

Single-Molecule Analysis of SARS-CoV-2 Binding to C-Type Lectin Receptors

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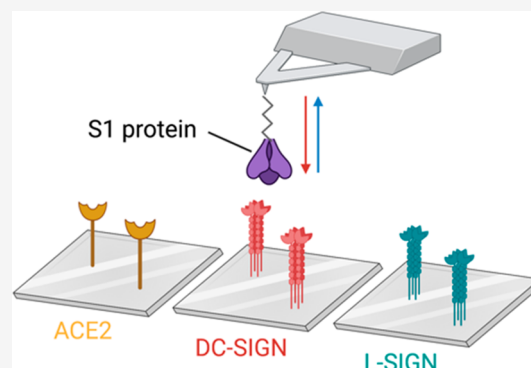
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ABSTRACT: Despite intense scrutiny throughout the pandemic, development of efficacious drugs against SARS-CoV-2 spread remains hindered. Understanding the underlying mechanisms of viral infection is fundamental for developing novel treatments. While angiotensin converting enzyme 2 (ACE2) is accepted as the key entry receptor of the virus, other infection mechanisms exist. Dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN) and its counterpart DC-SIGN-related (DC-SIGNR, also known as L-SIGN) have been recognized as possessing functional roles in COVID-19 disease and binding to SARS-CoV-2 has been demonstrated previously with ensemble and qualitative techniques. Here we examine the thermodynamic and kinetic parameters of the ligand–receptor interaction between these C-type lectins and the SARS-CoV-2 S1 protein using force–distance curve-based AFM and biolayer interferometry. We evidence that the S1 receptor binding domain is likely involved in this bond formation. Further, we employed deglycosidases and examined a nonglycosylated S1 variant to confirm the significance of glycosylation in this interaction. We demonstrate that the high affinity interactions observed occur through a mechanism distinct from that of ACE2.

KEYWORDS: SARS-CoV-2, DC-SIGN, L-SIGN, single molecule, kinetics, atomic force microscopy, protein glycosylation



The impacts of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) and its associated illness COVID-19 devastated the globe and altered the trajectory of contemporary society.¹ With >6 million reported deaths² and infections continuing to cause debilitating disease and damaging economies,^{3,4} the continued development of treatments and prophylaxis remains a high priority.¹ To better facilitate the rapid development of therapeutic measures, detecting and characterizing key components involved in viral landing, binding, and cellular invasion is critical for identifying druggable targets.^{5,6}

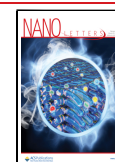
Early during the pandemic, angiotensin converting enzyme 2 (ACE2) was identified as an important entry receptor for SARS-CoV-2,⁷ leading to an explosion of research regarding this interaction.^{8–10} However, ACE2-independent cellular infection has been reported,^{11,12} and entry via the gastrointestinal route has been demonstrated to occur in cell populations which lack ACE2 expression.¹³ Moreover, biopsy results have shown that the virus is present in tissues deficient in ACE2,⁹ highlighting the involvement of alternative host receptors across systemic infection. Due to their interactions with other viruses,^{14,15} dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN) and its counterpart DC-SIGN-related (DC-SIGNR, also known as

L-SIGN)¹⁶ are being investigated as potential mediators of transinfection and cellular entry. The pair are homologous C-type lectins,¹⁷ which share 79.5% sequence identity. Both are known to bind carbohydrates in a Ca²⁺ dependent manner via a carbohydrate recognition domain (CRD).¹⁸ However, while closely related, the pair possess distinct physiological functions, tissue distributions, and interact with dissimilar ligands. DC-SIGN is predominantly expressed on immune and dendritic cells,¹⁹ whereas L-SIGN is more commonly expressed on epithelial and endothelial cells.^{14,20} Of particular note for this study, L-SIGN lacks one of the two internalization motifs and possesses a substitution (Val351 for Ser363) causing it to preferentially bind mannose, whereas DC-SIGN favors fucose and is a cycling receptor.²¹ Both receptors are known to bind N-linked high mannose oligosaccharide via their CRD, which is believed to be the source of their interactions with the SARS-CoV-2 spike (S) protein.^{15,18,22}

