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How viruses hijack the SIGN's

The development of effective therapies to combat the threat of emerging viruses requires a thorough understanding of the mechanisms governing virus tropism. Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) was identified as a C-type lectin that mediates the initial interaction between dendritic cell and T cell, as well as serving additional roles in immunology. DC-SIGN and the related L-SIGN were also found to play a role in the *cis* and *trans* infection of cells by a wide array of viruses. Their role in *cis* infection can be explained either by functioning as attachment factor or, in some cases, as true entry receptors. Though differentiating the two experimentally is very difficult, the best approach seems to be overexpression of either lectin in permissive, completely non-susceptible cells and determine whether their expression can make these cells susceptible. Furthermore, it might be prudent to change the current nomenclature and instead address attachment factors and entry receptors as first and second line receptors, respectively.

Introduction

Viruses remain a major health care problem in developing countries and the developed world alike. Even in the Western world emerging viruses remain a constant looming threat, illustrated most notably by the seasonal epidemics and occasional pandemic outbreaks of influenza virus. Nowadays also more exotic viruses are waiting at our doorstep: global warming and increased mobility of people have facilitated the spread of arthropod vectors and the viruses they host to new, susceptible populations. [(1)] A striking example of this is the introduction of West Nile Virus (WNV) in North-America during the last decade: the Asian tiger mosquito (*Aedes albopictus*), the arthropod vector for WNV, was introduced into the US presumably via hitchhiking on airplanes or cargo transport and quickly established a foothold in the US. In 1999 the first case of WNV induced West Nile encephalitis was found in New York, the virus was presumably introduced via the import of infected birds from either Israel or Egypt. Since then the virus has spread to over 47 states and caused the death over 1000 people. [(2)] Epidemics like this are, however, not restricted to WNV, but have also been seen for viruses like Rift Valley Fever, Sindbis Virus and Blue Tongue virus. [(1)]

For the successful attachment and infection of cells viruses rely on the expression of cell-specific receptors. These receptors are often either (glycosylated) proteins or glycans. Considering the impact of newly emerging infectious diseases and the spread of already existing viruses into new territories, understanding the details of receptor–ligand interaction and its role in viral tropism are of utmost importance. This thesis will look at the roles of **D**endritic **C**ell-**S**pecific **I**ntercellular adhesion molecule-3-**G**rabbing **N**on-integrin (DC-SIGN) and a closely related protein (L-SIGN) as viral receptors and will critically review previous research.

Discovery of DC-SIGN

The process of antigen uptake by dendritic cells (DC) and subsequent presentation to T cells is the main link between the innate and adaptive immune system and plays a pivotal role in the combating of infections. [(3)] For the presentation of antigen by DC to T cells a close proximity of both cell types is required. Early studies by Steinman *et al* showed DC surrounded by lymphocytes in a rosette shape fashion, a first indication that adhesion molecules are likely to play an important role in this interaction. [(4)] There were numerous candidate adhesion and costimulatory molecules with their ligands that could mediate such an interaction, such as LFA-3/CD2 and LFA-1/ICAM-1, -2 or -3. [(5)] The multitude of adhesion receptors and ligands that contribute simultaneously to DC-T cell interaction hampered initial blocking experiments. However, the group

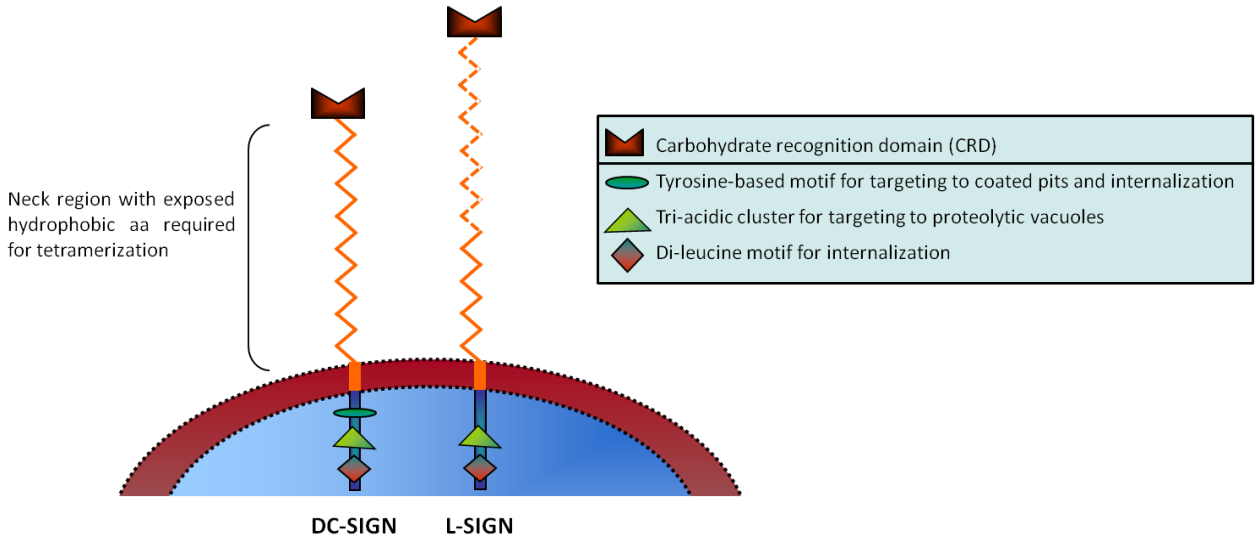

Fig 1. Domain structure of DC-SIGN and L-SIGN.
The CRD is attached to the neck region and is positioned to point outward from the cell membrane to allow most efficient binding with ligands. The neck region, important in receptor multimerization, consists of 7.5 repeats in the case of DC-SIGN and between 4 and 10 for L-SIGN (with variable repeats depicted as dashed lines). DC-SIGN encodes three distinct amino acid motifs on its cytoplasmatic domain that play a role in endocytosis. Whereas L-SIGN also encodes the di-leucine motif and the tri-acidic cluster, it lacks the tyrosine based motif.

