Plasma Membrane DC-SIGN Clusters and Their Lateral Transport: Role in the Cellular Entry of Dengue Virus

Ken Jacobson, Laurie Betts, Ping Liu, Marc Ridilla, Aravinda de Silva, and Nancy L. Thompson

One of us (KJ) was profoundly influenced by his interactions with Professor Gregorio Weber who is rightfully the father of fluorescence in biochemistry and molecular and cell biology. Gregorio served as the outside reader on KJ's Ph.D. thesis that described the fluorescence polarization of perylene in lipid bilayer vesicles as a measure of membrane fluidity. KJ and his young family subsequently traveled to Urbana-Champaign in the summer of 1972 where he worked in the Weber laboratory at the University of Illinois interacting with the Professor and a number of people in the lab at that time, including two graduate students, Dave Jameson and Joe Lakowicz, and two postdoctoral fellows, Dick Spencer and George Mitchell. The summer in Urbana was also productive in that phase fluorimetry was used to measure the rotation of lysozyme bound to acidic phospholipid vesicles for the first time. For KJ and his family, it was a delightful experience, the benefits of which carry forward to today.

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Abstract DC-SIGN (a single-pass transmembrane protein and C-type lectin) is a major receptor for a variety of pathogens on human dendritic cells including dengue virus (DENV), which has become a global health threat. DENV binds to cell-surface DC-SIGN and the virus/receptor complexes migrate to clathrin-coated pits where the complexes are endocytosed; during subsequent processing, the viral genome is released for replication. DC-SIGN exists on cellular plasma membranes in nanoclusters that may themselves be clustered on longer length scales that appear as microdomains in wide-field and confocal fluorescence microscopy. We have investigated the dynamic structure of these clusters using fluorescence and super-resolution imaging in addition to large-scale single particle tracking. While clusters themselves can be laterally mobile there appears to be little mobility of DC-SIGN within clusters or exchange of DC-SIGN between the clusters and the surroundings. We end this account with some outstanding issues that remain to be addressed with respect to the composition and architecture of DC-SIGN domains and some highly unusual aspects of their lateral mobility on the cell surface that may accompany and perhaps facilitate DENV infection.

Keywords DC-SIGN • Dengue virus • Lateral mobility • Membrane nanodomains • Super-resolution microscopy • TIRF microscopy

Contents

1 Introduction

Immature dendritic cells (DC) express many antigen-capture receptors and these include those of the C-type lectin family. These receptors enable DCs to bind and internalize antigen efficiently [1, 2]. One such pathogen is the mosquito-borne dengue virus (DENV), which has been recognized as a global health threat because nearly ½ billion people may be infected per year. One fifth of these develop systemic infection and out of those, 500,000 experience life-threatening symptoms

[3, 4]. DENV is a small (~50 nm) flavivirus that is bounded by a lipid membrane packed with envelope glycoproteins, termed E-proteins.

A major receptor for DENV on DCs is the C-type lectin DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) [5, 6]. DC-SIGN is termed a pattern-recognition receptor for microbial surfaces [7] because it recognizes glycosylation patterns, specifically mannose or fucose containing structures, expressed by glycosylated components on the surfaces of numerous virions, bacteria, yeast, and parasite species. The binding of pathogens to DC-SIGN triggers diverse immune responses [8].

DC-SIGN is a single pass, 44 kDa (without glycosylation), type II transmembrane protein. Its extracellular region contains the carbohydrate recognition domain (CRD) which is connected to the transmembrane domain by a region containing up to seven and a half repeats of a 23 amino acid helical domain. The short cytoplasmic domain contains three internalization motifs. Tetramerization of DC-SIGN, facilitated by the tandem repeats in the extracellular region, greatly enhances DC-SIGN's binding affinity to high mannose carbohydrates when compared to that of monomeric CRDs [9]. Indeed, DC-SIGN forms multimers as shown by biochemical and biophysical assays [9–11]. The structural features of DC-SIGN and a conceptualization of its binding to multivalent, pathogenic ligands are shown in Fig. 1.