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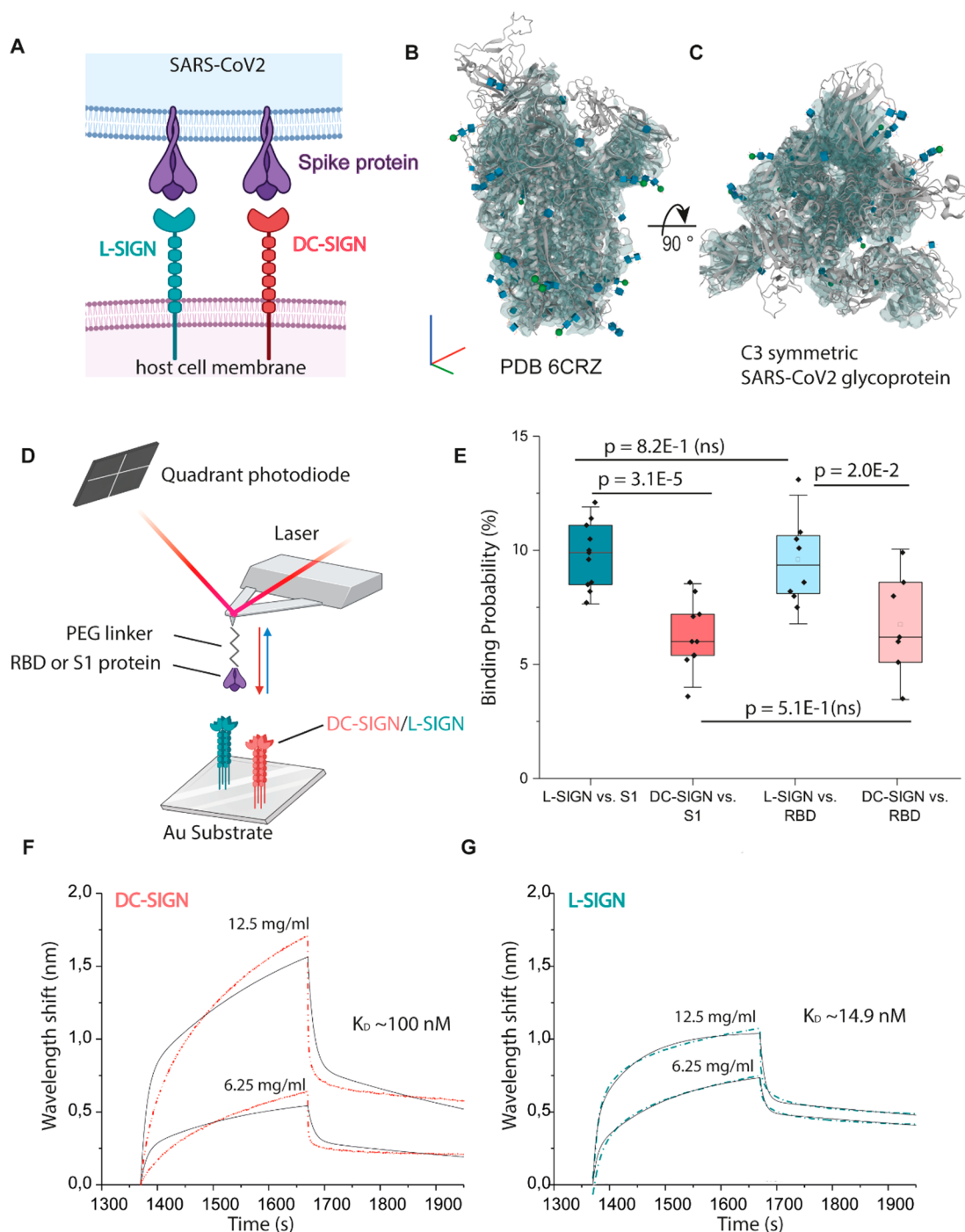


Figure 1. Binding of SARS-CoV-2 to DC-SIGN and L-SIGN. SARS-CoV-2 binds cell surface receptors through the exposed trimeric S protein (A). Electron density map of the S1 trimer with glycans rendered in blue as seen from the side (B) and from the top of the RBD³³ (C). Example of the experimental setup for binding assessment of model surfaces (D). A laser is focused on the back of the AFM cantilever, being reflected into a photodiode, which allows for detection of forces acting on the functionalized tip as it is approached and retracted from the surface, bringing RBD or S1 with DC-SIGN or L-SIGN; attached to the tip via a PEG linker was either the S1 protein, or the isolated RBD domain. On the model surface with which the tip was interacting was grafted either L-SIGN (teal) or DC-SIGN (red). The resulting BPs (E) between S1 (left) or RBD (right) functionalized tip and model surfaces (D) coated with L-SIGN (blue shades) or DC-SIGN (red shades); $n > 8$ for each condition. Significance as represented by p value was calculated using a student t test. BLI sensograms showing the kinetics of the interaction between DC-SIGN (F) and L-SIGN (G) with S1 protein expressed in HEK cells; fitting is indicated by dark lines.