of Carl Figdor developed a method using fluorescent beads coated with ICAM-3 to circumvent this problem. This method lead to the identification of a novel protein that mediated the interaction between DC and T cells via binding to ICAM-3 expressed on resting T cells. In a 2000 Cell paper they named this protein **D**endritic **C**ell-**S**pecific **I**ntercellular adhesion molecule-3-**G**rabbing **N**on-integrin (DC-SIGN). [6]

A gene closely related to DC-SIGN was identified subsequently and named **L**iver/Lymph node-**S**pecific **I**CAM-3-**G**rabbing **N**on-integrin (L-SIGN, or DC-SIGN Related, DC-SIGNR). The two genes share 77% identity at the amino acid level and are thought to have arisen from a gene duplication event. [7]

Since their discovery an overwhelming amount of articles has been published on the SIGN's and their role in immunology and, surprisingly enough, in virology. This thesis will shortly discuss the functions of DC-SIGN and the closely related L-SIGN in immunology and then look more into detail at their role in virology.

Structure and tissue distribution of DC-SIGN and L-SIGN

DC-SIGN is a type II transmembrane lectin receptor which is abundantly expressed on immature DC (iDC) that are present in peripheral tissues, but it is also found, albeit down-regulated, on mature or activated DC (mDC) in lymphoid tissues such as lymph nodes, tonsils and spleen. [8] However, it is not expressed on certain DC-types such as follicular DC or on skin-resident Langerhans DC. Alveolar macrophages are also known to express DC-SIGN as well as activated B cells. [9]

L-SIGN is expressed on a completely different set of cell types: it is not found on DC but on the surface of endothelial cells in the liver, lungs, lymph node sinuses, and placental villi, alveolar cells in the lungs and on capillaries in the lamina propria of the terminal ileum. [10]

Both DC-SIGN and L-SIGN contain three distinct domains: an intra-cellular, N-terminal signaling domain containing recycling and internalization motifs, a trans-

membrane domain important for anchoring in the membrane and an extra-cellular C-terminal domain involved in ligand recognition. (Fig 1)

The extra-cellular domain of DC-SIGN is composed of a flexible neck region to which the carbohydrate recognition domain (CRD) is linked, the latter protrudes away from the cell. Binding of the CRD to its carbohydrate ligand depends on the presence of calcium-binding pockets, making it a calcium-dependent lectin. A remarkable difference between the CRDs of DC-SIGN and L-SIGN is the substitution of Val351 in DC-SIGN with Ser363 in L-SIGN. This single amino acid difference is responsible for their different ligand affinities by eliminating the Van der Waals interaction between Val351 and the 2-hydroxy group of fucose. Though both receptors bind to N-linked high-mannose oligosaccharides, DC-SIGN binds preferentially to fucose whereas L-SIGN binds with higher affinity to mannose. [(11)]
The neck region of DC-SIGN is composed of 7.5 repeats, each containing 23 amino acid residues, which fold in an α-helical conformation interspersed with non-helical regions. The neck region is involved in tetramerization of the receptor on the cell surface, which is important for high-avidity binding to glycans. [(12)] Hydrophobic residues in the neck region are believed to stabilize these oligomers and project the CRD outward from the cell. This way the CRDs are positioned optimally for interaction with their ligand. Moreover, by oligomerization of DC-SIGN the specificity of the CRDs for multiple repetitive units on host molecules is increased drastically. [(13)]
Tetramer formation was also shown to be important for the binding to closely spaced (approx. 5 nm between sugar binding sites) oligosaccharides on the envelopes of viruses and membranes of parasites. By using a number of truncated forms of DC-SIGN it was demonstrated that its ability to oligomerize depends on the number of helical repeats in the neck region: whereas at least 6 repeats are needed for tetramerization, 5.5 repeats result in equilibrium between tetramer and dimer formation and two repeats give equilibrium between dimer and monomer. [(14)]
Whereas the number of neck region repeats is constant for DC-SIGN, the number of repeats in L-SIGN ranges between 4 and 10. [(15)] Since homo-oligomerization of the neck region of SIGN's seems crucial for high-affinity ligand binding, heterozygous expression of L-SIGN polymorphs with a different number of repeats could prevent efficient multimerization and thus affect ligand-binding affinity. [(7)]

The intra-cellular domain of DC-SIGN contains a di-leucine motif essential for internalization, a tri-acidic cluster known to play a role in targeting to proteolytic vacuoles and a tyrosine-based motif involved in signal transduction. [(16)] The presence of these domains and the observation that ligands are released upon lowering of the pH indicates a dual ligand-binding role for DC-SIGN: mediating both adhesion *and* endocytosis. By releasing its bound ligand at endosomal pH it can act as a recycling endocytic receptor. [(17)]
L-SIGN on the other hand also contains the di-leucine motif and the tri-acidic cluster but lacks the tyrosine-based motif. [(18)] Furthermore it was shown that L-SIGN does not dissociate with its ligand upon lowering of the pH, indicating that L-SIGN functions only as an adhesion receptor and not as an endocytic receptor. [(19)]

