

VU Research Portal

Langerhans cells and dendritic cells in innate defense against pathogens

de Jong, M.A.W.P.

2010

document version

Publisher's PDF, also known as Version of record

[Link to publication in VU Research Portal](#)

citation for published version (APA)

de Jong, M. A. W. P. (2010). *Langerhans cells and dendritic cells in innate defense against pathogens*.

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal ?

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

E-mail address:

vuresearchportal.ub@vu.nl

Dendritic cells mediate herpes simplex virus infection and transmission through the C-type lectin DC-SIGN

M.A.W.P. de Jong^{1*}, L. de Witte^{1*}, A. Bolmstedt², Y. van Kooyk¹,
T.B.H. Geijtenbeek¹

¹ Department of Molecular Cell Biology and Immunology, VU Medical Center, Amsterdam, The Netherlands. ² Department of Clinical Virology, University of Göteborg, Göteborg, Sweden.

* These authors contributed equally

**Journal of
General Virology**

Journal of General Virology 2008;89(10):2398-409

Abstract

Dendritic cells (DCs) are essential for the induction of specific immune responses against invading pathogens. Herpes simplex virus (HSV) is a common human pathogen that causes painful but mild infections of the skin and mucosa, and which results in latency and recurrent infections. Of the two HSV subtypes described, HSV-1 causes mainly oral-facial lesions, whilst HSV-2 is associated with genital herpes. DCs are involved in HSV-induced immune suppression, but little is known about the molecular interactions between DCs and HSV. This study demonstrated that HSV-1 and -2 both interact with the DC-specific C-type lectin DC-SIGN. Further analyses demonstrated that DC-SIGN interacts with the HSV glycoproteins gB and gC. Binding of HSV-1 to immature DCs depended on both DC-SIGN and heparan sulfate proteoglycans. Strikingly, HSV-1 infection of DCs was almost completely inhibited by blocking antibodies against DC-SIGN. Thus, DC-SIGN is an important attachment receptor for HSV-1 on immature DCs and enhances infection of DCs *in cis*. In addition, DC-SIGN captures HSV-1 for transmission to permissive target cells. These data strongly suggest that DC-SIGN is a potential target to prevent HSV infection and virus dissemination. Further studies will show whether these interactions are involved in HSV-induced immune suppression.

Introduction

Herpes simplex virus (HSV) is a double-stranded DNA virus that belongs to the family *Alphaherpesviridae*. HSV is a common human pathogen that causes painful but mild infections of the skin and mucosa, resulting in cold sores and blisters. Two subtypes of HSV have been described: HSV-1, which mostly causes oral-facial lesions, and HSV-2, which is associated with genital herpes. HSV is transmitted by direct body contact with infected lesions or body fluids and enters the body at the mucosal tissues or lesions of the skin. The primary target cells for HSV are the epithelial cells and keratinocytes. During primary infection, HSV enters the cutaneous sensory neuron and travels to the dorsal root ganglia where it establishes the characteristic lifelong latent infection. At times when the immune system is suboptimal, such as under conditions of stress, trauma, exposure to UV light, fatigue or a common cold, the virus escapes immune surveillance and migrates via the peripheral nerve towards the epithelium or skin, where it causes recurrent herpes simplex lesions (reviewed by (1;2)).

In addition to painful cold sores and blisters, HSV can cause ocular herpes stromal keratitis, making it the major infectious cause of blindness in the western world (discussed in (3)). In addition, HSV can cause life-threatening infections in individuals with inadequate cellular immune responses, such as newborns or immunocompromised patients. This indicates that, under normal conditions, dissemination of HSV throughout the body is prevented by the immune system. In addition, HSV has evolved strategies to escape our immune system and cause latent infection.

As sentinels of the immune system, dendritic cells (DCs) have an important regulatory function. Due to their specific location in the subepithelium and dermis,

DCs are a target for invading pathogens such as human immunodeficiency virus type 1 (HIV-1), human cytomegalovirus (HCMV) and measles virus (MV) (4-6). These viruses target DCs for infection, dissemination and immune evasion by targeting the DC-specific C-type lectin DC-SIGN.

HSV-1 has developed several mechanisms to escape immune surveillance by DCs. DCs express the HSV entry receptors HVEM (Hve-A) and nectin-2 (Hve-B), as well as heparan sulfates, which mediate attachment of HSV-1. DCs are productively infected with HSV (7-10), resulting in apoptosis (11). Moreover, HSV-1 infection strongly affects DC function by interfering with DC maturation, including downregulation of co-stimulatory molecules as well as CD83 and CD1 molecules and major histocompatibility complex class I (10;12). HSV-1 infection also decreases interleukin (IL)-12 production and lowers the allostimulatory capacity of DCs (13;14). The C-type lectin DC-SIGN expressed by DCs plays an important role in attachment and dissemination of various viruses (4-6). In addition, Halary *et al.* demonstrated that herpesvirus family member HCMV interacts with DC-SIGN; however, the authors concluded that HSV-1 glycoproteins do not bind to DC-SIGN (5).

To get a better understanding of HSV pathology and to determine targets to enhance the immune response to HSV, it is essential to understand the molecular interactions of HSV with DCs. Here, we investigated the receptors involved in the interaction between human monocyte-derived DCs and HSV. We demonstrated that HSV-1 and -2 interact with DC-SIGN via glycoproteins gB and gC. Binding of HSV to DCs depended on both heparan sulfate proteoglycans and DC-SIGN. Our data demonstrate that DC-SIGN not only increases DC infection by HSV-1 but also captures HSV-1 for transmission to permissive target cells. Thus, DC-SIGN is an attachment receptor for HSV-1 that contributes to the infectivity and transmission of HSV.

Results

Recombinant DC-SIGN interacts with both HSV-1 and -2

DC-SIGN is a receptor for various viruses, including the herpesvirus family member HCMV (5). HSV contains multiple envelope glycoproteins that are potential ligands for DC-SIGN. We therefore investigated whether this C-type lectin interacts with HSV-1 and -2. HSV virions were coated on plates and binding of recombinant DC-SIGN was analysed by ELISA. Both viruses were efficiently coated on the plate as determined using an antibody against HSV (Figure 1A,B, right panels). Recombinant DC-SIGN efficiently interacted with both HSV-1 and -2 in a concentration-dependent manner (Figure 1A,B, left panels). The interaction was specific for the lectin domain of DC-SIGN, as pre-incubation with the polycarbohydrate mannan blocked the binding (Figure 1A,B). To exclude the possibility that DC-SIGN specifically interacts with laboratory strains, we investigated the binding of DC-SIGN to a clinical isolate. Both attenuated and clinical HSV strains strongly bound to DC-SIGN and this interaction was specific, as mannan could completely block the binding (Figure 1C). These results demonstrated that soluble DC-SIGN interacts with both HSV-1 and -2. To investigate further the role of DC-SIGN with HSV, we used the HSV-1 isolate Syn17⁺.