The SARS-CoV-2 S protein is a large (180 kDa) type I transmembrane glycoprotein, existing as multiple trimers on the viral surface.^{23,24} Each monomer of the spike consists of two subunits, S1 and S2.^{24,25} The S2 subunit mediates membrane fusion and serves as an anchorage point to the

virion, whereas the distal S1 is predominantly associated with receptor interactions and facilitates attachment and binding to the cellular interface.^{10,25} The S1 subunit contains the receptor binding domain (RBD), which is known to interact with ACE2 and is the main target of most neutralizing antibodies.²⁶ The

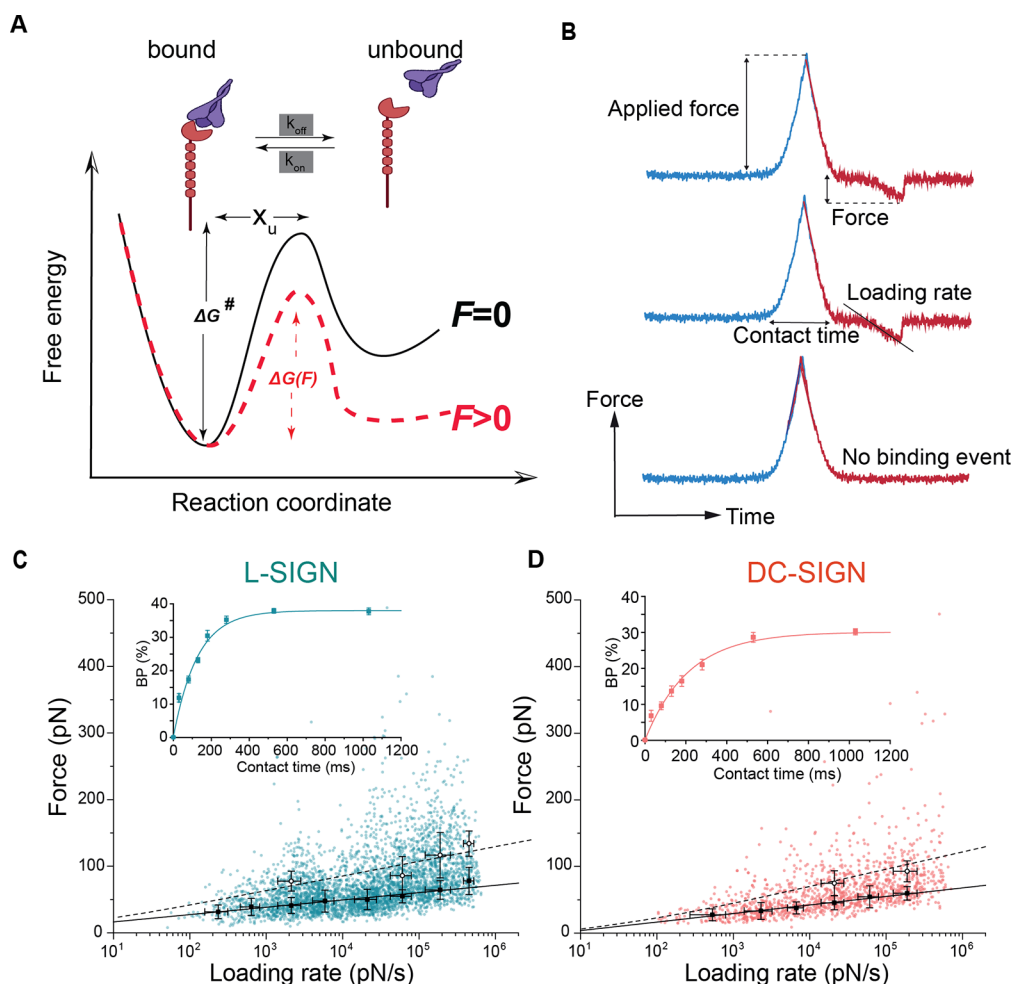


Figure 2. Binding kinetics of S1 protein with L-SIGN and DC-SIGN. Free energy landscape of ligand–receptor binding dynamics as given by a two-state Bell–Evans model for single bond rupture (A). Retrieval of information from adhesion events (B), showing the determination of the force (B, top) and loading rate ($\Delta F/\Delta t$) (B, middle) from curves that display binding events (B, top and middle), and an example of a retrieved curve with no binding event (B, bottom). DFS plots constructed from retrieved data for L-SIGN (C) and DC-SIGN (D) with contact time versus BP graphs (inset); solid line represents a least-squares fit of a monoexponential decay. DFS plots show the distribution of rupture forces as a function of loading rate (LR) for all data points. Solid line represents the Bell–Evans fit for single noncovalent bonds, whereas the Williams–Evans fit for a second uncorrelated bond is indicated by a dashed line.

N-terminal region of S1, located in the RBD, is formed from four β -sheets with connecting loops and is highly glycosylated, with N-glycans being common modifications in particular.^{23,27} Overall the S protein possesses 22 N-linked glycosylation sites, of which 13 are present in the S1.²⁴ O-glycosylation is also present however the exact number of sites remains poorly defined, although 2 have been reported within the RBD.²⁷ A sequence analysis has revealed that there is no evidence of mutations in the SARS-CoV-2 variants of concerns at the reported or predicted glycosylation sites on the spike proteins, although change in their conformation due to the amino acids impacted may alter the glycosylation profile.²⁷ Previous studies have shown that interactions between S1 and both DC-SIGN and L-SIGN can be blocked via glycomimetics,^{22,28} supporting glycosylation of the spike protein as the driver of this interaction.