Surface expression of DC-SIGN seems to be organized into distinct molecular clusters in lipid microdomains as shown by studies using transmission electronic microscopy and near-field scanning optical microscopy. This spatial organization of DC-SIGN into high-avidity binding domains is thought to improve binding to viral particles and bacteria containing multivalent binding sites. Furthermore it may facilitate the interaction of DC-SIGN with intracellular signaling molecules that are recruited to the same membrane domains. [(20)]

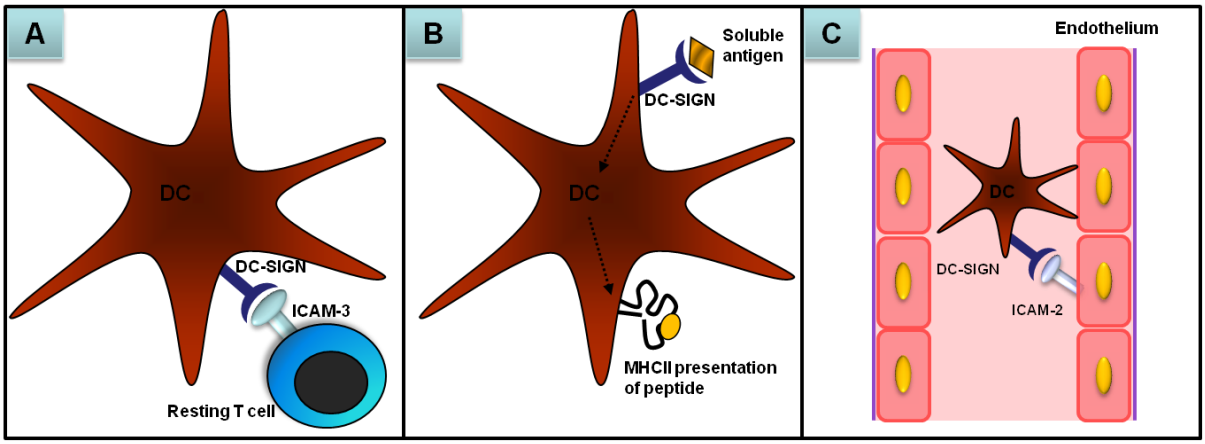

Fig 2. DC-SIGN's distinct roles in immunology.
(A) DC-SIGN interacts with ICAM-3 expressed on resting T cells to form the immunological synapse and allow the TCR to scan the DC MHC repertoire for the correct antigen. (B) DC-SIGN can bind soluble antigen, mediate its uptake into the lysosomal pathway after which the peptide is processed for presentation in the context of MHCI. (C) Rolling and tethering to the endothelial layer lining the blood vessels is mediated by the interaction between ICAM-2, expressed on endothelial cells, and DC-SIGN. After specific chemotactic signals, DC-SIGN can also mediate migration across the endothelial layer into the tissue.

Functions in immunology

DC-SIGN was originally identified as a receptor that mediates the interaction between DC and T cells during priming, however, subsequent research showed that it had also many additional roles in immunology. (Fig 2)

I: Immunological synapse formation

T cells are activated when their T cell receptor (TCR) binds the appropriate peptide complexed to an MHC molecule on the surface of a DC. The initial interaction between DC and T cell is transient and allows the T cell time to scan the DC peptide-MHC complex-repertoire with its TCR. This initial interaction is not depended on the presence of the correct antigen. The cell-cell complex required for this scanning process is called the immunological synapse and is governed by the recruitment of specific adhesion receptors to the interaction interface. [21] It was shown that the on resting T cells highly expressed ICAM-3 plays a role in this transient interaction because it rapidly clusters after formation of the immunological synapse. The interaction partner for ICAM-3 was identified as DC-SIGN by the fluorescent bead assay and subsequent blocking experiments. The current model is that the initial interaction between resting T cell and DC is mediated via the ICAM-3–DC-SIGN interaction, after which stronger interactions are resulting from the recruitment of other adhesion molecules to the synapse. [6]

II: Interactions with blood endothelium

DC are professional antigen presenting cells that function as a bridge between the innate and adaptive immune system. These cells patrol the peripheral tissues and have a sentinel function against invading pathogens. [22] In order to perform this task, DC in the blood have to migrate into peripheral tissue either to replenish resident DC or in response to chemotactic signals that result from an inflammatory milieu. The interaction of DC with the endothelial cells lining the blood vessels is very important for this process. DC-SIGN is known to mediate the tethering and rolling of cells expressing DC-SIGN by binding ICAM-2, a glycoprotein expressed on the surface of endothelial cells. [23] Since DC-SIGN is rapidly upregulated on monocytes in

response to GM-CSF and IL-4, both pro-inflammatory cytokines produced at the site of inflammation, the DC will be able to adhere and home to the site of infection. [6] It was later also shown that DC-SIGN not only mediates rolling and tethering to the endothelium but also plays a role in adhesion and subsequent transendothelial migration.

III: Pathogen recognition

For the initiation of an immune response invading pathogens have to be picked up by DC and presented to T cells. A key role in this process is played by the class of Pathogen Recognition Receptors (PRR) that recognize conserved molecular patterns expressed on many pathogens, including lipopolysaccharide, peptidoglycan and certain carbohydrates. [24] DC express a wide array of sugar-binding C-type lectins that function as PRP. Most of these proteins have a specificity for mannose-containing carbohydrates. However, they all recognize specific branching and positioning of these sugars, giving them a complementary role instead of a redundant one. [25]

DC-SIGN was shown to rapidly internalize after binding of soluble ligand, a process mediated by the di-leucine motif. Upon internalization the DC-SIGN-ligand complex is targeted to lysosomes by the presence of the tri-acidic cluster. [26] When the pH lowers, the complex dissociates and the ligand is processed for subsequent presentation in the context of MHCII to $CD4^+$ T cells. This process seems to be important for the uptake of e.g. viruses and indicates a role for DC-SIGN in pathogen recognition and subsequent antigen presentation in order to elicit an immune response. [17]

Functions of L-SIGN

Though a substantial amount of information is available on DC-SIGN, far less is known about the functions of L-SIGN. Similar to DC-SIGN it is able to bind high-mannose oligosaccharides as well, but in contrast it is expressed only on certain endothelial cells. Since endothelial cells are thought to play a similar role to macrophages and DC in antigen capture from the blood it is to assume that L-SIGN functions as an antigen receptor for these cells. [10] In this way L-SIGN present on endothelial cells in the liver can remove antigen from the circulation in a manner similar as DC do in lymphoid organs. [25]

Friend or Foe?