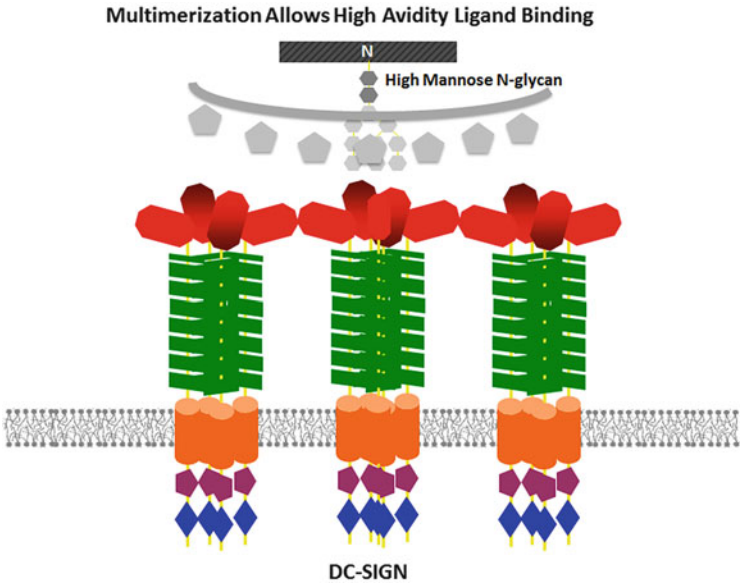

Fig. 1 Conceptualization of DC-SIGN interaction with pathogens that express mannose residues on their surface. Individual DC-SIGN proteins are depicted as clustered in tetramers in which the C-terminal portions of the ectodomains (*red*) contain the carbohydrate recognition domains (CRD) that are connected to the "neck" repeat sections (*green*). The transmembrane domains are colored *orange* and the short cytoplasmic domains containing the internalization motifs are colored in *mauve* and *blue*

Once DENV binds to cell-surface DC-SIGN clusters, the virus/receptor complexes migrate to clathrin-coated pits where the complexes are endocytosed [12]. Processing through the endosomal pathway leads to a low pH – induced fusion of the viral and endosomal membranes. This fusion event releases the viral genome into the cytoplasm for subsequent translation and replication.

DC-SIGN clusters exhibit a number of quite unusual properties in both their structure and mobility in the plasma membrane and this is the main subject of this review. These properties leave open a number of outstanding questions that are enumerated at the end.

2 Structure of DC-SIGN Clusters

Previous studies have shown that DC-SIGN on the surface of fixed DCs is organized in clusters on the nanometer scale; these studies employed transmission electron microscopy and near-field scanning optical microscopy [13, 14]. Clustering of DC-SIGN could improve binding to viral particles or bacteria by providing high-avidity platforms for these multivalent entities. Heterogeneity in the cluster size or nanoarchitecture could provide a variety of structurally different binding platforms tuned for recognizing different pathogen types.

2.1 Clusters Imaged at Light Microscope Resolution

DC-SIGN clusters imaged in wide-field or confocal microscopy show a variety of sizes ranging from the diffraction limit to over a micron in dimension (Fig. 2, left panel). Interestingly, similar sizes are seen when DC-SIGN is endogenously expressed in DCs or when it is expressed in a permanently transfected NIH 3 T3 cell line (termed MX DC-SIGN) (Fig. 2, right panel) [15].

We measured, employing total internal reflection fluorescence microscopy (TIRFM), the copy number occupancy of DC-SIGN domains [16]. Our approach was based on comparing the intensities of fluorescently labeled microdomains, in which DC-SIGN was labeled with primary monoclonal antibodies (mAbs) or expressed as GFP fusions in NIH 3 T3 cells, with those of single antibodies. In microdomains that range in dimension from the diffraction limit (slightly greater than 200 nm) to over 1 μm, the number of DC-SIGN molecules ranges from only a few to over 20 in both DCs and NIH 3 T3 cells. However, microdomains that appear at the diffraction limit typically contain only 4–8 molecules of DC-SIGN in either immature DCs or NIH 3 T3 cells. In fact, these small domains are capable of binding DENV leading to infection of host cells [12]).