While binding and infectivity behaviors associated with the SARS-CoV-2 interaction with DC-SIGN and L-SIGN have been strongly evidenced,²² direct single molecule-based examination of crucial elements such as specificity and kinetics of this interaction remains underdeveloped. Here we utilize

atomic force microscopy (AFM)²⁹ and employ single-molecule force spectroscopy (SMFS)^{30,31} to probe this interaction and shed unique insight on the behaviors exhibited.³² We demonstrate that the SARS-CoV-2 S1 specifically binds DC and L-SIGN, likely through the glycans of the RBD. Further, we discern the kinetic parameters associated with this interaction. We confirm that this interaction is tied to the glycosylation state of the S1 protein and that it is independent from the mechanism of binding for ACE2. These results are confirmed using the ensemble technique biolayer interferometry (BLI). Here we explore glycan-associated binding of the SARS-CoV-2 S1 protein with DC-SIGN and L-SIGN at a molecular level via AFM, unveiling the underpinnings of an alternative mechanistic function for the viral spike in gaining access to cells without the necessary involvement of ACE2.

Binding of S1 Protein and RBD to DC-SIGN and L-SIGN Is Specific. Given the function of DC-SIGN and L-SIGN as C-type lectins, binding of SARS-CoV-2 spike protein to cell surface receptors (Figure 1A) is most likely mediated by their CRD domains. Due to the highly glycosylated S1 and RBD domain being the most exposed region of the SARS-

Table 1. Kinetic Parameters for S1 Complexation with DC-SIGN and L-SIGN from SMFS^a

	k_{on} ($\mu\text{M}^{-1}\text{s}^{-1}$)	k_{off} (s^{-1})	K_{D} (nM)	x_{u} (nm)
L-SIGN	3.6 ± 0.2	0.07 ± 0.03	20 ± 17	0.86 ± 0.09
DC-SIGN	2.1 ± 0.1	0.91 ± 0.40	428 ± 210	0.74 ± 0.05
ACE2	0.0641 ± 0.0096	0.008 ± 0.005	119 ± 90	0.81 ± 0.05

^aAverage values with standard error are given for k_{on} and k_{off} and results presented for K_{D} and x_{u} are presented as mean and standard deviation. Data for ACE2 reproduced from ref 34.

CoV-2 virion, it is highly likely that these regions and their associated glycans^{27,33} are responsible for the behaviors that have been reported (Figure 1B,C). To verify the interactions previously reported and identify whether the supramolecular interactions formed between SARS-CoV-2 S protein and the two lectins were specific in nature, we initially explored the binding behaviors using AFM to probe model surfaces (Figure 1D). By coating gold surfaces with either L-SIGN or DC-SIGN, and functionalizing the AFM tip with S1 or RBD, we were able to assess the binding probability (BP) and ability of antibodies to interfere with binding behaviors. Using force–distance AFM (FD-AFM), the tip was cyclically approached and retracted as the cantilever raster scans the surface, while the signal was collected from the reflection of a laser from the back of the tip into a photodiode, with the displacement of the signal indicating the forces associated with adhesion.³² The retrieved force–distance (FD) curves were then used to categorize pixels that contain no binding and specific binding, and consequently the proportion of the curves displaying adhesion events were used to calculate BPs for each interaction (Figure 1E).³⁴

When tips were functionalized with either S1 or RBD, L-SIGN exhibited a higher BP ($9.8 \pm 1.4\%$) than did DC-SIGN ($6.3 \pm 1.4\%$). While distinct from each other, both DC-SIGN and L-SIGN showed no significant difference in BP regardless of whether tips were functionalized with S1 or RBD. While the N-terminal domain (NTD) of the SARS-CoV-2 spike has the highest density of glycosylation sites, this result confirms that the RBD also plays a significant role in the binding toward DC-SIGN and L-SIGN receptors. The specificity of these interactions was verified by blocking the interaction with antibodies against the surface grafted protein (Figure S1), wherein a significant reduction in relative BP was observed for both C-type lectins ($\sim 70\%$ reduction in BP). Moreover, removal of the RBD from the tip using EDTA to disrupt the His-Ni²⁺-NTA bond similarly resulted in loss of binding (Figure S1). Taken together, the high binding probability and the mitigation of binding through exposure to blocking antibodies for either partner illustrate that the interactions obtained are a specific phenomenon that may play a role in the biology of COVID-19 given the prevalence and distribution of these receptors *in situ*. These results support prior reports of a potential role of DC-SIGN and L-SIGN in the biology of SARS-CoV-2 binding and transmission.²²

To better understand the molecular basis for the differential recognition of glycans between DC-SIGN and L-SIGN, we performed BLI, a label-free ensemble biophysical technique, to ascertain the kinetics of a biomolecular interaction based on the principles of optical interference.³⁵ From the results obtained, it was quite evident that L-SIGN binding to S1 protein ($K_{\text{D}} \sim 15$ nM) is better facilitated than is the case for DC-SIGN ($K_{\text{D}} \sim 100$ nM) (Figure 1F,G). Although both K_{D} values correspond to a range relevant to high-affinity interactions, the slower dissociation kinetics observed for L-

SIGN indicates the formation of a stronger and more stable complex with S1 than does DC-SIGN. These results are in line with previous reports, with the L-SIGN exhibiting higher affinity to S1 protein.²² In order to better understand these behaviors, we sought to further examine the underlying kinetics and elaborate upon the dynamics of noncovalent bond formation between SARS-CoV-2 S1 and both L-SIGN and DC-SIGN through use of dynamic force spectroscopy (DFS).