DC-SIGN is clearly an important lectin receptor, governing crucial aspects of DC biology; it not only facilitates the interaction between DC and T cell, but is also important in the migration of DC and the uptake of antigen. During the discovery of DC-SIGN it became, however, clear that this lectin was identical to a protein identified earlier as a GP120 binding protein on the surface of DC. [27, 28] This was the first clue that DC-SIGN not only functions in normal immunological processes, but is also an important determinant of viral infections. Nowadays it is known that DC-SIGN can function as either an attachment factor or viral entry receptor to mediate infection *in cis* or to allow dissemination from the virus' entry site and mediate subsequent infection *in trans*. (Fig. 3) We will now look at both these misuses and critically assess the experimental approaches used to support these claims.

When talking about viral receptors a subdivision into two types can be made: attachment factors and entry receptors. Whereas the first type is important in targeting large amounts of virus to a cell, entry receptors are necessary *per se* to actually mediate entry into the cell. Though attachment factors may seem redundant at first glance they are actually very important to establish successful infection of a target cell because they present a temporal window for the entry receptor and its ligand to induce viral uptake. So in this thesis I will use the widely accepted definition that a receptor is crucial for uptake and infection of a permissive cell whereas an attachment factor is not needed for infection *per se*,

however, it greatly enhances infection rate by facilitating close proximity virus-cell contact and giving the receptor more time to mediate entry.

Infection *in cis*

In order to infect a cell viruses must achieve the delivery of their genetic material into a permissive cell, i.e. a cell that supports viral replication. To bind and ultimately enter a target cell viruses make use of a diverse set of receptors expressed on the surface of these cells, well known examples are sialic acid as a binding partner for hemagglutinin expressed by influenza virus and CD4 as binding partner for HIV's GP120. Cells that are both permissive to viral infection *and* express the correct set of receptors to allow entry are called susceptible. [(29)] DC-SIGN and L-SIGN also present attractive binding partners for many viruses because they bind with high affinity to glycans rich in mannose, a structure found on the surface of many viruses.

One of the first articles describing the role of DC-SIGN in *cis* infection by a virus was published in 2002 by Alvarez *et al* and concerned Ebola virus. [(30)] This virus encodes a single heavily glycosylated viral surface protein, GP, which is thought to be a major factor governing cellular and tissue tropism. One important determinant for Ebola infection is the folate receptor-α, a highly conserved protein in mammals that is expressed in epithelial and parenchymal cells of many organs. Though it plays a role in the entry into some cell types, it does not so in all. Since very strict biosafety protocols make working with Ebola very difficult, a common approach to circumvent this problem is the use of pseudo-typed recombinant retroviral particles in which GP is expressed in the context of a lentiviral core. In the article they stably transfected the erythroleukemic cell line K562 with DC-SIGN and subsequently incubated them with GP-pseudotyped lentivirus. They measured infectivity 48 hr post-infection with a luciferase assay and showed that the virus can infect cells expressing DC-SIGN with a factor 10 higher than non-transfected K562 cells. The effect of DC-SIGN expression was abolished when cells were first incubated with the DC-SIGN-specific monoclonal antibody MR-1. These results indicate that Ebola indeed utilizes DC-SIGN as either attachment factor or receptor, however, it cannot distinguish between the two. Next they repeated these experiments now using the susceptible but non-permissive Jurkat cell line which are regarded as being receptor deficient. Transfection with either DC-SIGN or L-SIGN and subsequent incubation with the GP-pseudotyped lentivirus indeed resulted in infected Jurkat cells whereas no control cells were positive in the luciferase assay. To see if it was truly due to the presence of either DC-SIGN or L-SIGN they repeated these experiments now using anti-DC-SIGN or anti-L-SIGN antibodies, incubating with these blocking antibodies indeed prevented infection. The ability to confer permissivity to a completely non-permissive cell line seems to imply that both DC-SIGN and L-SIGN can function as true entry receptors. However, contrary with these results is a study by Simmons *et al.* that showed that DC-SIGN and L-SIGN can function only as attachment factor and not as entry receptor. In this paper they transduced primary $CD4^+$ T cells with either DC-SIGN, L-SIGN or CD8. The latter was used as control protein. Expression of DC-SIGN or DC-SIGNR did not result in infection with a GP-pseudotyped lentivirus, although control 293T cells were efficiently infected. Furthermore, Vesicular Stomatitis Virus (VSV)-G pseudotyped lentivirus infected the T cells efficiently, confirming that the block on infection took place at the level of virus entry. [(31)]