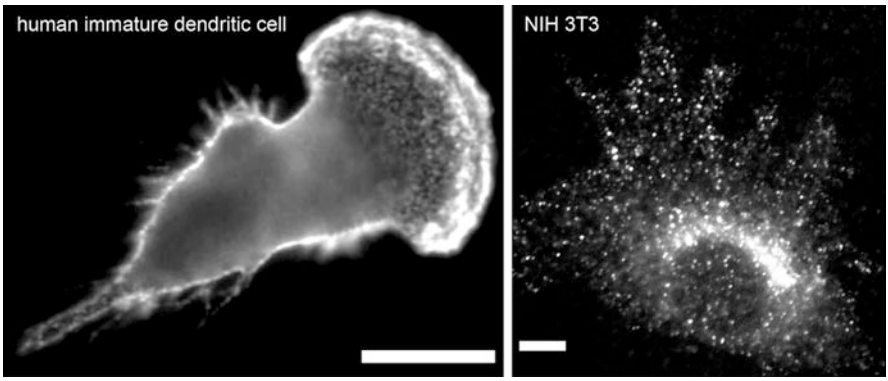

Fig. 2 DC-SIGN spontaneously forms microdomains when endogenously expressed in the plasma membranes of DCs (*left*) or permanently expressed in the plasma membranes of MX DC-SIGN cells (*right*). Indirect immunofluorescence on fixed cells. Bars = 10 μm. Reproduced by permission of the Company of Biologists

2.2 Clusters Imaged with Super-Resolution Microscopy

Larger DC-SIGN microdomains are remarkably stable [17], but the fact that inner leaflet lipid markers can diffuse through them (see below) suggests that they have an internal substructure rather than being densely packed with this C-type lectin (CTL). We therefore investigated the lateral distribution of DC-SIGN within microdomains by using a super-resolution imaging technique, Blink microscopy. Blink uses reducing/oxidizing buffers and a tuned excitation intensity to adjust the fluctuating emission of fluorophores on antibodies [18] so that only a few emit during a given image frame within the total movie acquisition time; thus, observed fluorophores are well separated in space (for single frames) so that their locations can be more precisely determined and a full map of fluorophore positions can be constructed from the movie. Blink is one type of molecular localization super-resolution microscopy.

Blink images of DC-SIGN in fixed DCs revealed a frequent presence of *several small nanodomains*, about 75 nm wide, which appeared as *single microdomains* by TIRFM (Fig. 3). Another CTL, CD206, and influenza hemagglutinin (HA) are similarly clustered in small (~80 nm diameter) nanodomains on the plasma membrane. Spatial analysis of nanodomain centroids from Blink images indicated that DC-SIGN and CD206 nanodomains are localized randomly on the plasma membrane, and two-color Blink imaging showed that these CTLs were largely restricted to separate nanodomains, despite their apparent co-localization by wide-field microscopy. By contrast, HA nanodomains are not randomly distributed, and clustered on length scales up to 1 μm.

We estimated that DC-SIGN nanodomains contain between one and three tetramers, as a lower limit, by comparing the number of Blink localizations from nanodomains and single antibodies (Fabs). Given the measured average

