Igβ (CD79) heterodimer, which couples the receptor to downstream signaling pathways. Igα and Igβ contain an ITAM motif, which becomes phosphorylated by Src-family tyrosine kinases after receptor engagement. This allows for recruitment and activation of the cytosolic Syk/Zap70 family of protein tyrosine kinases, leading to downstream signaling [126]. In resting B cells, BCRs are located into nonraft areas of the plasma cell membrane. Upon antigen binding, BCR molecules oligomerize and translocate rapidly into lipid rafts, where BCR signaling is facilitated. This was not only determined using biochemical methods [127] but was also visualized in living cells by fluorescent microscopy [128, 129]. Partitioning into lipid rafts is intrinsic to the BCR itself and independent of BCR signaling or the actin cytoskeleton [130].

CD19 forms together with complement receptor CD21, tetraspanin CD81, and Leu-13—an essential coreceptor complex for the BCR. This coreceptor complex lowers the threshold for B cell activation, as activated CD19 recruits signaling molecules to the BCR [131]. Interestingly, BCR, which is coligated to the CD19/CD21 complex, has a longer retention time in lipid rafts, resulting in the formation of a stable signaling-active complex at the plasma membrane [132]. Subsequently, BCR internalization and targeting of the BCR to MHC class II peptide-loading compartments are required for antigen presentation. This process was found to depend on the association of phosphorylated clathrin with lipid rafts [133]. The BCR is able to continue signaling after being endocytosed, providing a means to activate different kinases at different subcellular locations [134]. The role of tetraspanin CD81 in the BCR coreceptor complex is critical, as CD81 is required for stabilizing mature CD19 on the plasma membrane of human and murine B cells [135–139]. Moreover, CD19, CD21, and the BCR fail to translocate into DRMs in CD81-deficient cells after coligation of CD21 and the BCR [33]. Palmitoylation of the cytoplasmic tail of CD81 is increased upon coligation of the BCR and CD19/CD21/CD81 complex, which is needed for the stabilization of protein complexes in lipid rafts [140] and TEMs. Thus, CD81 is critically involved in BCR localization to microdomains and thereby, promotes BCR signaling. The finding that CD81 can localize to TEMs and lipid rafts exemplifies again the dynamics and molecular exchange that occurs between different types of microdomains.

An additional means through which the BCR can be compartmentalized is via the cortical actin cytoskeleton, which acts as a barrier to BCR diffusion, compartmentalizing the BCR from activating coreceptors, including CD19, thereby preventing spontaneous BCR signaling in resting B cells. Disruption of the actin network is sufficient to induce robust signaling in B cells in the absence of BCR stimulation [141]. After B cell activation, however, the cortical actin cytoskeleton needs to be remodeled for BCR clusters and rafts to coalesce and to form stable signaling platforms [142, 143]. Ezrin-Radixin-Moesin proteins, which link the actin cytoskeleton with plasma membrane proteins, are transiently dephosphorylated upon BCR stimulation. This results in dissociation of the plasma membrane from the underlying actin cytoskeleton and a transient increase in BCR diffusion [142, 144]. Recently, Mattila and colleagues [145] elegantly showed that signaling through the BCR is regulated by combined action of the actin cytoskeleton and the CD81 tetraspanin web in primary B cells. With the use of super-resolution microscopy techniques and CD19- and CD81-deficient B cells, they deciphered the role of the tetraspanin network in BCR signaling induced by cytoskeleton disruption. In contrast to the release of the BCR, CD19 diffusion is not altered upon disruption of the cytoskeleton. Instead, CD19 is restrained by the tetraspanin network, shown by the significantly faster diffusion of CD19 in CD81-deficient cells. CD81 thus holds coreceptor CD19 in place to interact with mobile BCR nanoclusters released after cytoskeleton disruption. Whether BCR signaling is initiated in lipid rafts or whether initial signaling needs to occur before reorganization of the actin cytoskeleton allows the BCR microdomains to move into stable raft-signaling platforms still needs to be addressed. Taken together, these studies demonstrate that the interplay among different microdomains, including lipid rafts and TEMs, is essential for efficient signaling via the BCR. In the following section, the relevance of membrane microdomains in different diseases will be discussed together with the possibilities to target these structures as novel therapeutics.