Single-Molecule Binding Kinetics Indicate That L-SIGN Binds S1 with Higher Affinity Than DC-SIGN.

Through controlling the mechanical energy input into the system and the time allowed for noncovalent bonds to form through moderating retraction speed and contact time, respectively, we gained insight into the thermodynamics and kinetic parameters that belie the binding between S1 and both DC-SIGN and L-SIGN. To explore the binding free-energy landscape (Figure 2A) of the receptor–ligand complex, we extracted the force and loading rate from retrieved curves (Figure 2B) and use these in conjunction with contact time data to obtain the kinetic off and on rates (k_{off} and k_{on}) for the binding behaviors observed. By fitting the acquired data (see Methods in the Supporting Information), with the Bell–Evans model for single bond rupture,³⁶ we obtained a k_{off} value for both L-SIGN (Figure 2C) and DC-SIGN (Figure 2D), yielding values of 0.91 ± 0.40 s^{−1} and 0.07 ± 0.03 s^{−1} for each when paired with S1 trimer (data are summarized in Table 1). For both complexes, the k_{on} values are quite close (~ 2 – 4 μM^{-1} s^{−1}) and comparable to values previously observed in the literature for C-type lectins.³⁷

From the retrieved K_{D} values, it appears that L-SIGN possesses a similar affinity to S1 as we have previously reported for ACE2;³⁴ however, the k_{on} and k_{off} values show that the strength and mechanical stability of this interaction are quite different. While L-SIGN possesses a higher k_{on} indicating greater potential for bond formation, it also possesses a higher k_{off} meaning that this complex dissociates faster when compared to ACE2. The x_{u} values obtained for both receptors indicates similar conformational mobility in the binding interaction, which supports the notion that the homologues bind S1 in the same manner, likely the CRD, though with different affinities as should be expected given the dissimilarity in their endogenous glycan scavenging behaviors. As L-SIGN can be found in a heterodimer with ACE2,³⁸ this raises an intriguing possibility that in cells positive for both L-SIGN and ACE2, L-SIGN could function as an initial attachment point before being outcompeted by ACE2, which forms a stronger and longer-lived attachment to the S1 RBD. The shorter-lived bonds formed indicate that the glycan binding behavior exhibited may be exploited by SARS-CoV-2 to aid in finding other entry receptors, especially given that L-SIGN is lacking one of the internalization sequences present in its DC-SIGN homologue. While the DC-SIGN exhibited a K_{D} value within the submicromolar range, its affinity is significantly less than