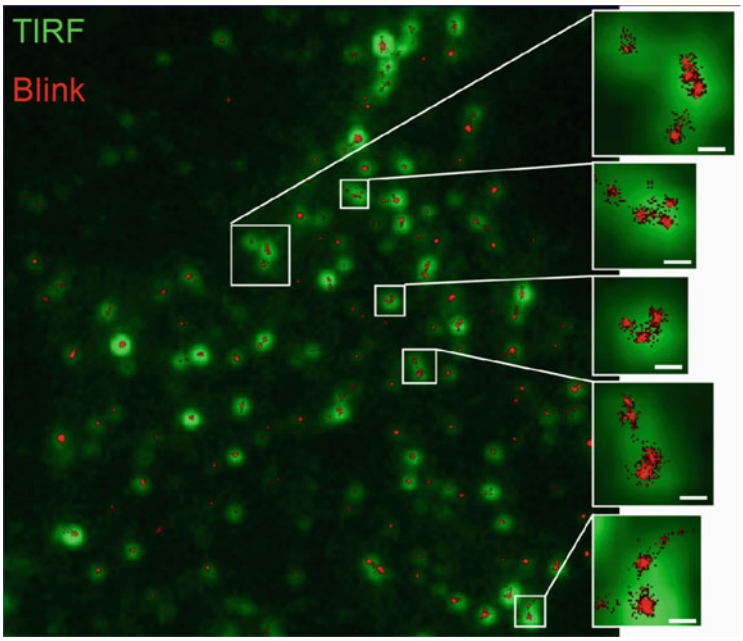

Fig. 3 Blink spatial localization super-resolution microscopy images of DC-SIGN expressing MX DC-SIGN cells. *Green* represents the TIRFM image while *red* represents the Blink image showing the centroids of DC-SIGN molecular localizations. *Insets* show magnifications of specific areas on the large image and show that more than one nanocluster is often contained within a microdomain as visualized in TIRFM images. (Bars = 100 nm)

nanodomain size and the known DC-SIGN size, the estimated DC-SIGN copy number occupancies strongly suggest that other proteins and lipids are present in nanodomains (see Figure 7 in [18], and below). Thus, the nanodomains themselves most likely possess an intricate underlying architecture.

2.3 Additional Structural Considerations

We undertook a mutational approach to investigate which domains/motifs of DC-SIGN might be responsible for clustering and how the microdomains form and remain stable [19]. Four mutants, expressed in NIH 3 T3 cells and either unlabeled or as GFP fusions, were generated for use with confocal imaging and fluorescence recovery after photobleaching (FRAP) studies to assess the existence, size, and stability of resultant microdomains. Deletion of the cytoplasmic portion had little effect on microdomain formation or stability, implying that DC-SIGN clustering is not mediated by a direct interaction with cytoskeletal structures. A point mutation preventing potential N-linked glycosylation at Asn80 also failed to reduce microdomain stability, thereby ruling out any significant contribution from

galectin–glycoprotein crosslinking in microdomain formation. By contrast, deletion of the seven and a half tandem repeats, which are thought to mediate tetramerization by forming coiled-coil α-helices, resulted in enhanced membrane diffusion and nearly complete recovery in FRAP measurements. A more profound effect – the complete loss of observable microdomains on the cell surface – was observed following removal of the CRD; the deletion mutants instead showed a diffuse and homogeneous distribution within the membrane and nearly full lateral mobility. This result suggests that the CRD might interact directly with components of the extracellular matrix or with transmembrane adaptor proteins to indirectly link to the cytoskeleton. A plausible possibility is that pathogens may compete with these putative stabilizing interactions to facilitate their attachment to DCs and more rapid movement to sites of internalization.

3 Lateral Mobility of Clusters and DC-SIGN Molecules Within Clusters

We initially found that most bright DC-SIGN clusters on DCs and NIH 3 T3 cells are apparently immobile when examined for relatively short times [15]. Moreover, FRAP measurements on these clusters revealed that little or no recovery occurred after many seconds, indicating that exchange of DC-SIGN molecules between the clusters and the surrounding membrane was minimal (Fig. 4, left panel). By contrast, recovery of a lipid probe, PM tracker, on the inner leaflet was substantial suggesting that at least some lipids could move through the DC-SIGN clusters as imaged at light microscope resolution (Fig. 4, right panel). Lateral mobility and partial or complete loss of DC-SIGN microdomains could be effected by certain mutations as described above.