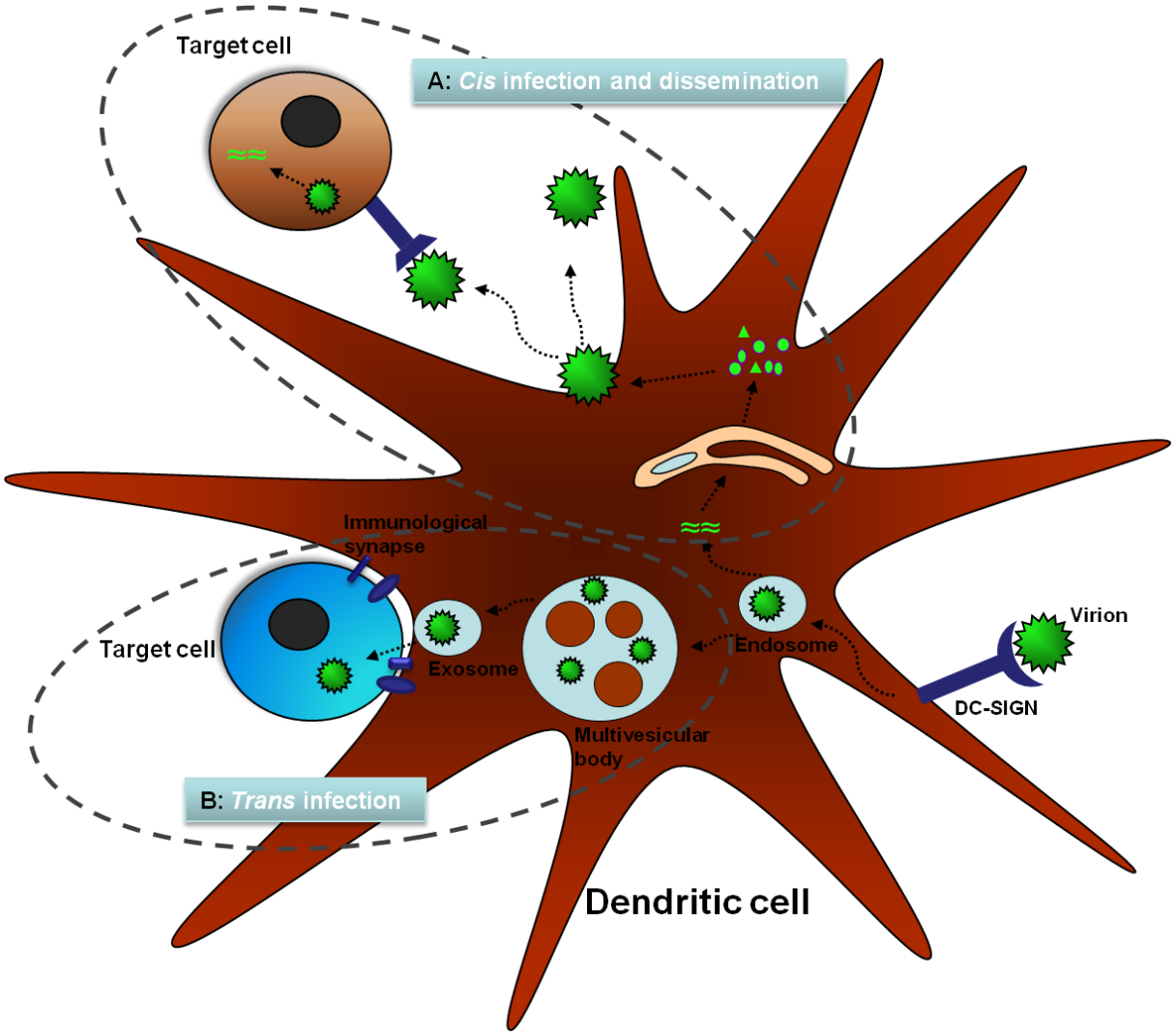

Fig 3. Mediation of *cis* and *trans* infection by DC-SIGN.
DC-SIGN can bind a wide array of viruses via its CRD. Following binding the virus is taken up into the DC via receptor mediated endocytosis after which it can either (A) fuse with the endosomal membrane and escape into the cytosol or (B) be stored in a multivesicular body (MVB). In the first scenario the virus can start an infective cycle of the DC leading to the production of new virus (*cis* infection). Newly produced virus can bud from the DC and infect other target cells. In the second scenario the virus is able to prevent entering the lysosomal pathway and can survive within a MVB for up to 4 days. During this time the DC will migrate from the periphery to the lymph nodes where the virus can leave the DC in an exosomal manner and can infect target cells via the immunological synapse (*trans* infection).

In the case of human cytomegalovirus the previous approach was also used: the authors were able to make low-permissive cells highly permissive by transfecting them with DC-SIGN. The authors, however, do not state that DC-SIGN is the elusive CMV receptor but rather an attachment factor. They base this statement mainly on the fact that CMV also targets cells that lack DC-SIGN expression. To test whether DC-SIGN can definitely not function as an entry receptor the experiment should be repeated with a DC-SIGN transfected non-permissive cell line.