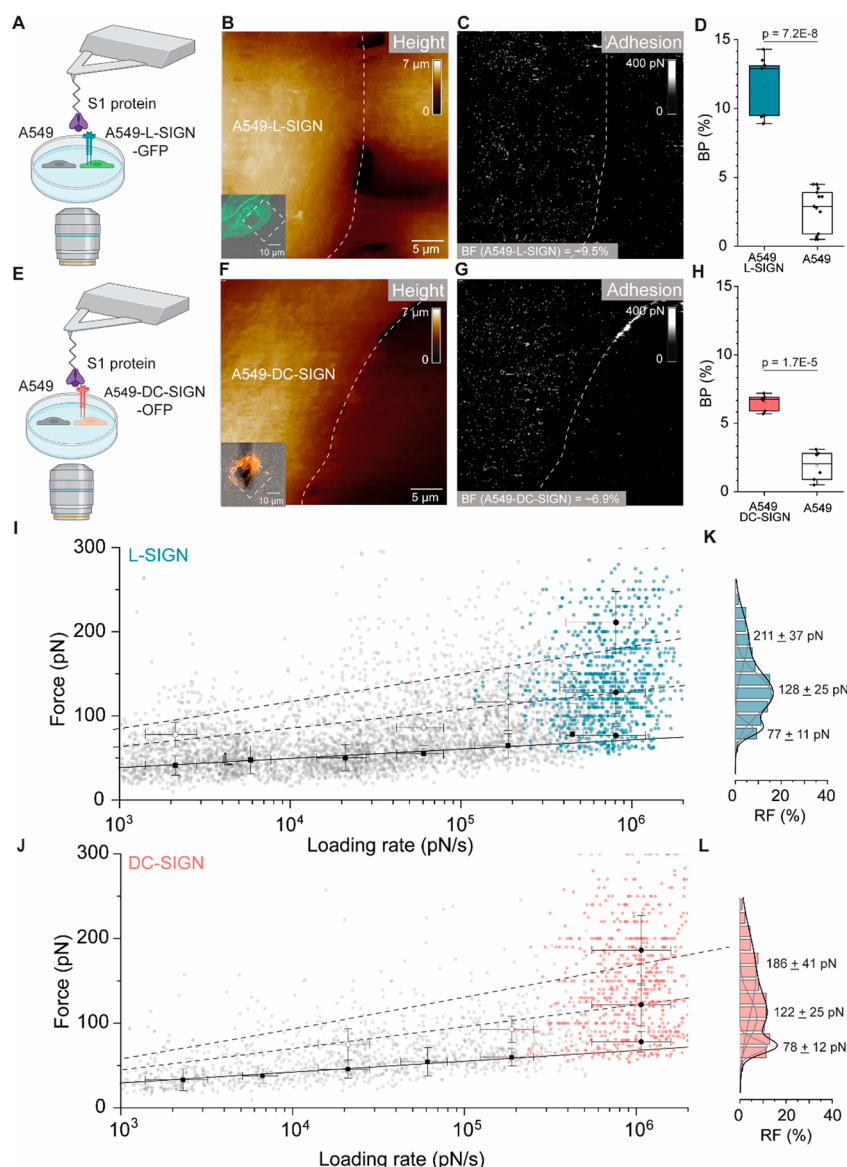


Figure 3. Probing S1 binding to L-SIGN or DC-SIGN on live A549 cells. Binding of S1 is probed on A549 cells and A549 cells transfected with L-SIGN GFP (A). An example height map (B) with corresponding region indicated on a confocal micrograph (inset), GFP fluorescence in green; scale bars are 5 and 10 μm , respectively. Edge of the positive cell being examined is indicated with a dotted line in the height map. Grayscale adhesion map of the same cell (C) showing binding on the positive cell (left-hand side) and less on the negative cells (right-hand side), scale is 0–400 pN. Corresponding binding probability results for L-SIGN positive and negative cells (D), showing significantly higher binding on positive cells ($n = 9$ for L-SIGN expressing cells, $n = 13$ for control cells, individual points are representative of the result from an individual cell, hollow marker indicates the average BP, whiskers denote the data range, bisecting lines show the median, and the interquartile range is shown by the length of the box). Experimental setup for live cell experiments to assess DC-SIGN binding on transfected A549 cells (E). Height map and confocal micrograph (inset) of a DC-SIGN-OFP positive and negative cell (F), adhesion map of this region (G), and binding probability calculated from live cell adhesion maps (H) showing significantly higher binding probability for transfected cells ($n = 6$ in the case of both DC-SIGN expressing and control cells, individual points are representative of the result from an individual cell, hollow marker indicates the average BP, whiskers denote the data range, bisecting lines show the median, and the interquartile range is shown by the length of the box). Corresponding DFS plot for L-SIGN incorporating the data retrieved from living cell experiments (I) and resulting histograms (J). Corresponding DFS plot for DC-SIGN incorporating the data retrieved from living cell experiments (K) and resulting histograms (L).

either L-SIGN or ACE2, which could support its purported role in transinfection, as the binding strength may not be sufficient to induce internalization, thus it could represent an attachment to incidentally encountered circulating dendritic cells and macrophages, rather than a direct infective route. Although this interaction is specific, as a weaker interaction (by almost 10 times) than L-SIGN or ACE2, means the virus could easily be dislodged from the surface of DC-SIGN positive cells

should it encounter a more viable binding partner or entry receptor.

Validation of Binding Interactions on Living Lung Carcinoma Cells. In order to validate that the interactions described thus far are relevant to physiological processes, we sought to assess whether the binding behaviors described occur in a cellular context. As such, A549 cells were transfected with either L-SIGN-GFP (Figure 3A) or DC-SIGN-OFP (Figure 3E), and AFM tips were functionalized with S1 as per prior