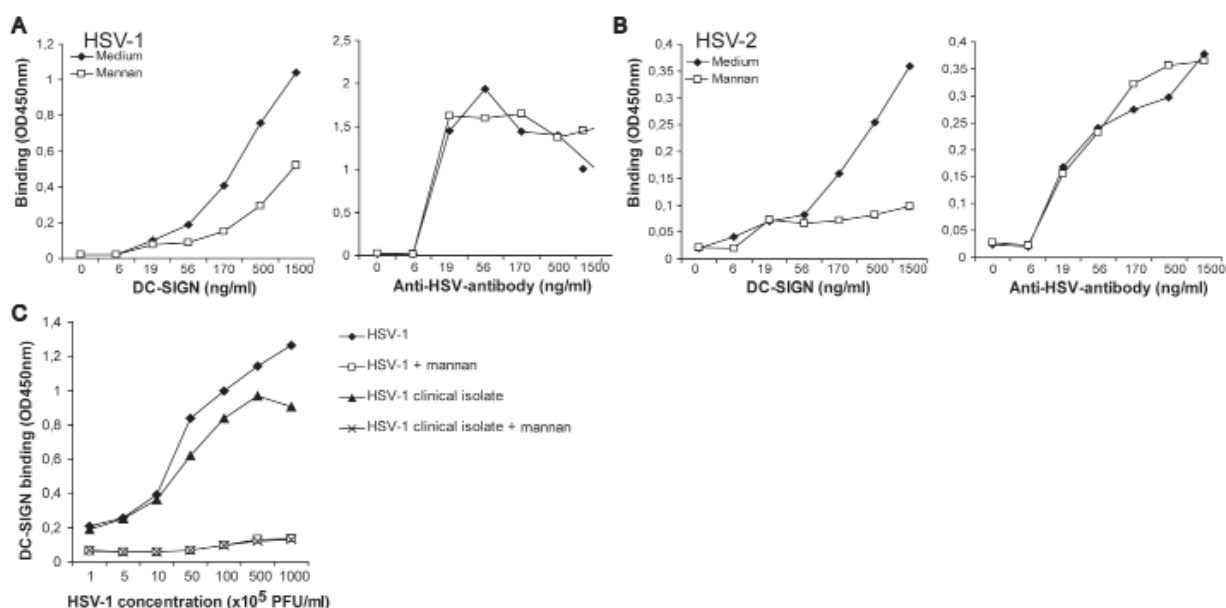


Figure 1 HSV-1 and HSV-2 interact with soluble DC-SIGN. (A,B,C) HSV-1 (KOS321), a clinical isolate of HSV-1 and HSV-2 (333) were coated onto ELISA plates (1×10^7 PFU/ml, unless depicted otherwise) and the binding of DC-SIGN-Fc was determined by ELISA. Specificity of the DC-SIGN-Fc HSV interaction was determined by measuring binding in the presence of mannan. As a control for coating the viruses were detected by an antibody against HSV gB (B11D8). One representative experiment out of three is shown.

HSV-1 gB and gC interact with DC-SIGN

The envelope of HSV-1 contains 12 different glycoproteins. gB and gC have been shown to mediate binding of HSV-1 to target cells, whereas gB, gD and gH/L are important for entry of the virus into target cells (15). We investigated the interaction of DC-SIGN with gB and gC using a soluble DC-SIGN binding assay, as these glycoproteins are involved in binding to target cells. Soluble DC-SIGN interacted with both coated HSV-1 virions and the purified glycoproteins gB and gC (Figure 2A). The interaction was specific for the binding site of DC-SIGN, as the calcium chelator EGTA and the polycarbohydrate mannan both completely inhibited the interaction (Figure 2A). Next, cell lines expressing high levels of DC-SIGN and low levels of heparan sulfates (data not shown) were used to determine the interaction of gB and gC with cellular DC-SIGN. gB and gC were coated onto fluorescent beads and interaction with mock-transfected K562 and K562/DC-SIGN cells was determined. K562/DC-SIGN cells efficiently interacted with gB and gC, in contrast to the mock-transfected K562 cells (Figure 2B). The interaction was specific for DC-SIGN, as mannan, EGTA and antibodies against DC-SIGN inhibited the interaction, similar to the HIV-1 gp120-DC-SIGN interaction (Figure 2B).

Both DC-SIGN and heparan sulfates are involved in HSV-1 binding to DCs

The above results showed that HSV-1 gB and gC interact with cellular DC-SIGN. As DCs are among the first antigen-presenting cells to interact with invading HSV, we investigated whether DC-SIGN plays a role in this interaction. In contrast to K562 cells, DCs express both DC-SIGN and the HSV-1 attachment receptors, heparan sulfates (15). We therefore investigated the role of DC-SIGN and heparan sulfates in HSV-1 binding to DCs. Both gB and gC interacted with DCs, and a blocking antibody

against DC-SIGN completely blocked the interaction of gB with DCs (Figure 3A). Strikingly, the antibody against DC-SIGN inhibited the gC interaction to a lesser extent than the gB interaction, suggesting that gC also binds to other receptors (Figure 3A). These data demonstrated that DC-SIGN interacts with HSV-1 through gB and, to a lesser extent, through gC.

Next, we investigated the interaction of HSV-1 with DCs and the involvement of heparan sulfates and DC-SIGN. DCs were treated with heparinase III, which specifically digests cellular heparan sulfate chains on DCs (16). Heparan sulfate expression was found to be strongly decreased after treatment (Figure 3B). This was further confirmed by the increased expression of the 3G10 epitope, representing heparinase-digested heparan sulfates on heparinase-treated DCs (Figure 3B). The interaction of whole HSV-1 particles with DCs was explored further using HSV-1 particles coated on fluorescent beads. DCs efficiently interacted with HSV-1 (Figure 3C) and this was partially mediated through DC-SIGN, as antibodies against DC-SIGN inhibited the interaction (Figure 3C). However, binding was not blocked to background levels, suggesting that other receptors, such as heparan sulfates, also play a role. Indeed, heparinase treatment decreased the binding of HSV-1 to DCs and, in combination with blocking antibodies against DC-SIGN, binding was further blocked (Figure 3C). These data showed that both heparan sulfates and DC-SIGN mediate the binding of HSV-1 to DCs.

DC-SIGN is not an entry receptor for HSV-1 but enhances infection *in cis*

To determine whether DC-SIGN is an entry receptor for HSV-1, we used DC-SIGN-transfected CHO cells, expressing high levels of DC-SIGN (Figure 4A). CHO cells were not susceptible to HSV-1, in contrast to the permissive GMK cells, which were efficiently infected (Figure 4B). Even at high viral input, expression of DC-SIGN did not confer susceptibility to HSV-1, demonstrating that DC-SIGN is not an entry receptor for HSV-1 (Figure 4B). Next, we investigated the function of DC-SIGN in HSV-1 infection of permissive cells. Parental Raji cells are infected with HSV-1 as

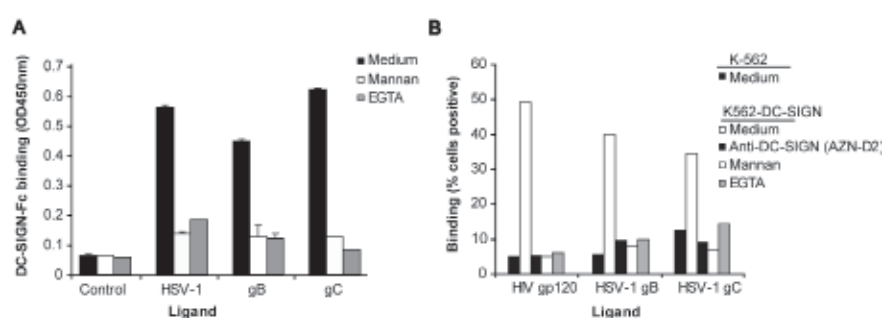


Figure 2. HSV-1 gB and gC interact with DC-SIGN. (A) DC-SIGN-Fc interaction with HSV-1, HSV-1 gB and gC was determined by a DC-SIGN-Fc-binding ELISA. Specificity of the DC-SIGN-Fc HSV-1 interaction was determined by measuring binding in the presence of mannan or EGTA. One out of four representative experiments is shown. Error bars represent standard errors of triplicates. (B) The interaction of purified gB and gC or HIV-1 gp120 with either parental K562 cells or K562-DC-SIGN transfectants was measured by fluorescent bead binding assay. Specificity of the DC-SIGN interaction was determined by measuring binding in the presence of a blocking antibody against DC-SIGN (AZN-D2), mannan or EGTA. One representative experiment out of two is shown.

determined by gB expression on the cell surface. However, Raji cells expressing DC-SIGN were infected more efficiently by HSV-1, demonstrating that DC-SIGN enhances HSV-1 infection *in cis* (Figure 4C). The increased levels of infection were inhibited by mannan, demonstrating that the enhancement was specific for DC-SIGN. Thus, DC-SIGN functions as an attachment receptor for HSV to enhance HSV infection *in cis*.