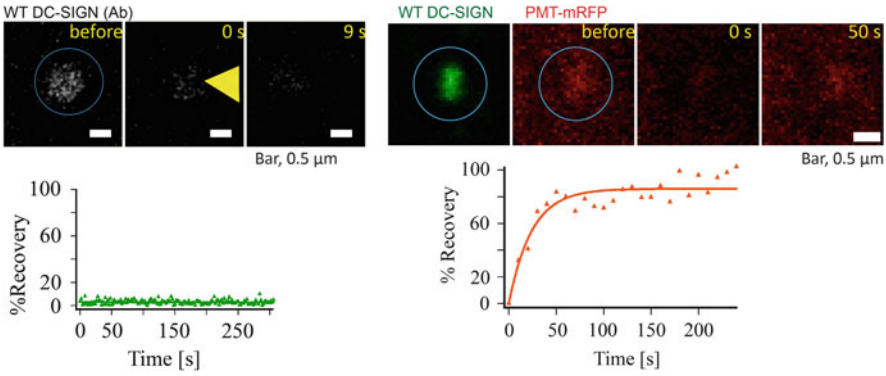

Fig. 4 Different exchange mobilities of DC-SIGN and an inner leaflet plasma membrane probe (PMT-mRFP) in the plasma membranes of MX DC-SIGN cells. *Top panels*: confocal images of the initial area followed by bleaching and recovery (times in seconds). *Bottom panels*: FRAP curves for DC-SIGN (*left panel*) and plasma membrane tracker – mRFP (*right panel*)

We investigated DC-SIGN and HA lateral mobility within membrane domains using both line scan fluorescence correlation spectroscopy and single particle tracking with defined-valency quantum dots [17]. Both techniques indicated essentially undetectable lateral mobility for DC-SIGN within microdomains. By contrast, HA retained appreciable lateral mobility within its domains on the cell surface.

More recently, we have employed u-track to investigate the mobility of both native and DENV-bound DC-SIGN clusters. U-track is a single particle tracking software package [20] that enables large numbers of clusters to be tracked simultaneously from live cell TIRFM videos. Using a moment scaling spectrum approach, particle trajectories can be divided into sub-diffusive, diffusive, and super-diffusive (likely directed) motion categories [20]. These data and their analysis have revealed some remarkable results. First, we found that many DC-SIGN clusters are laterally mobile in agreement with previous studies [21]. We believe that our earlier failure to find such mobility most probably resulted from a previous focus on brighter clusters and shorter time scales. Second, DENV binding, even only to a few DC-SIGN clusters, induces a *global* cellular response in which both DENV-loaded and DENV-unloaded DC-SIGN clusters exhibit dramatically increased lateral mobility; this is probably the result of cytoskeletal rearrangement proximate to the plasma membrane and has the possible consequence of enhancing DENV-loaded DC-SIGN cluster encounters with clathrin-coated pits [22]. Also, a small but significant fraction of DC-SIGN clusters *in the plasma membrane* undergo rapid, microtubule-based directed transport towards the cell center and the velocities are increased after dengue binding [22]. This activity may be required to bring captured pathogens from the leading margins of DCs back to the perinuclear zone for subsequent internalization and processing.

4 Outstanding Questions

Given that nanoclustering of membrane proteins appears to be a pervasive motif [23], our results for DC-SIGN raise four important questions not only for DENV virology but also for membrane biology in general.

4.1 Question 1: What Is the Architecture and Composition of DC-SIGN Clusters?

An initial statistical analysis of DC-SIGN nanodomain locations found no long-range order; i.e., no evidence against a random spatial arrangement in the membrane plane [18]. On the other hand, distinct microdomains having a large range of sizes appear at the level of light microscope resolution [15]. This range in sizes of

DC-SIGN clusters begs the question of whether a) the density of nanoclusters is in the range where individual nanoclusters convolute with the microscope point spread function to produce an apparent size distribution with a larger average size or b) there is a hierarchical, local, and "invisible" ordering of DC-SIGN nanoclusters such that they can arrange in structures that appear distinct in wide-field or confocal images. If such long-range order exists, what is the mechanism for coordinating these structures?