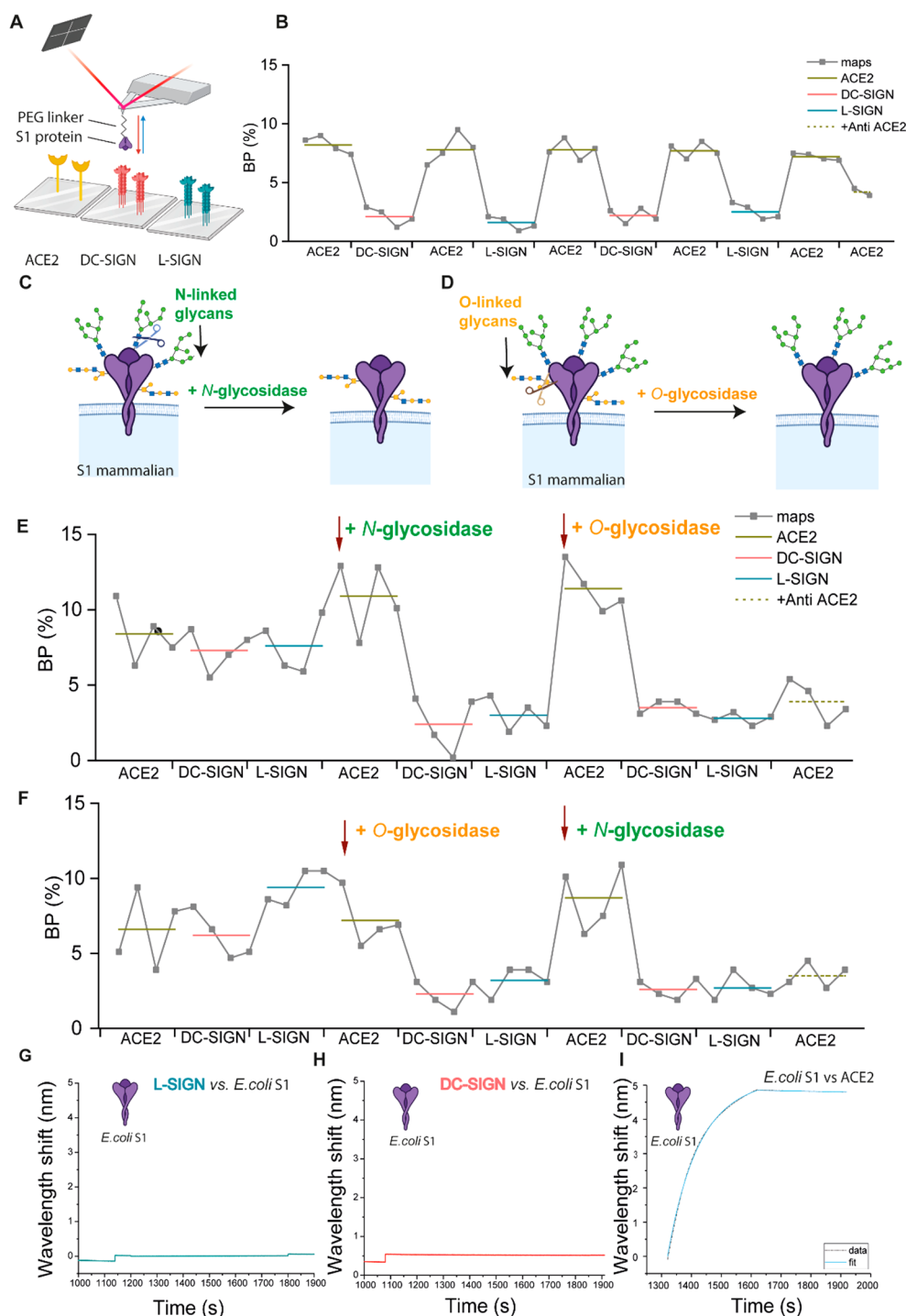


Figure 4. Evaluating the role of glycosylation in binding behaviors observed between S1 and DC/L-SIGN. A multisurface experimental set up was utilized (A), wherein the BP was observed across multiple surfaces with the same tip, with ACE2 acting as a positive control. When S1 derived from *E. coli* was attached to the AFM tip (B), binding was conserved with ACE2 (brown lines), however was severely impacted for both DC-SIGN (red lines) and L-SIGN (teal lines). These findings were corroborated using BLI (B) wherein binding was not observed for DC-SIGN or L-SIGN, but maintained for ACE2. Enzymatic removal of glycans (C and D) and impact on BP was performed using a similar experimental setup, and binding was assessed for all three surfaces before and after the addition of N-glycosidase and O-glycosidase (E), or in the reverse order (F) (anti-ACE2 mAb being used as a control in both cases). To confirm that the importance of glycans only applies to certain interactions, BLI was performed using *E. coli* derived S1 with L-SIGN (G), and DC-SIGN (H) performing poorly and exhibiting very little wavelength shift due to lack of binding to the sensor, whereas ACE2 (I) gave a much larger shift, as S1 bound the receptor and thus coated the probe.

experiments (Figure 3A,E). Using this approach, cells expressing the fluorescent protein directly adjacent those which did not were imaged using combined confocal-AFM microscopy, allowing direct comparison between cells based on

expression status, with control data being built into each image due to the nonexpressing population. Using the combined confocal-AFM setup, height topographies for samples containing L-SIGN or DC-SIGN expressing cells (Figure 3B,F,

respectively) could be correlated to fluorescence images (Figure 3B,F, inset) and adhesion maps (Figure 3C,G) used to calculate BPs for receptor positive and negative populations (Figure 3D,H).