DC-SIGN enhances infection of DCs *in cis*

Next, we investigated the role of DC-SIGN in HSV-1 infection of DCs. Immature DCs were incubated with different concentrations of HSV-1 and infection was measured by staining for HSV-1 gB. As shown previously (10), immature DCs were efficiently infected with HSV-1 at different viral inputs (Figure 5C,D), whilst the viability of the cells was not significantly decreased (data not shown). Uninfected DCs showed high levels of DC-SIGN expression and low CD86 levels (Figure 5A, left panel). Infection of DCs with HSV-1 partially increased CD86 expression, but it

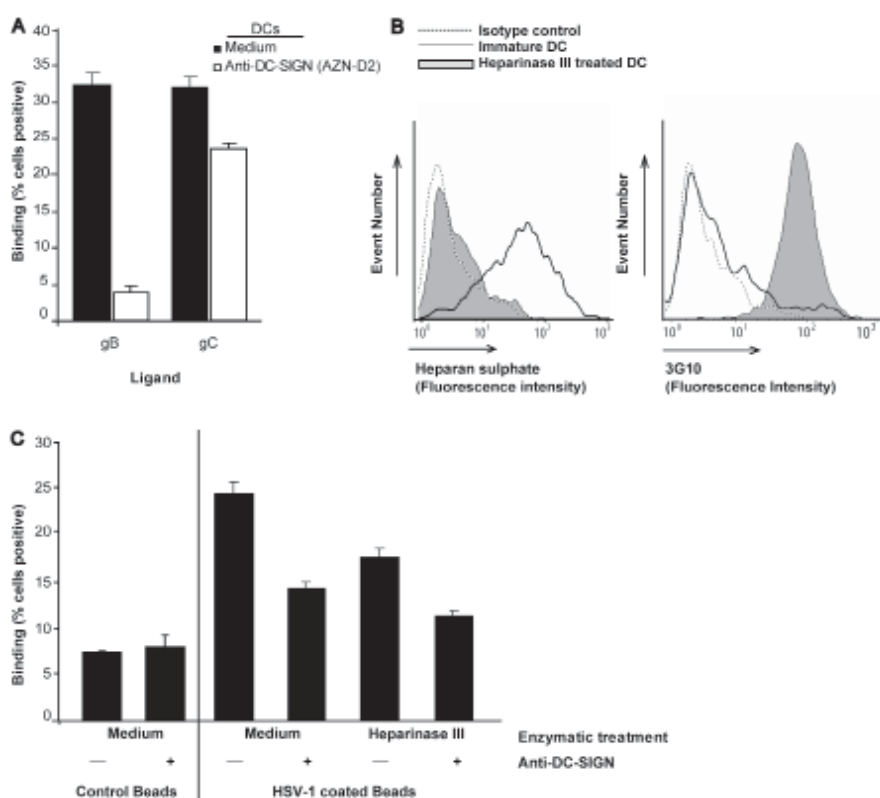


Figure 3. HSV-1 interacts with DCs through DC-SIGN and heparan sulfates. (A) HSV-1 gB or gC binding to DCs was measured using the fluorescent bead adhesion assay. Specificity of the binding for DC-SIGN was determined by measuring the adhesion in the presence of anti-DC-SIGN (AZN-D2). One representative experiment out of three is shown. Error bars represent standard errors of duplicates. (B,C) Immature DCs were treated with Heparinase III. (B) The treatment was analyzed by staining with an antibody against heparan sulfates (10E4) or an antibody against cleaved heparan sulfates (3G10) and subsequent analysis of expression by flow cytometry. Empty and filled histograms represent untreated and Heparinase III treated DCs, respectively. The dashed lines represent the isotype control. (C) DCs were pre-incubated with mannan (1 mg/ml) and binding of HSV-1 coated beads or control beads was measured using the fluorescent bead adhesion assay. Error bars represent standard errors of duplicates. One representative experiment out of three is shown.

was not as strong as that observed with LPS-matured DCs. The expression of DC-SIGN was reduced compared with uninfected cells, whereas the downregulation of DC-SIGN by LPS-matured DCs was stronger than HSV-infected DCs (Figure 5A, middle and right panels).

To determine the role of DC-SIGN in HSV infection of DCs, we investigated mRNA expression of the HSV-1 thymidine kinase (*tk*) gene. Infection of DCs with HSV-1 induced the expression of *tk* mRNA after 6 h as measured by quantitative real-time PCR (Figure 5B). Expression was significantly blocked with mannan or antibodies against DC-SIGN, whilst isotype control antibodies did not affect DC infection. These data strongly suggested that DC-SIGN increases HSV-1 infection of DCs.

To confirm these findings at the protein level, DCs were infected with HSV-1 at different m.o.i. in the presence of mannan, blocking antibodies against DC-SIGN or an isotype control. Strikingly, HSV-1 infection was strongly decreased by blocking DC-SIGN with either mannan or anti-DC-SIGN antibodies, whilst isotype antibodies did not have an effect (Figure 5C,D). These data showed that DC-SIGN enhances HSV-1 infection of DCs *in cis*.

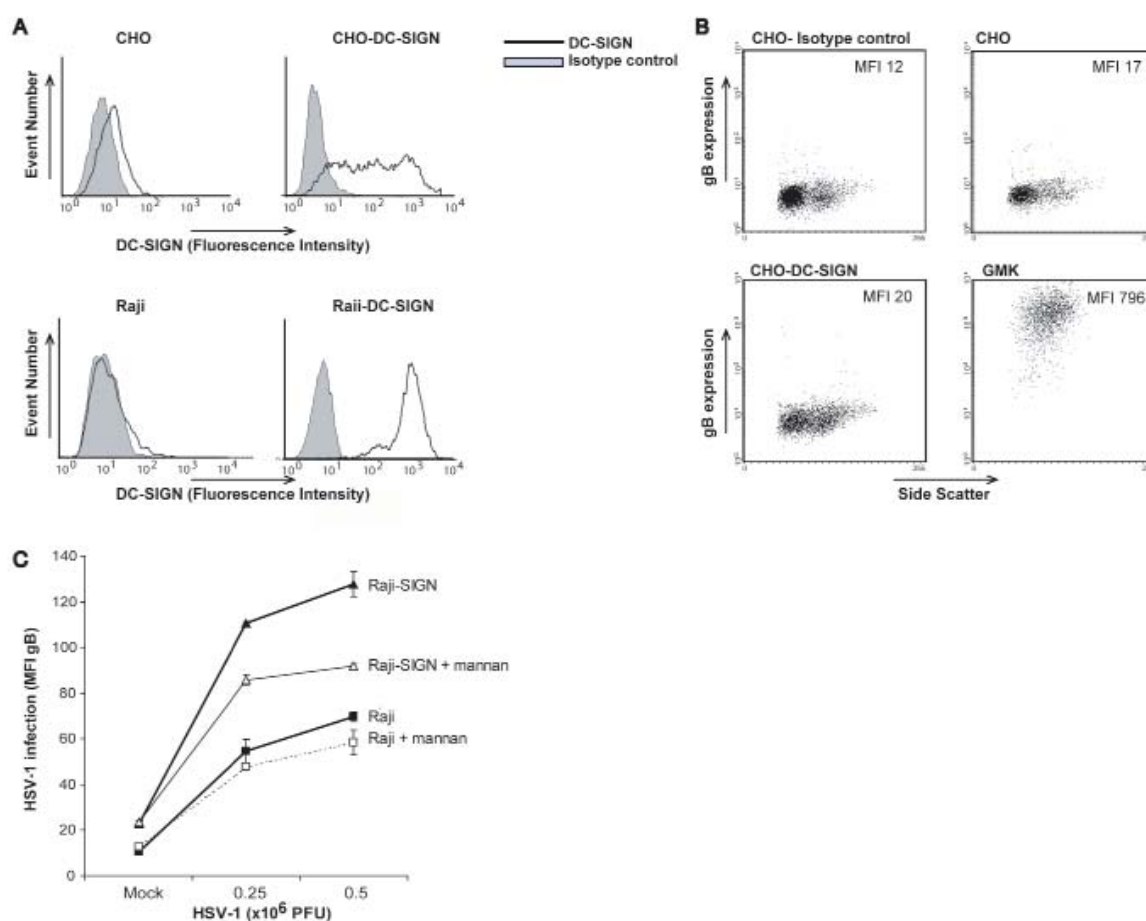


Figure 4. DC-SIGN mediates infection of immature DCs with HSV-1 *in cis*. (A) CHO- and Raji-DC-SIGN transfectants were analyzed for DC-SIGN expression by flow cytometry. Open histograms represent specific staining; filled histograms the isotype controls. (B) GMK, CHO and CHO-DC-SIGN cells (2×10^5 cells) were infected with a high concentration of HSV-1 (1×10^7 PFU) (C) Raji and Raji-DC-SIGN cells (5×10^4 cells) were pre-treated with mannan and infected with the depicted concentrations