IMPLICATIONS OF MICRODOMAINS IN IMMUNE-RELATED DISEASES

Prevailing evidence suggests that certain pathogens use rafts and tetraspanin microdomains to gain entry and to survive inside of the cell. Pathogens can use different strategies to take advantage of microdomains in the membrane. For example, bacteria, such as *Salmonella typhimurium* and *Legionella pneumophila*, exploit lipid rafts to create phagosomes that allow them to survive inside APCs (reviewed in ref. [146]). Several bacteria enter phagocytic cells through raft-associated proteins (such as CD55 and CD48) that prevent trafficking to lysosomes, thus avoiding degradation and antigen presentation. Although there is no evidence for the presence of PRRs in caveolar structures, a role for caveolae in pathogen uptake has been reported. For example, enrichment of GPI-anchored proteins (such as CD48) into caveolae has been documented that can bind bacterial antigens and thereby, facilitate pathogen

entry into host cells [147]. In addition, caveolin proteins have been reported to inhibit downstream signaling pathways of TLRs [148].

Also, certain viruses, including HCV and HIV-1, use rafts and tetraspanin microdomains for virus assembly and budding [20] (reviewed in refs. [149, 150]). HCV binds directly to the extracellular loop of tetraspanin CD81 to invade not only APCs but also lymphocytes and hepatocytes. The clinical relevance of the HCV–CD81 interaction was shown by the capacity of CD81-specific antibodies to mediate protection to HCV infection in vivo [151]. Similarly, blocking CD55 could prevent host-pathogen interactions [146]. In addition, cholesterol-sequestering agents that interfere with cholesterol biosynthesis (statins) may have therapeutic value for patients with infectious disease and sepsis [152]. Another novel approach is the use of recombinant soluble tetraspanin large extracellular domain proteins that may interfere with tetraspanin microdomain assembly [153], exemplified by their potency to inhibit CCR5-tropic HIV-1 infection of macrophages [154]. Taken together, collaboration among different PRRs increases the specificity of recognition, extends their signaling competence, and enables the host to respond to almost any type of infection. At the same time, certain pathogens have evolved mechanisms to take advantage of membrane microdomains for their own benefit. The challenge is to translate this knowledge into new therapeutic strategies for emerging infectious diseases.

The broad influence and the complex role of the tetraspanin web on cell function are reflected in the diversity of phenotypes observed in human tetraspanin deficiencies. Tetraspanin transmembrane 4 superfamily 2 is mutated in families with X-linked mental retardation [155], and mutations in tetraspanin-12 cause familial exudative vitreoretinopathy [156–158]. A mutation in CD151, leading to loss of the integrin-binding domain, causes defects in assembly of basement membranes in kidney and skin. These patients suffer from renal failure, pretibial epidermolysis bullosa, sensorineural deafness, and β-thalassemia minor [159]. In the immune system, tetraspanin proteins play a crucial role, as demonstrated by human tetraspanin deficiencies in CD53 and CD81. Lack of CD53 expression on neutrophils was found to be the cause of an immunedeficiency syndrome in a family that suffered from opportunistic infections and reactivation of chronic silent infections. Although the mechanism underlying this defect in immune cell function was not studied, a role for CD53 in the negative regulation of the immune response was postulated [160]. Furthermore, a patient with a gene defect in CD81 was reported with severe nephropathy and hypogammaglobulinemia. Absence of CD81 expression as a result of a homozygous splice-site mutation resulted in a complete lack of CD19 expression on B cells. Rescue experiments by transduction of WT CD81 into the patient B cells showed that the CD81-deficient B cells are able to produce CD19, but the maturation and subsequent membrane expression of CD19 are defected in CD81-negative B cells. As a consequence, BCR-mediated stimulation is affected, resulting in impaired antibody responses and memory B cell formation [139]. Taken together, defects in individual tetraspanins can result in a wide variety of complex phenotypes, and one could speculate that unidentified mutations in tetraspanin proteins could be the cause of unexplained human diseases.