For Measles Virus (MV) it is known that both CD46 and CD150 play an important role in attachment and entry of the virus into permissive cells. By using a set of cell lines

expressing different combinations of CD46, CD150 and DC-SIGN the group of Theunis Geijtenbeek was able to prove that whereas CD46 and CD150 function as entry receptors, DC-SIGN functions solely as an attachment factor. Interestingly, they also showed the *in vivo* relevance of DC-SIGN mediated infection of DC by MV by using blocking antibodies. By blocking DC-SIGN on iDC these cells became resistant to MV infection, illustrating that attachment factors can be truly important for virus infections. [32]

In the case of Sindbis virus, an arthropod-borne virus (arbovirus), the hardly permissive THP-1 cell line was transfected with DC-SIGN, L-SIGN or the Δ35 mutant. The latter is a truncated form of DC-SIGN that lacks the cytoplasmic domain holding the internalization motifs, thereby abrogating endocytosis of the lectin upon ligand binding. [33] Interestingly transfection with either of the full-length SIGN's leads to a strong increase of infection but transfection with the endocytosis-deficient variant hardly increased infectivity. This implies an important role for DC-SIGN's endocytotic properties in infection. However, in their in their flow cytometry data there seems to be a discrepancy between overexpression of the full-length protein when compared to the mutant protein. This could also be due to a loss of affinity of the antibody for the mutant protein. [34]

Similar experiments using the THP-1 cell line have also been performed for another arbovirus, namely dengue virus (DV). Similar results were obtained: also for this virus there seemed to be an important role for endocytosis of the receptor in infection. [35] The authors speculate that DV is able to mediate fusion with the compartmental membrane after acidification of the endosome, as was reported earlier. [36] The observation, that also some infection occurs in the context of the Δ35 mutant, is speculated to be due to random bulk phase endocytosis. This cell line process is known for doing this. The group of Dominique Schols repeated these experiments using DC-SIGN transfected Raji cells which are regarded as being susceptible but completely non-permissive. Also in these experiments it was shown that DC-SIGN can function as an entry receptor. [37]

In conflict with these findings is the paper published by Lozach *et al*. from 2005 that shows that DV infection of DC-SIGN transfected HeLa cells is independent of the internalization motifs. [38] Instead of using truncation mutants they used single amino acid substitution mutants that showed similar expression levels as wild type DC-SIGN. These conflicting findings might be explained by the use of different cell lines expressing other sets of surface proteins, different endocytotic routes or simply the level of overexpression.

In the case of WNV both DC-SIGN and L-SIGN can function as attachment factor. It is, however, very interesting to note that this virus seems to have an unexpected preference for L-SIGN as was shown in K562 cells transfected with either of the two lectins. [39] The SIGN's are not regarded as true entry receptors for WNV, though, because this virus is known to undergo membrane fusion upon lowering of the pH without the need for target proteins or carbohydrates. [40]

Infection *in trans*

We have seen that for a wide array of viruses DC-SIGN and L-SIGN present a means to gain entry into a DC and establish an infectious cycle. For other viruses it is known that infection *in trans* plays a more important role. The first article describing this phenomenon concerned HIV and was published by the group of Theunis Geijtenbeek as an accompanying article to the 2000 Cell paper that reported the discovery of DC-SIGN. [28] They incubated iDC with HIV for 2 hr, washed away unbound virus and then cultured them in the presence of activated T cells. Strikingly more T cells became infected when virus was pre-incubated with iDC when compared to T cells to which the same viral load was

added, implying that iDC actually enhance the infection of T cells. Since DC also express many other lectins they used DC-SIGN specific antibodies to abrogate this effect to show that it is indeed a DC-SIGN-specific effect. To further prove that the observed effect was indeed specific they also transfected THP-1 cells with DC-SIGN, incubated them with HIV, washed and then added them to HEK293T cells expressing CD4 and CCR5, the receptors for HIV. In this experiment they again showed that the CD4/CCR5 expressing cells became infected. The model that the authors propose for *trans* infection postulates that virus binds to DC and retains its infectivity while being shuttled by the DC from the periphery to the lymph nodes. Once in the lymph nodes the virus is able to leave the DC and is free to infect permissive cells present there.

In order for *trans* infection to play a role *in vivo* HIV must be able to retain its infectivity for an extended period of time, because DC take time to migrate from the mucosal tissue to the lymph nodes. They performed a time-course experiment in which they incubated DC-SIGN expressing cell lines with HIV, washed them thoroughly and then cultured them up to 7 days before adding activated T cells. They showed that the virus was able to infect the T cells up until 4 days, whereas cell-free virus lost its infectivity within one day. [28]

The cellular environment of DC-SIGN plays an important role in its ability to mediate HIV uptake and *trans* infection. This is illustrated by the observations that DC-SIGN expressing Raji cells are able to retain HIV in an infectious form and mediate *trans* infection, whereas no transmission of virus was observed for cell lines K562 and HEK293 after transfection with DC-SIGN. [41] The process seems to depend on the intracellular shuttling of the virus after uptake: after Hepatitis C virus is bound to DC-SIGN it is retained in early endosomes in the DC-SIGN$^+$ Raji cells and can still mediate *trans* infection, whereas in DC-SIGN$^+$ K562 cells the virus directly entered the lysosomal pathway where it was subsequently degraded. Furthermore, Lewis X, a carbohydrate blood group antigen that can also bind DC-SIGN, is targeted directly to lysosomes independently of the cell line that is expressing DC-SIGN. [42] These data indicate that viruses may alter the internalization pathway of DC-SIGN to avoid entering the lysosomal pathway in DC and certain B-cell lines but not in other cell lines such as K562 cells. Apparently the necessary machinery for remaining infectious within a cell are missing in some cell lines and one must therefore be very careful to draw conclusions about *trans* infection.

Confocal studies by the group of Dan Littman delved deeper into the mechanism of *trans* infection by proving that binding of GP120 to DC-SIGN leads to co-localization of HIV with endosomal markers. Inhibition of DC-SIGN mediated endocytosis by expressing either of the truncation mutants Δ35 or Δ20 prevented *trans* infection. These results imply that HIV is taken up into the DC, stored in multivesicular bodies and released again in an exosomal manner during formation of the so-called 'infectious synapse' during which DC and T cell are undergoing close interaction.