Binding probabilities for receptor positive cells matched well with those obtained from model surfaces, with L-SIGN (Figure 3D) exhibiting higher binding ($11.6 \pm 2.2\%$) than DC-SIGN (Figure 3H) ($6.5 \pm 0.6\%$). In both cases, receptor negative cells gave a binding probability of $\sim 2\%$. By extracting the force and loading rate from retrieved curves, data from live cell experiments could be superimposed on model surface information, allowing for comparison between the pair (Figure 3I,J). The good correlation observed between the data acquired on model surfaces and directly on living cells demonstrates the physiological relevance of this type of interaction in a biological context. Interestingly, in a cellular context both L-SIGN and DC-SIGN displayed up to three uncorrelated bonds according to Williams–Evans prediction (Figure 3I,J).

S1 Binding to Both DC-SIGN and L-SIGN Are Tied to Glycosylation State. In order to demonstrate the key role of the glycosylation state for S1 in these interactions, we developed a new AFM binding assay based on contiguous surface probing experiments using the same functionalized AFM tip. First, an S1 variant derived from *E. coli*, and thus lacking glycosylation,³⁹ was used to demonstrate that binding to ACE2 remains intact, whereas without these glycans there was little binding to surfaces grafted with DC-SIGN or L-SIGN (Figure 4A,B). These results illustrate that the binding behaviors exhibited arise from mechanistic differences within the system, i.e., different components of the S1 protein are involved in bond formation, and that these differences come from glycosylation patterns of the viral protein and not from other factors associated with experimental setup.

This experiment was then repeated with HEK-derived S1, possessing both N-linked and O-linked glycans, with sequential exposure to N-glycosidase and O-glycosidase in either order (Figure 4C–F). Binding to both DC and L-SIGN was observed to be reduced in response to both glycosidases, whereas ACE2 was not impacted by glycosidase exposure (Figure 4E,F). Deglycosylation was confirmed by electrophoresis and Western blot (Figure S2). Intriguingly, the removal of N-glycans from the S1 trimer improved the binding probability on the ACE-2 surface, highlighting that it interacts via a different mechanism with this receptor than it does either DC-SIGN or L-SIGN. As the order of glycan removal did not appear to drastically differ between experimental set-ups, this implies that the small number of both O- and N-glycans contribute to the interactions described on model surfaces and in living cells.

Finally, we performed control experiments using BLI to probe the interaction between S1 derived from *E. coli* and both DC-SIGN and L-SIGN to validate observations from the contiguous surface experiments. In keeping with the results of the AFM experiments, we observed no appreciable wavelength shift in the association trace because of negligible interaction with the nonglycosylated S1 from *E. coli* (Figure 4G,H).⁴⁰ As per other prior reports,²⁷ we found that S1 from *E. coli* has high affinity for ACE2 receptors ($K_D \sim 1.7$ nM) (Figure 4I) (Figure S3) asserting that ACE2 recognition by S1 of SARS-CoV2 is not driven by the glycans projecting from the S1 surface, but by a number of stabilizing salt-bridge and hydrophobic interactions.¹⁰ This highlights the importance of glycans to

serve as ligands for carbohydrate-specific receptors, thus aiding SARS-CoV2 to anchor onto cellular surfaces via tight binding with DC-SIGN and L-SIGN receptors.

Given the similar binding behaviors observed with both S1 and RBD on model surfaces, and the behaviors exhibited in contiguous model surface experiments exploring the impacts of deglycosylation, it appears that the small number of glycans present within the RBD are very important to SARS-CoV-2 binding of both DC-SIGN and L-SIGN. It is possible that glycans appended to the S1 within the RBD are granted better exposure and thus improved availability for binding on account of this viral protein being the most protrusive, hence both S1 and RBD yielding similar results across the experimental series presented. The similar behavior could also be linked to the S1 being more likely to attach to the cantilever via the N-terminus, thus leaving the RBD the most exposed and consequently most likely site available to interact with receptors. It is worth noting that in the case of ACE2 surfaces, the binding subtly increases in response to glycan loss, implying that glycan modifications could be associated with shielding from one receptor whereas improve scavenging by other cell populations. It would be of interest to explore whether different glycomic profiles map well to favoring certain entry pathways.

Here we explored the interactions between DC-SIGN and L-SIGN and the SAR-CoV-2 S1 protein and specifically the RBD of this capsid element at a single molecule level for the first time. We demonstrated that (i) L-SIGN possesses higher affinity than does DC-SIGN and (ii) this interaction is biologically relevant and (iii) is driven by glycosylation of the viral protein. We show through an experimental series that these interactions are mechanistically distinct and possess differing underlying kinetics than the better understood target ACE2. This research highlights the importance of delving into the interactions of viruses with alternative entry receptors, as the virus continues to mutate and evolve, the role of putative binding partners, the mechanics of their interactions, and links between each partner will facilitate the production of prophylactic and therapeutic agents.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.nanolett.2c04931>.

Materials and methods. Figure S1, bar graph of control and blocking experimental results for model surfaces. Figure S2, gel images confirming deglycosylation. Figure S3, additional sensograms for K_D determination (PDF)

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Notes

The authors declare no competing financial interest.

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