DCs capture HSV-1 for transmission in a DC-SIGN-dependent manner

DC-SIGN has been shown previously to be important in viral transmission to target cells (4-6). Therefore, we investigated whether DCs are also capable of transmitting HSV-1 via DC-SIGN. DCs were inoculated with HSV-1 for 2 h at 4 or 37°C and washed extensively to remove unbound HSV-1. Co-localization of HSV-1 gB with CD1a and DC-SIGN was analysed by immunofluorescence confocal microscopy. We did not observe any internalized HSV-1 in DCs incubated with HSV-1 at 4°C and viral particles were detected at the cell surface only sporadically (data not shown). In contrast, large numbers of viral particles were observed both intracellularly and at the cell surface of DCs incubated at 37°C (Figure 6A). HSV-1 partially co-localized with DC-SIGN at the cell surface (Figure 6A). These data demonstrated that HSV-1 is internalized by DCs within 2 h and suggest that DC-SIGN on DCs is involved.

Next, we investigated whether DCs transmit HSV-1 to other target cells. DCs were incubated with a recombinant HSV-1 virus (strain v44) expressing VP16 linked to GFP (HSV-1-GFP), which results in GFP expression following infection (17). Cells were washed extensively and added to permissive target cells (GMK or Jurkat cells). Transmission was followed over time by flow cytometry. Infection of the target cells (Jurkat and GMK) could be detected within 18 h of co-culture and the infection increased over time (Figure 6C,D). Immunofluorescence analyses suggested that transmission occurred in a cell-cell contact-dependent manner, as foci of HSV-1-infected cells were observed (Figure 6B). Strikingly, pre-incubation of DCs with mannan partially inhibited the transmission of HSV-1 to target cells. These data demonstrated that DCs capture HSV-1 and are able to transmit the virus to target cells in a DC-SIGN-dependent manner.

Discussion

DCs are the first antigen-presenting cells to encounter HSV-1 and -2 at the site of infection, mucosal tissue in the oral and genital areas, respectively. HSV has been shown to escape immune surveillance by DCs. Together with the incidence of HSV-1/-2 co-infections with HIV-1 and the increased risk of acquiring HIV-1 in the presence of HSV infection, it is important to understand the molecular interactions between HSV and DCs.

Here, we investigated the interaction of HSV and DCs. We demonstrated that HSV-1 glycoproteins gB and gC, as well as HSV-1 virus particles, interact with both soluble and cellular DC-SIGN. The interaction of DCs and gB was primarily mediated by DC-SIGN, whereas both heparan sulfates and DC-SIGN were involved in binding of gC to DCs. Our data demonstrated that DCs capture HSV-1 through DC-SIGN, which leads to efficient infection of DCs and virus transmission to permissive cells.

DC-SIGN is a receptor for various viruses, including HIV-1, Ebola virus, MV, dengue virus and, interestingly, the herpesvirus family member HCMV (4-6). Here, we demonstrated that DC-SIGN is also involved in HSV-1 interactions with DCs. Both gB and gC bound to soluble and cellular DC-SIGN. This is in contrast to a previous report that demonstrated that HSV-1 gB did not bind to DC-SIGN (5). The recombinant HSV-1 gB used by those authors was produced in insect cells by