Similar coculture assays as performed for HIV also proved DC-SIGN's role in *trans* infection by Ebola virus, MV and SARS-CoV. [30, 43, 44] Interestingly the internal-ization motifs of DC-SIGN always seems to play a crucial role in *trans* infection because in all cases the Δ35 mutant is unable to induce *trans* infection.

The principle of *trans* infection has also been shown for L-SIGN. Using L-SIGN transfected HEK293 cells the group of Robert Doms showed that this lectin is also able to transfer HIV and SIV to permissive T cells. They hypothesize that this mechanism may play a role in the crossing of endothelial barriers by these lentiviruses. [45]

In a very interesting study by Chan *et al.* it was shown that L-SIGN can also mediate *trans* infection by SARS-CoV and that this ability depends on the combination of L-

SIGN polymorphisms that is expressed. Whereas in cells expressing L-SIGN alleles encoding the same number of tandem repeats in the neck region, i.e. homozygous expression, the lectin binds with high affinity to the virus which leads to subsequent viral degradation. On the contrary, cells expressing L-SIGN alleles encoding different numbers of tandem repeats, i.e. heterozygous expression, bind with lower affinity to SARS-CoV and the virus is degraded less efficiently. The heterozygous cells also show a markedly increased tendency for *trans* infection. The authors also performed a genetic risk association study which showed that individuals homozygous for L-SIGN repeats are less likely to develop SARS than heterozygous individuals. [46]

Discussion

Approaches to identify viral receptors

There are several methods than can be utilized to prove that a certain surface structure functions as a viral receptor. Until the mid 80's virus receptors were characterized enzymatically, for example by removal of cell surface molecules (sialic acid by neuraminidase, proteins by proteases, heparin sulphates by heparinases), or by competition (retroviruses). [47] The development of monoclonal antibodies greatly boosted the identification of viral receptors. By blocking specific structures on the plasma membrane those structures important for infection could be pinpointed, because blocking them would prevent infection of the cell. However, by itself this approach cannot distinguish between an attachment factor and an entry receptor.

A more elegant approach is transfection of the gene encoding a putative receptor into susceptible, non-permissive cells and see if they can be made permissive. A final approach is to produce recombinant soluble receptor and incubating this with the virus prior to adding the virus to permissive cells. If the soluble protein is indeed a receptor it will bind to its ligand on the viral particle and prevent it from binding to cells. These three approaches are also regarded as criteria for substantiating that a putative receptor is a bona fide receptor important in virus entry.

Though it is relatively easy to prove that a certain cell structure functions as a virus receptor, it is very difficult to experimentally prove whether it is an attachment factor or an entry receptor. The most unambiguous approach seems to be the use of permissive but completely non-susceptible cells made susceptible by transfection with the putative entry receptor. To check whether a cell line is adequate it should first be tested if it is able to support viral replication (permissivity), this can be done by directly bringing the virus' genetic material into the cell by transfection (e.g. by electroporation or lipofectamine). If the cell line supports replication of the virus upon transfection but does not so upon exposure to live virus, it can be regarded as being permissive but non-susceptible. This approach was used for example in the case of Ebola to show that the SIGN's only function as attachment factors. [31] To show that the effect is indeed specific, a control experiment should always be performed in which the effect of receptor expression is undone by incubating with blocking antibodies. The main problem with this approach is that an appropriate cell line is essential: cells that are susceptible at a very remote level might still create a confounder effect leading to misidentification of a structure as entry receptor instead of a attachment factor. Unfortunately adequate cell line are not always available.

The most black and white approach to truly prove a receptor to be an entry receptor remains the elucidation of the mechanism of membrane fusion by means of structural studies.

Depending on the definition of entry receptor versus attachment factor it might be best to refer to the different structures involved in virus binding and infection in a sequential manner. What we call attachment factors are structures that bind large amounts

of viral particles to the membrane of cells, and would be more accurately called 'first line receptors'. In the case of viruses like MV and sindbis virus DC-SIGN belongs to this category. Also opsonization of virus with antibody and subsequent targeting to Fc receptors, a process termed Fc receptor mediated enhanced infection, may be regarded as belonging to this category. The 'second line receptors' are the classical receptors that not only bind virus but are also needed for uptake and membrane fusion. This model would imply that loss of first line receptors would lead to a substantial decrease in infectivity, though infection is still possible. On the other hand loss of a second line receptor would lead to a complete loss of infection. [48] Some viruses, like WNV, may only have need for first line receptors, because they undergo membrane fusion upon acidification of the endosome without help from any cell expressed structures that function as second line receptor. [40]

Role of endocytotic properties

DC-SIGN is an endocytotic receptor that allows the binding and subsequent uptake of virus into a cell. Also L-SIGN is thought to have at least some endocytotic properties. It is attractive to assume that viruses exploiting DC-SIGN or L-SIGN as entry receptor utilize this pathway to enter a cell via an endocytotic vesicle and then escape at some point into the cytoplasm. Since the internalization motifs are all present on the cytoplasmic tail of the lectin a truncation mutant in which the internalization motifs are absent is an additional way to prove an entry receptor: in the case of an attachment factor endocytosis of the receptor is less likely to play a role, and thus expression of the Δ35 mutant should show no difference with the full-length protein, however, for an entry receptor depending on a SIGN's endocytotic properties loss of the internalization motifs would prevent infection. In the latter case it is feasible that some infection remains due to spontaneous endocytosis of receptor as seen in certain cell lines. However, in the study by Klimstra *et al* the cell surface expression of the mutant protein seems a factor three lower. [34] This can either be caused by a decreased affinity of the antibody for the mutant protein or by lower expression levels of the truncated protein. If it is indeed due to lower expression levels this makes comparison of results susceptible to confounder effects. An interesting approach to circumvent this problem would be to make use of full-length SIGN mutants in which single amino acids of the internalization motif are substituted. Since single amino acid substitutions are likely to have a less severe impact on protein expression and stability than truncation mutants would, this could present a less artificial means to study the role of DC-SIGN's endocytotic properties. The latter approach was used by Lozach *et al*. and proved successful. [38]