Proteomics (and lipidomics) will be invaluable in sorting out the molecular constituents that give rise to DC-SIGN clustering on several different length scales and whether these components of clusters are involved in pathogen recognition and/or internalization. Thus, for example, we have shown that annexin VI associates with DC-SIGN by proteomic and immunoprecipitation analysis, but knock-down of annexin VI does not affect DENV infection of DC-SIGN expressing human lymphoid cells [24]. Nevertheless, this study is a paradigm for investigating what proteins associate with DC-SIGN and how these proteins affect DC-SIGN cluster structure and pathogen recognition properties required for mediating DENV infection.

4.2 Question 2: What Is the Relationship Between DC-SIGN Cluster Size and Pathogen Processing?

DC-SIGN mediates the binding and internalization of pathogens ranging in size from small viruses like DENV to yeast. What are the structures of DC-SIGN cluster/pathogen complexes and possible structural rearrangements of the complex components during initial recognition; after binding but before internalization; during internalization; or after internalization and during intracellular pathogen processing? Forexample, we have shown by super-resolution microscopy (dSTORM) that single DENV particles can co-localize with apparent single DC-SIGN tetramers [16]. Whether or not these minimal DENV/cluster complexes can proceed to facilitate productive cellular infection is at present unknown. In another example, we have shown that DC-SIGN nanoclusters accumulate in the region of contact between DCs and yeast zymosan as a consequence of pathogen recognition [25].

Associated with this broad question is the issue of whether DC-SIGN is a complete pathogen receptor leading both to attachment and entry into host cells or whether a co-receptor(s) is involved. For DENV, the possible existence of a co-receptor is the subject of an active and ongoing controversy. Co-localization microscopy showed that both full-length DC-SIGN and DC-SIGN without its cytoplasmic tail (containing the internalization motifs) are internalized along with dengue [12]. Although the absence of the DC-SIGN cytoplasmic region reduced both dengue binding and endocytosis, cell infection was not abrogated but only reduced. Thus, DC-SIGN appears able to act in concert with a co-receptor

containing cell entry motifs. However, productive DENV entry may also occur adventitiously, employing constitutive endocytosis mechanisms all of which meet in the early endosome. It is likely that proteomic analysis (see above) will give important leads in the search for putative co-receptors that DENV may require (or sometimes employ) for cellular entry via DC-SIGN.

4.3 *Question 3: How and Why Does Global Activation of DC-SIGN Cluster Mobility Occur as Triggered by DENV?*

What is the mechanism of global activation of DC-SIGN diffusion? The enhanced mobility might in part be a consequence of pathogen-mediated release from stabilizing interactions of the CRD with pericellular matrix components, as suggested above [19]. The global character of the phenomenon suggests that transmembrane signal transduction might affect the subjacent membrane skeleton fence, a presumed regulator of lateral diffusion. Changes in lipid composition or organization are also possible, although the effect is large enough (fourfold) to suggest that membrane core modification would not alone be an adequate explanation. Is enhanced mobility after viral exposure exhibited by all membrane proteins or only a subset (e.g., solely DC-SIGN or only proteins that are clustered)? It is likely that answers to these questions will significantly advance our basic understanding of the structure and dynamics of the cortical cytoskeleton subjacent to the plasma membrane as well as interactions of plasma membrane components with the cytoskeletal and the pericellular matrix.

With respect to DENV virology, can global activation be triggered by DENV binding to its many other receptors or is it specific to binding to DC-SIGN, and what is the cell-type specificity? Does the mobility enhancement directly accelerate the DENV uptake mechanism in a biologically significant manner, or is the phenomenon primarily a reflection of another cellular event (e.g., cytoskeletal rearrangement) that occurs as an intrinsic part of the anti-viral response?

4.4 *Question 4: How Does Directed Transport of DC-SIGN Clusters in the Plane of the Plasma Membrane Occur and What Is Its Role in DENV Entry and Infection?*

What role does superdiffusion (i.e., directed transport) of DC-SIGN clusters play in pathogen processing in DCs and what is the mechanism of such unusually rapid cell-surface transport? Will understanding this phenomenon alter our current view of membrane cytoskeletal interactions?

In all, the investigation of DC-SIGN and its relation to DENV entry has proved to be a fertile ground for those interested in the continuing mysteries of the plasma membrane and its associated structures.

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