TARGETED THERAPY OF MICRODOMAINS

In the last decade, evidence has accumulated that microdomains can be targets for novel treatment opportunities. For example, targeting components of lipid rafts as therapeutic intervention for cancer has been explored by inhibitors of the EGFR that is overexpressed in breast cancer [161]. In malignancies of the immune system, the B cell marker CD20 has been targeted by rituximab, which was shown to be effective against non-Hodgkin's lymphoma and CLL (reviewed in ref. [162]). In lymphoma B cell lines, it has been shown that upon binding of rituximab on the cell surface, CD20 molecules are redistributed to lipid rafts, resulting in raft stability and organization and subsequently, induction of apoptosis via Src kinase-dependent pathway [163, 164]. Rituximab also inhibits BCR signaling by preventing relocalization into lipid rafts [165]. Although the underlying mechanisms have not been investigated thoroughly, lipid raft constituents are being used to predict rituximab treatment affectivity. For example, the expression of the raft-associated GM1 differs among various primary B cell lymphomas, and low GM1 expression has been shown to lead to unresponsiveness of lymphoma patients to rituximab treatment [166].

Polyunsaturated fatty acids from fish oil can have a potential therapeutic effect for patients suffering from inflammatory or autoimmune diseases, such as colitis or asthma [167]. Although the exact underlying mechanisms remain to be defined, polyunsaturated fatty acids can enhance B cell function in vivo by increasing MHC class II expression and diminishing GM1 microdomain clustering [168, 169]. Lipids in microdomains can also serve as a therapeutic target, exemplified by mouse anticholesterol antibodies that are specific for clustered cholesterol on the cell surface. Binding of this antibody to B cell lymphoma cells in vitro has been reported to recruit MHC class II and CD80 molecules into microdomains, resulting in effective antigen presentation to T cells [170].

TEMs are already targeted by specific antibodies or soluble tetraspanin large extracellular domain proteins to interfere with viral and bacterial infections, as described above. Moreover, tetraspanins are currently under detailed investigation as new targets for therapy of hematopoietic malignancies. CD37 is highly expressed on B cells and is therefore a candidate therapeutic target for B cell malignancies, like CLL. Excitingly, CD37-specific, anti-CD37 SMIP has been shown to induce apoptosis and antibody-dependent cellular cytotoxicity in lymphoma/leukemia cells in vitro [171], and humanized anti-CD37 SMIP is currently in Phase I/II clinical trials for relapsed CLL and non-Hodgkin's lymphoma (http://www.
clinicaltrial.gov/). Anti-CD37 SMIP induces association of CD37 with phosphorylated signaling proteins (pSHP1, pLyn, and pSrc) within DRMs of CLL patient cells and thereby, regulates CLL cell death directly [81]. Recently, dual-ligand immunoliposomes have been tested as a new concept for targeted drug delivery and apoptosis in B-CLL cells [172]. These liposomal nanoconstructs express two types of antibody ligands with high affinity for B-CLL cells, such as CD19, CD20, and

CD37. The antigen-expression levels on the cell surface vary among patients, and this study showed that a combination of anti-CD37 with anti-CD20 or anti-CD19 led to higher binding and delivery efficiency of immunoliposomes than the single-antibody immunoliposomes. Taken together, these studies show the potential of targeting microdomains and provide a solid basis for further research and development of new therapies.

CONCLUDING REMARKS

It is well-established that the spatial organization of proteins and lipids in the plasma membrane into multimolecular complexes is fundamental for inter- and intracellular communication. In APCs, specialized membrane microdomains are emerging as critical players that regulate fundamental immunological processes, including pathogen recognition, antigen presentation, T cell activation, and antibody production. Although we are only at the beginning of understanding the biology of membrane microdomains in APCs, we anticipate that advanced microscopy techniques will provide comprehensive, new insights into the formation, dynamics, and signaling capacity of these highly specialized membrane structures. In particular, pioneering studies at the single-molecule level in living cells have revealed that the dynamic behavior of components of lipid rafts and TEMs is heterogeneous, ranging from rapid diffusion to confinement. This may provide cells with the capacity to regulate delicately the formation of immunoreceptor signaling complexes in the plasma membrane that are essential for APC function. Indeed, recent studies have shown that tetraspanin proteins regulate the mobility of their interacting immunoreceptors in the plasma membrane [59, 60]. As evidence is now accumulating that the development of different human diseases is dependent on early-stage, nanoscale changes of plasma membrane molecules, we envisage the exploitation of membrane microdomains as novel, therapeutic targets for treatment of infectious and malignant diseases.

AUTHORSHIP

M.Z., C.M.d.W., A.C., and A.B.v.S. designed and wrote the review. A.B.v.S. coordinated the manuscript.

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DISCLOSURES

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KEY WORDS:
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