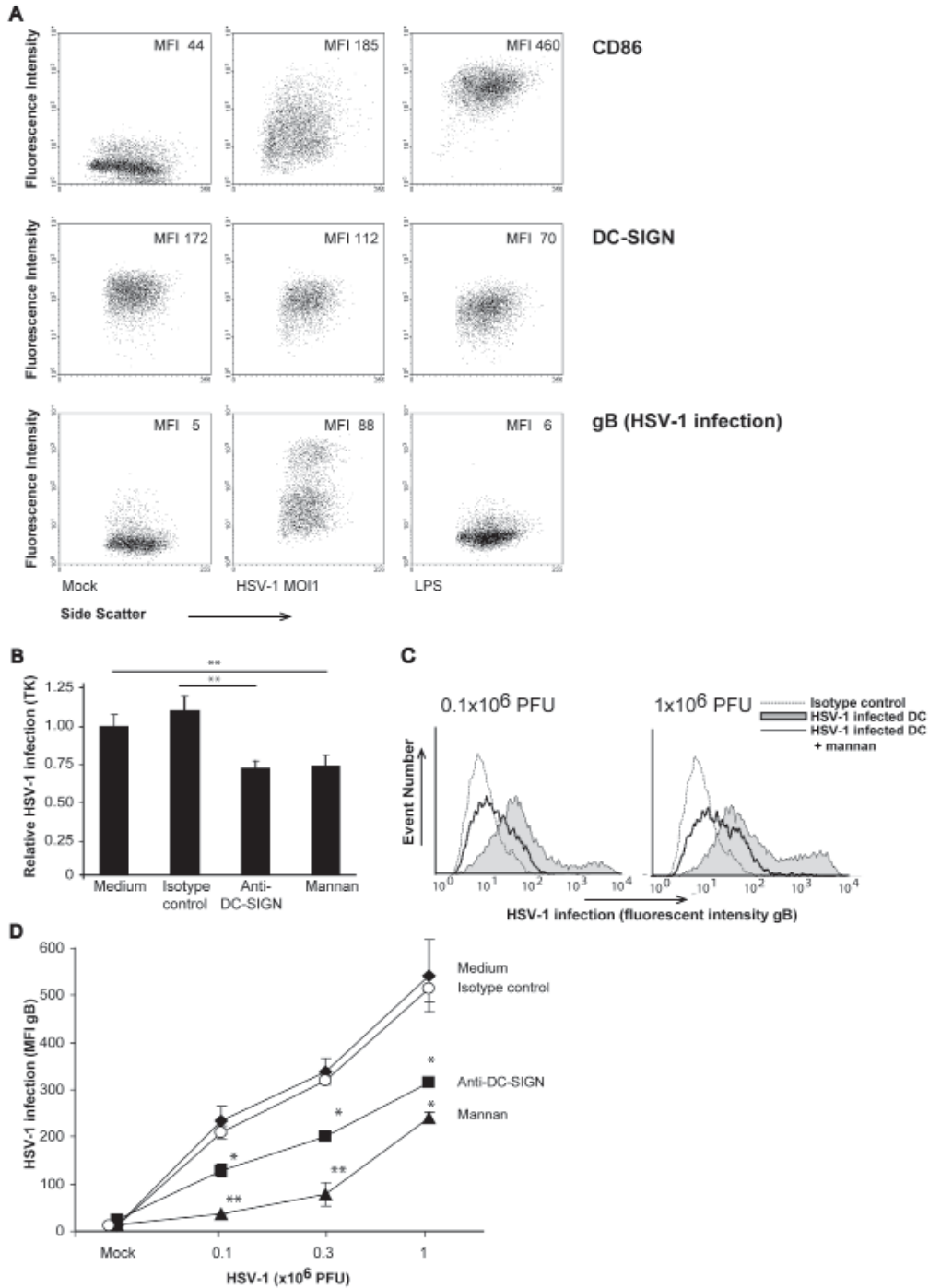


Figure 5. DC-SIGN enhances infection of immature DCs with HSV-1. (A,C,D) Immature DCs (5×10^4 cells) were infected with different concentrations of HSV-1 for 24 hours (A) CD86, DC-SIGN and HSV-1 gB staining on immature, LPS-matured and DCs (1×10^6 PFU) was measured by flow cytometry. (B) Immature DCs (1×10^5 cells) were pre-treated with mannan, anti-DC-SIGN or isotype control antibody before infecting them at MOI 1. After 6 hours, mRNA levels of thymidine kinase was measured by quantitative real-time PCR. Medium condition infected with HSV-1 MOI 1 was set at 1 for analysis. (C,D) To determine the level of HSV-1 infection, HSV-1 gB expression was measured by flow cytometry. Cells were pre-treated with mannan, anti-DC-SIGN or an isotype control antibody to determine the contribution of DC-SIGN to DC infection. Error bars represent standard deviation of duplicates. One representative experiment out of two is shown. Significance was determined using a one-way analysis of variance (ANOVA) with the post hoc Bonferroni test using Graphpad prism software. Asterisks represent p-value compared to the medium condition, * $p < 0.05$, ** $p < 0.01$.

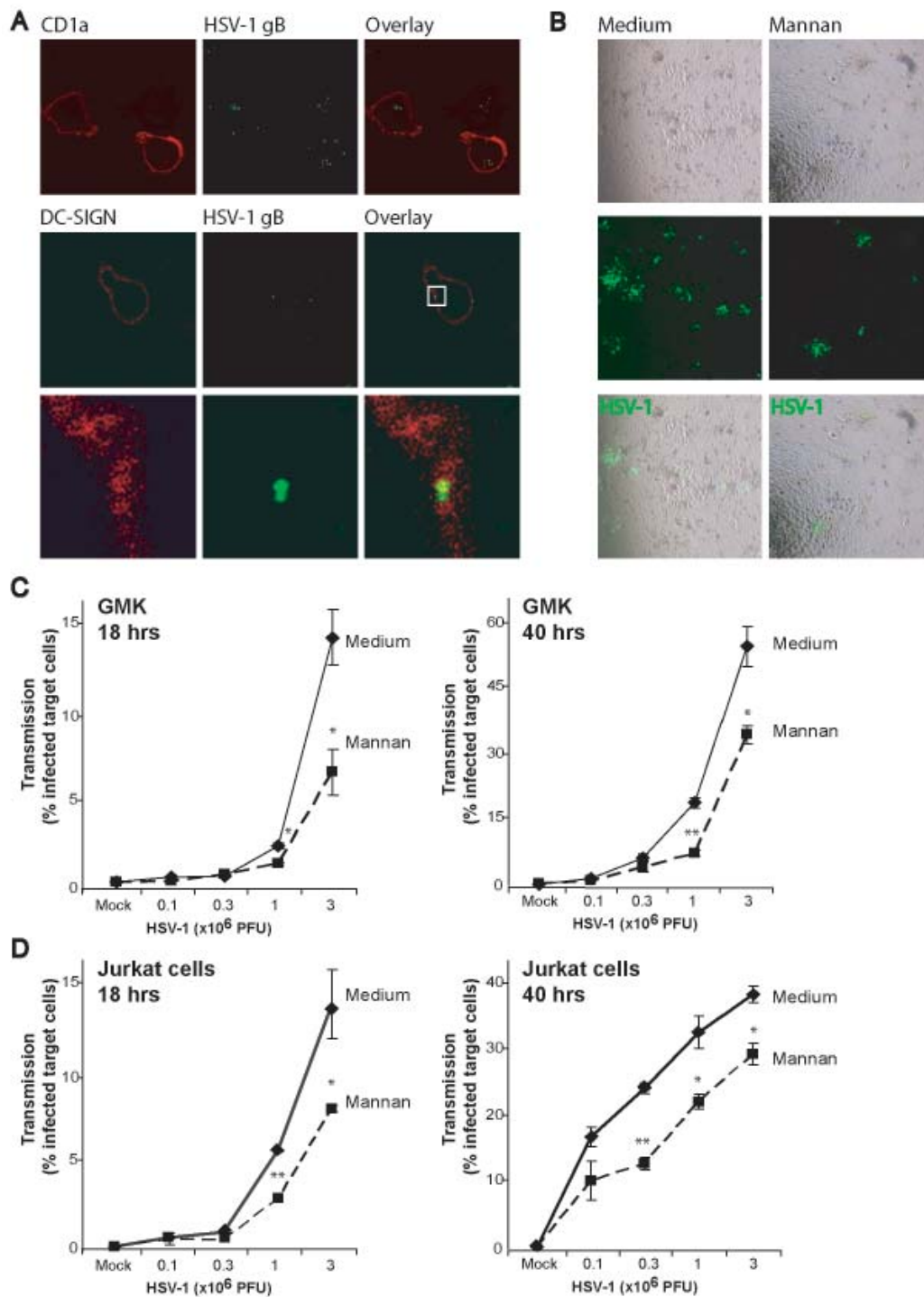


Figure 6. HSV-1 is captured and transmitted by DC-SIGN on DCs. (A) DCs were incubated for 2 hours with HSV-1 (MOI 5) either at 4°C or 37°C, washed extensively and stained intracellular for HSV-1 gB, CD1a and DC-SIGN. Confocal microscopy was performed at a magnification of 630x with 4x zoom. (B,C,D) For the transmission assay, 10⁴ DCs were pre-incubated with mannan, and infected with HSV-1-GFP (MOI 1) for 2 hours. Cells were washed extensively and added to 5x 10⁴ GMK and Jurkat cells. (B) Immunofluorescence analyses are after 18 hours of transmission to GMKs at a magnification of 40x combined with bright field pictures (C,D) Infection of target cells was analysed by GFP expression after 18 (left) and 40 hours (right) for GMK (C) or Jurkat (D) cells. DCs were excluded by using CD1a staining. Error bars represent standard deviation of duplicates. One representative experiment out of two is shown. Significance was calculated by a two-sided unpaired Student t-test. Asterisks represent p- value compared to the medium condition, * p < 0.05, ** p < 0.01.

overexpression, whereas gB and gC used in our experiments were purified from eukaryotic cells. Thus, differences in the cell type and method of production might account for the differences observed. Indeed, infection by HSV-1 induces glycosylation changes (18;19) and insect cells lack the glycosylation components necessary to generate glycosylations present in eukaryotic cells (20). Thus, gB and gC produced in eukaryotic cells represent the glycosylations observed in HSV-1 particles, which are recognized by DC-SIGN. However, we cannot exclude the possibility that other surface glycoproteins, such as gE and gD, can also mediate binding to DCs via DC-SIGN or other receptors.

In addition to HSV-1 binding to DC-SIGN, we detected binding of recombinant DC-SIGN to HSV-2, suggesting a similar interaction between HSV-2 and DC-SIGN on DCs. As gB is highly conserved throughout the family *Herpesviridae*, it is highly likely that HSV-2 also interacts with DCs via DC-SIGN, as HSV-1 gB and HSV-2 gB show 87% nucleotide sequence identity (21-25) and 83% amino acid sequence identity in their protein-coding regions (26).

Heparan sulfates are glycosaminoglycan side chains of cell-surface proteoglycans that have been shown to mediate attachment of HSV (15). On DCs, removal of heparan sulfates and blocking of DC-SIGN decreased HSV-1 binding, demonstrating that both DC-SIGN and heparan sulfates are involved in the binding of HSV-1 to DCs. This is similar to what we have observed previously for HIV-1, in which syndecan-3 on DCs, together with DC-SIGN, is important for attachment of HIV-1 to DCs (16). Therefore, in analogy with HIV-1, it is tempting to speculate that the core protein containing the heparan sulfate side chains involved in HSV-1 binding is the heparan sulfate proteoglycan syndecan-3.