Interestingly L-SIGN lacks the tyrosine based motif in its cytoplasmic domain and was shown to be unable to induce endocytosis of bound ligand as was shown by its lacking ability to mediate internalization of neoglycoprotein. [19] This implies that endocytosis of the lectin and its bound virus cannot play a role in either *cis* or *trans* infection by L-SIGN. For all viruses for which it is known that both DC-SIGN and L-SIGN can induce *cis* infection it is known that endocytosis always plays a role in the case of DC-SIGN, except in the case of DV. It therefore remains puzzling how L-SIGN can mediate infection. It would be interesting to see whether L-SIGN truncation mutants or substitution mutants in which the two remaining internalization motifs are disabled are still able to induce *cis* infection. Unfortunately no article has yet delved into this.

Complementary entry routes may exist

Many of the viruses described here have a broader tropism than just SIGN expressing cells. This does, however, not mean that these lectin cannot be an entry receptor by definition. For example, in a study by Dominique Schols *et al* on DV they were

able to block infection of DC-SIGN expressing Raji cells by using the mannose-specific plant lectins HHA and GNA, which bind to mannose residues on the virion. Both lectins also had a dose dependent effect in blocking the infection of IL-4 treated monocytes which have a high expression of DC-SIGN. However, when they used these same lectins to block infection of the permissive cell line Vero-B, which are DC-SIGN negative, there was no antiviral effect observed. [37] These results imply that viruses might utilize additional mechanisms to a enter a cell dependent on the cell-type involved.

The redundancy of the role of DC-SIGN in virus infection is also reflected in HIV-1. This virus was shown to be able to bind to certain cell surface receptors other than CD4 or the co-receptors. The syndecans, which are a family of heparin sulfate proteoglycans, can also promote HIV-1 attachment when expressed together with CD4 and chemokine receptors. Members of this family are present on endothelial cells and macrophages and were shown to efficiently capture and transmit HIV-1 to permissive cells via gp120, in a manner similar to DC-SIGN. [49]

Similar process as observed for DV and HIV-1 may also play a role in determining the cellular tropism of Ebola virus, which is known to be able to infect a broad range of cell types.

Virus origin influences binding affinity

Interestingly it was shown for both SV and WNV that virus grown in mosquito cells was much more efficient in binding to DC-SIGN and L-SIGN than virus grown in human cell lines. This illustrates the difference in glycosylation patterns between virus grown in the arthropod vector and in the human host. [34]

Virus grown in different cell types can not only differ in glycosylation patterns but also in the proteins that they might incorporate in their envelop during budding. During HIV infection of a cell certain cell surface molecules can become upregulated, which subsequently become incorporated into newly produced virus particles. This process seems to be tightly regulated because other molecules remain on the host cell's surface and do not become incorporated into the virus. In the case of HIV it was, for example, shown that interactions between ICAM-1, incorporated in the virion, and LFA-1, expressed on T cells, can enhance HIV attachment and infection of certain target cells. [50]

***In vivo* relevance**

Some of the studies presented here made use of α-DC-SIGN blocking antibodies in combination with iDC. This approach might reveal clues whether or not DC-SIGN is indeed an important factor governing viral pathogenesis. Although it is unlikely that antibody-based blocking is a 100% effective, resulting in some residual infection even if the targeted receptor is the entry receptor, it is an interesting approach to show that DC-SIGN truly plays a role in the infection of iDC. If blocking DC-SIGN on iDC has only a negligible effect on infection efficiency it is most likely that the cell expresses more receptors, implying a redundant role for the lectin in virus targeting and pathogenesis.

All the studies presented here have been performed *in vitro* using either transfected cell lines or natural SIGN expressing cells. Studying whether the SIGN's truly play a role *in vivo* would be very interesting. Investigating their contribution in a natural host is, however, very challenging because blocking experiments are difficult to perform and complete knock-out models will most likely result in artifacts, because also formation of the immunological synapse is prevented. Philosophizing about the importance of the SIGN's *in vivo* is possible, though. When arguing from the viral point of view it is very attractive to bind to DC-SIGN, because DC are abundantly present in the tissues where viruses penetrate the host, e.g. the respiratory tract for MV, mucosal tissues for HIV and the skin for arboviruses like

DENV. Not only are DC present at all these entry sites but they are also highly mobile cells that allow trafficking of the virus throughout the body to other tissues. Additionally, L-SIGN is expressed on endothelial cells in the lungs and could therefore present an early target for viruses that transmit via aerosols. In this respect we can conclude that both SIGN's would make attractive targets for viruses *in vivo*, especially to mediate dissemination from the peripheral entry sites to other organs, most notably the lymph nodes.

Conclusion

The most straightforward approach to differentiate a viral attachment factor from a viral entry receptor is overexpression of the putative receptor in permissive cells that are completely non-susceptible and see whether this makes them susceptible to infection with the studied virus. Though it is difficult to identify an unambiguous *in vivo* role for DC-SIGN and L-SIGN during viral pathogenesis it seems likely that they do play an important role in the infection of DC and the dissemination of the virus throughout the body, whether this is depending on *cis* or *trans* infection. DC-SIGN and L-SIGN may therefore prove suitable targets for rational drug design in order to prevent infection of the SIGN expressing cells and thereby slowing down viral spreading.

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