Both HSV-1 gB and gC are important for binding of HSV-1 to target cells (15;27). Although binding significantly enhances the efficiency of HSV-1 infection, it is not essential for infection, in contrast to the entry receptors. Using soluble DC-SIGN and DC-SIGN-transfected cell lines, we demonstrated that both gB and gC interacted with DC-SIGN. The interaction of gB with DCs was completely dependent on DC-SIGN, whereas gC binding to DCs was partially mediated by DC-SIGN and heparan sulfates. These results suggest that gB has a higher affinity for DC-SIGN and gC for heparan sulfates. Indeed, it was shown previously that gB has a lower affinity for heparan sulfates than gC (28). We conclude that, although gB and gC are not essential for viral entry, they do enhance viral attachment to target cells.

DC-SIGN plays an important role in DC infection by different viruses as it enhances the infection of DCs *in cis* for various viruses, including HIV-1 and MV (4;6). DC-SIGN is highly expressed on DCs and efficiently captures the glycan structures on the viral envelope glycoproteins. This interaction enhances the contact of the virus with its entry receptors, resulting in infection. This process is thought to be important for entry receptors that have a low affinity for the viral glycoproteins or are expressed at low levels on DCs. Here, we demonstrated that HSV-1 targets DC-SIGN for viral attachment and DC infection *in cis*, as the infection of DCs with HSV-1 could be inhibited by mannan and antibodies against DC-SIGN at both the mRNA and the protein levels. Notably, mannan blocked HSV-1 infection more efficiently than DC-

SIGN antibodies. This could indicate the presence of an additional mannose-specific receptor on DCs or that mannan is more efficient in inhibiting DC-SIGN function. Upon infection of DCs with HSV-1, we observed two populations of infected DCs that differed in their expression of gB, suggesting that not all DCs are productively infected.

Using a CHO cell line, which is naturally non-permissive to HSV entry, we demonstrated that DC-SIGN by itself is not an entry receptor, as transfection of CHO cells with DC-SIGN did not result in HSV susceptibility of the cell line. Recently, Satoh *et al.* demonstrated that transfection of a CHO cell line with a novel identified entry receptor resulted in HSV infection, verifying that the CHO cell line is able to sustain viral infection(29).

Our data demonstrated that DC-SIGN is also involved in HSV-1 capture and subsequent transmission to permissive target cells. Immunofluorescence analyses strongly suggested that HSV-1 is retained in vesicles and is transmitted efficiently to permissive target cells, as transmission was observed within 18 h. However, we cannot exclude the possibility that *de novo* virus production is also involved. These data suggest that DCs might facilitate dissemination throughout the body during primary infection or during complications such as herpes encephalitis.

Infection of DCs by HSV-1 downregulates the immunostimulating phenotype of DCs (10;30). Therefore, our data strongly suggest that HSV-1 targets DC-SIGN to infect DCs and escape immunity. Recently, we have shown that pathogens such as *Mycobacterium tuberculosis* and HIV-1 target DC-SIGN to modulate Toll-like receptor signalling and thereby immune responses (31;32). Therefore, HSV-1 interaction with DC-SIGN might also result in modulation of immune responses, especially as HSV-1 has been shown to trigger Toll-like receptors (33). Further studies are needed to investigate the effect of DC-SIGN binding of HSV-1 on DC signalling pathways.

In conclusion, we demonstrated that HSV-1 targets DC-SIGN for efficient infection of DCs and viral transmission to target cells. Our data suggest that DC-SIGN intervention strategies might prevent HSV-1 infection and dissemination.

Materials and methods

Antibodies, cells, proteins and viruses. Monoclonal antibodies (mAbs) used were for all experiments as follows: mAbs against DC-SIGN (AZN-D1 and AZN-D2; (34;35), HSV-1 gB (B11D8) and gC (C12H12 and C4H12) (36), heparan sulfates (10E4; Seikagaku), digested heparan sulfates (F-69-3G10; Seikagaku), CD86 conjugated with phycoerythrin (PE) (HA5.2B7; Immunotech), CD1a conjugated with PE (NA1/34; Abcam), a mouse IgG1 isotype control (MG1-45; Biolegend), goat anti-human IgG conjugated with horseradish peroxidase (HRP; Jackson Immunoresearch) goat anti-mouse antibody conjugated with fluorescein isothiocyanate (FITC) (Zymed Laboratories).

Immature DCs were cultured as described previously (37). Briefly, purified human monocytes were differentiated into immature DCs in the presence of IL-4 (500 U ml⁻¹) and granulocyte-macrophage colony-stimulating factor (800 U ml⁻¹) (both from Schering-Plough). To generate mature DCs, immature DCs were incubated for 24 h with 10 ng lipopolysaccharide (LPS) ml⁻¹ derived from *Salmonella typhosa* (Sigma).

Parental and DC-SIGN-transfected CHO(38), K562 (39) and Raji cells (34;35) were generated and cultured as described previously. The HSV-1 strains Syn17⁺ and KOS321, an HSV-1 clinical isolate,

HSV-1 strain v44 containing a VP16-green fluorescent protein fusion protein (HSV-1-GFP) and HSV-2 strain 333 were grown on green monkey kidney (GMK) cells. A plaque titration assay was performed to determine viral titres (as p.f.u.). HSV-1 proteins gB and gC were purified as described previously (28): virus lysate (strain KOS321) was passed through columns containing anti-gC (C4H12) or anti-gB (B11D8) mAb. Subsequently, the columns were washed and the proteins eluted with 0.1 M glycine/HCl (pH 2.4). Following neutralization, the material was centrifuged to near dryness over a microcentrifugal concentrator with a 30 kDa cut-off (PallGelman Sciences) and then resuspended in PBS and centrifuged again. The final product was resuspended in a small volume of PBS and stored at -70°C.

Fluorescent bead adhesion assay. Streptavidin-coated beads (TransFluorSpheres, 488/645 nm, 1.0 µm; Molecular Probes) were incubated with a biotinylated F(ab')₂ fragment goat anti-mouse antibody (6 µg ml⁻¹; Jackson Immunoresearch), followed by overnight incubation with mAbs against HSV gB or gC at a concentration of 10 µg ml⁻¹ at 4°C. The beads were washed and incubated overnight with HSV-1 or purified HSV gB or gC at 4°C. HIV-1 gp120 beads were produced as described previously (40). An adhesion assay was performed as follows (34;35): 1x10⁵ cells were incubated with beads for 45 min at 37°C. To determine the specificity of adhesion, cells were pre-treated with mannan (1 mg ml⁻¹), EGTA (10 mM) or blocking antibodies against DC-SIGN (20 µg ml⁻¹) for 15 min at 37°C. Binding was measured by flow cytometry.

To investigate the role of heparan sulfates, immature DCs (1x10⁵ cells per well) were seeded in a total volume of 30 µl PBS and heparinase III (Prozyme) was added at a concentration of 0.1 IU ml⁻¹. The plate was incubated for 1 h at room temperature. After 1 h, the cells were washed with TSA [20 mM Tris/HCl (pH 7.6), 150 mM NaCl, 1 mM CaCl₂, 2 mM MgCl₂, plus 0.5% BSA] and used directly for subsequent experiments. The efficiency of digestion was evaluated by staining the cells with mAb 10E4 (against heparan sulfates) and mAb 3G10 (against Δheparan sulfate, which recognizes digested heparan sulfate chains). The cells were analysed by flow cytometry. A strong reduction in the mean fluorescence intensity of heparan sulfate staining and a positive staining of the population with mAb 3G10 confirmed efficient treatment.

DC-SIGN-Fc binding. Recombinant DC-SIGN consists of the extracellular portion of DC-SIGN (aa 64-404) fused at the C terminus to the human IgG1 Fc domain. DC-SIGN-Fc was produced in CHO K1 cells after transfection with the DC-SIGN-Sig-plgG1-Fc vector (5 µg per 1x10⁶ cells). The soluble DC-SIGN-Fc-binding ELISA was performed as described previously (41). Briefly, different concentrations of HSV-1, -2 or purified HSV-1 gB and gC were coated onto ELISA plates overnight at room temperature. Non-specific binding was blocked by incubating the plate with TSA for 1 h at 37°C. Soluble DC-SIGN-Fc supernatant was added for 1 h at 37°C. Unbound DC-SIGN-Fc was washed away and binding was determined using an HRP-conjugated goat anti-human Fc antibody. Specificity was determined (unless indicated otherwise) in the presence of mannan (1 mg ml⁻¹) or EGTA (10 mM). The amount of HSV coated onto the plate was detected using anti-gB and HRP-conjugated goat anti-mouse Fc antibodies.

HSV-1 infection and transmission. Immature DCs, Raji or Raji/DC-SIGN cells (5x10⁴ cells) were seeded in a round-bottomed 96-well plate in complete RPMI 1640. The cells were pre-incubated with medium, a blocking antibody against DC-SIGN (AZN-D1; 20 µg ml⁻¹), an isotype control (IgG1; 20 µg ml⁻¹) or mannan (1 mg ml⁻¹) for 1 h at 37°C in 5% CO₂ before infecting them with different concentrations of HSV-1 Syn17⁺. The virus/DC mixture was incubated at 37°C for 24 h. Next, the cells were stained with mAb against HSV gB for 30 min at 4°C, followed by a 1 : 50 dilution of FITC-conjugated goat anti-mouse IgG for 30 min at 4°C. Finally, the cells were fixed with 2% paraformaldehyde (PFA) in PBS and gB expression was measured by flow cytometry.

CHO, CHO/DC-SIGN and GMK cells (2×10^5) were seeded in a 96-well flat-bottomed plate for 24 h before infection with HSV-1 Syn17⁺ (1×10^7 p.f.u. per well). Cells were harvested after 48 h of infection and analysed for gB expression as described above.

For the transmission experiment, 1×10^4 DCs were pre-incubated with mannan (1 mg ml^{-1}) for 30 min at 37°C in 5% CO₂ before infecting them with different concentrations of HSV-1-GFP. After 2 h, the cells were washed extensively to remove unbound HSV-1 and added to 5×10^4 permissive target cells (GMK or Jurkat cells). After 18 and 40 h, immunofluorescent images were captured and cells were harvested. Staining with anti-CD1a-PE mAb was performed to exclude DCs. Cells were fixed with 2% PFA in PBS and infection was analysed by measuring the amount of GFP by flow cytometry.

HSV-1 mRNA transcription. DCs (1×10^5) were pre-incubated with medium containing a blocking antibody against DC-SIGN (AZN-D1; $20 \text{ } \mu\text{g ml}^{-1}$), isotype control (IgG1; $20 \text{ } \mu\text{g ml}^{-1}$) or mannan (1 mg ml^{-1}) for 30 min at 37°C in 5% CO₂ before infection with HSV-1 Syn17⁺ at an m.o.i. of 1. After 6 h, the cells were washed extensively with PBS and mRNA was isolated using an mRNA capture kit (Roche). cDNA was synthesized using a reverse transcriptase kit (Promega). For quantitative real-time PCR analysis, PCR amplification was performed in the presence of SYBR Green, as described previously (42). Specific primers for HSV-1 thymidine kinase and GAPDH were designed using Primer Express version 2.0 (Applied Biosystems). Transcription was adjusted for GAPDH transcription and relative mRNA expression of HSV-1-infected control samples was set at 1.

Immunofluorescence microscopy. DCs were incubated with HSV-1 Syn17⁺ at an m.o.i. of 5 for 2 h at 37 or 4°C. The cells were washed extensively, fixed with 3% PFA in PBS and permeabilized with 0.1% saponine in PBS. After blocking with 2% BSA and 0.1% saponine in PBS, the cells were stained with anti-HSV-1 gB, mouse IgG2a anti-CD1a or anti-DC-SIGN (AZN-D1) for 45 min at room temperature. The cells were washed and the sections counterstained with isotype-specific Alexa Fluor-labelled anti-mouse antibodies for 30 min at room temperature. The cells were washed and mounted onto glass slides coated with 0.1% poly-L-lysine and analysed by confocal microscopy [Leica AOBS SP2 confocal laser-scanning microscope system containing a DMIRE2 microscope with glycerol objective lens (PL APO x63/NA1.30)]. Images were acquired using Leica confocal software version 2.61.

Statistical analysis. To compare the means of multiple groups, a one-way analysis of variance (ANOVA) was used. When the overall F-test was significant, differences were investigated further using a post-hoc Bonferroni test using Graphpad Prism software. To compare the statistical differences between groups, a two-sided unpaired Student's *t*-test was applied. For both tests, a *P* value of <0.05 was considered statistically significant.

Acknowledgements

We would like to thank Dr G. Davids for providing us with the 10E4 mAb, Dr P. O'Hare for supplying HSV-1-GFP (strain v44) and Dr G. Verjans for the clinical isolate of HSV-1. This work was supported by the Dutch Scientific Organization (NWO) grants 91746367 (LdW) and 91204025 (MAWPdJ).

References

1. Taylor,T.J., Brockman,M.A., McNamee,E.E., and Knipe,D.M. 2002. Herpes simplex virus. *Front Biosci.* **7**:d752-d764.
2. Roizman D, Knipe D, and Whitley,R. 2007. Herpes simplex viruses. In *Fields Virology*. Knipe DM, Howley D, Griffin DE, Lamb R, and Martin B, editors. Lippincott, Williams and Wilkins, Philadelphia, PA. 2501-2601.
3. Pepose,J.S., Keadle,T.L., and Morrison,L.A. 2006. Ocular herpes simplex: changing epidemiology, emerging disease patterns, and the potential of vaccine prevention and therapy. *Am. J Ophthalmol.* **141**:547-557.
4. Burleigh,L., Lozach,P.Y., Schiffer,C., Staropoli,I., Pezo,V., Porrot,F., Canque,B., Virelizier,J.L., renzana-Seisdedos,F., and Amara,A. 2006. Infection of dendritic cells (DCs), not DC-SIGN-mediated internalization of human immunodeficiency virus, is required for long-term transfer of virus to T cells. *J. Virol.* **80**:2949-2957.
5. Halary,F., Amara,A., Lortat-Jacob,H., Messerle,M., Delaunay,T., Houles,C., Fieschi,F., Arenzana-Seisdedos,F., Moreau,J.F., and Dechanet-Merville,J. 2002. Human cytomegalovirus binding to DC-SIGN is required for dendritic cell infection and target cell trans-infection. *Immunity* **17**:653-64.
6. de Witte,L., Abt,M., Schneider-Schaulies,S., van Kooyk,Y., and Geijtenbeek,T.B. 2006. Measles Virus Targets DC-SIGN To Enhance Dendritic Cell Infection. *J. Virol.* **80**:3477-3486.
7. Coffin,R.S., Thomas,S.K., Thomas,N.S., Lilley,C.E., Pizzey,A.R., Griffiths,C.H., Gibb,B.J., Wagstaff,M.J., Inges,S.J., Binks,M.H. et al 1998. Pure populations of transduced primary human cells can be produced using GFP expressing herpes virus vectors and flow cytometry. *Gene Ther.* **5**:718-722.
8. Salio,M., Cella,M., Suter,M., and Lanzavecchia,A. 1999. Inhibition of dendritic cell maturation by herpes simplex virus. *Eur. J Immunol.* **29**:3245-3253.
9. Kruse,M., Rosorius,O., Kratzer,F., Bevec,D., Kuhnt,C., Steinkasserer,A., Schuler,G., and Hauber,J. 2000. Inhibition of CD83 cell surface expression during dendritic cell maturation by interference with nuclear export of CD83 mRNA. *J. Exp. Med.* **191**:1581-1590.
10. Mikloska,Z., Bosnjak,L., and Cunningham,A.L. 2001. Immature monocyte-derived dendritic cells are productively infected with herpes simplex virus type 1. *J. Virol.* **75**:5958-5964.
11. Pollara,G., Speidel,K., Samady,L., Rajpopat,M., McGrath,Y., Ledermann,J., Coffin,R.S., Katz,D.R., and Chain,B. 2003. Herpes simplex virus infection of dendritic cells: balance among activation, inhibition, and immunity. *J Infect. Dis.* **187**:165-178.
12. Raftery,M.J., Winau,F., Kaufmann,S.H., Schaible,U.E., and Schonrich,G. 2006. CD1 antigen presentation by human dendritic cells as a target for herpes simplex virus immune evasion. *J Immunol.* **177**:6207-6214.
13. Kruse,M., Rosorius,O., Kratzer,F., Stelz,G., Kuhnt,C., Schuler,G., Hauber,J., and Steinkasserer,A. 2000. Mature dendritic cells infected with herpes simplex virus type 1 exhibit inhibited T-cell stimulatory capacity. *J Virol.* **74**:7127-7136.
14. Theodoridis,A.A., Prechtel,A.T., Turza,N.M., Zenke,M., and Steinkasserer,A. 2007. Infection of human dendritic cells with herpes simplex virus type 1 dramatically diminishes the mRNA levels of the prostaglandin E(2) receptors EP2 and EP4. *Immunobiology* **212**:827-838.
15. Spear,P.G. 2004. Herpes simplex virus: receptors and ligands for cell entry. *Cell Microbiol.* **6**:401-410.
16. de Witte,L., Bobardt,M., Chatterji,U., Degeest,G., David,G., Geijtenbeek,T.B., and Gallay,P. 2007. Syndecan-3 is a dendritic cell-specific attachment receptor for HIV-1. *Proc. Natl. Acad. Sci. U. S. A* **104**:19464-19469.
17. Barreca,C., and O'Hare,P. 2004. Suppression of herpes simplex virus 1 in MDBK cells via the interferon pathway. *J Virol.* **78**:8641-8653.
18. Olofsson,S., Khanna,B., and Lycke,E. 1980. Altered kinetic properties of sialyl and galactosyl transferases associated with herpes simplex virus infection of GMK and BHK cells. *J Gen. Virol.* **47**:1-9.
19. Nystrom,K., Biller,M., Grahn,A., Lindh,M., Larson,G., and Olofsson,S. 2004. Real time PCR for monitoring regulation of host gene expression in herpes simplex virus type 1-infected human diploid cells. *J Virol. Methods* **118**:83-94.
20. Altmann,F., Staudacher,E., Wilson,I.B., and Marz,L. 1999. Insect cells as hosts for the expression of recombinant glycoproteins. *Glycoconj. J* **16**:109-123.
21. Sarmiento,M., Haffey,M., and Spear,P.G. 1979. Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7(B2) in virion infectivity. *J Virol.* **29**:1149-1158.
22. Cai,W.Z., Person,S., Warner,S.C., Zhou,J.H., and DeLuca,N.A. 1987. Linker-insertion nonsense and restriction-site deletion mutations of the gB glycoprotein gene of herpes simplex virus type 1. *J Virol.* **61**:714-721.
23. Cai,W.H., Gu,B., and Person,S. 1988. Role of glycoprotein B of herpes simplex virus type 1 in

- viral entry and cell fusion. *J Virol.* **62**:2596-2604.
24. Little, S.P., Jofre, J.T., Courtney, R.J., and Schaffer, P.A. 1981. A virion-associated glycoprotein essential for infectivity of herpes simplex virus type 1. *Virology* **115**:149-160.
 25. Cheshenko, N., and Herold, B.C. 2002. Glycoprotein B plays a predominant role in mediating herpes simplex virus type 2 attachment and is required for entry and cell-to-cell spread. *J Gen. Virol.* **83**:2247-2255.
 26. Dolan, A., Jamieson, F.E., Cunningham, C., Barnett, B.C., and McGeoch, D.J. 1998. The genome sequence of herpes simplex virus type 2. *J Virol.* **72**:2010-2021.
 27. Herold, B.C., WuDunn, D., Soltys, N., and Spear, P.G. 1991. Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. *J. Virol.* **65**:1090-1098.
 28. Trybala, E., Liljeqvist, J.A., Svennerholm, B., and Bergstrom, T. 2000. Herpes simplex virus types 1 and 2 differ in their interaction with heparan sulfate. *J. Virol.* **74**:9106-9114.
 29. Satoh, T., Arii, J., Suenaga, T., Wang, J., Kogure, A., Uehori, J., Arase, N., Shiratori, I., Tanaka, S., Kawaguchi, Y. et al 2008. PILRalpha is a herpes simplex virus-1 entry coreceptor that associates with glycoprotein B. *Cell* **132**:935-944.
 30. Novak, N., and Peng, W.M. 2005. Dancing with the enemy: the interplay of herpes simplex virus with dendritic cells. *Clin Exp. Immunol.* **142**:405-410.
 31. Gringhuis, S.I., den Dunnen, J., Litjens, M., van het Hof, B., van Kooyk, Y., and Geijtenbeek, T.B. 2007. C-type lectin DC-SIGN modulates Toll-like receptor signaling via Raf-1 kinase-dependent acetylation of transcription factor NF-kappaB. *Immunity.* **26**:605-616.
 32. Hovius, J.W., de Jong, M.A., den Dunnen, J., Litjens, M., Fikrig, E., van der Poll, T., Gringhuis, S.I., and Geijtenbeek, T.B. 2008. Salp15 binding to DC-SIGN inhibits cytokine expression by impairing both nucleosome remodeling and mRNA stabilization. *PLoS. Pathog.* **4**:e31.
 33. Morrison, L.A. 2004. The Toll of herpes simplex virus infection. *Trends Microbiol.* **12**:353-356.
 34. Geijtenbeek, T.B., Torensma, R., van Vliet, S.J., van Duijnhoven, G.C., Adema, G.J., van Kooyk, Y., and Figdor, C.G. 2000. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell* **100**:575-585.
 35. Geijtenbeek, T.B., and van Kooyk, Y. 2003. DC-SIGN: a novel HIV receptor on DCs that mediates HIV-1 transmission. *Curr. Top. Microbiol. Immunol.* **276**:31-54.
 36. Bergstrom, T., Sjogren-Jansson, E., Jeansson, S., and Lycke, E. 1992. Mapping neuroinvasiveness of the herpes simplex virus type 1 encephalitis-inducing strain 2762 by the use of monoclonal antibodies. *Mol. Cell Probes* **6**:41-49.
 37. Sallusto, F., and Lanzavecchia, A. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* **179**:1109-18.
 38. van Gisbergen, K.P., Ludwig, I.S., Geijtenbeek, T.B., and van Kooyk, Y. 2005. Interactions of DC-SIGN with Mac-1 and CEACAM1 regulate contact between dendritic cells and neutrophils. *FEBS Lett.* **579**:6159-6168.
 39. van Gisbergen, K.P., Aarnoudse, C.A., Meijer, G.A., Geijtenbeek, T.B., and van Kooyk, Y. 2005. Dendritic cells recognize tumor-specific glycosylation of carcinoembryonic antigen on colorectal cancer cells through dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin. *Cancer Res.* **65**:5935-5944.
 40. Lekkerkerker, A.N., Ludwig, I.S., van Vliet, S.J., van Kooyk, Y., and Geijtenbeek, T.B. 2004. Potency of HIV-1 envelope glycoprotein gp120 antibodies to inhibit the interaction of DC-SIGN with HIV-1 gp120. *Virology* **329**:465-476.
 41. Geijtenbeek, T.B., van Duijnhoven, G.C., van Vliet, S.J., Krieger, E., Vriend, G., Figdor, C.G., and van Kooyk, Y. 2002. Identification of different binding sites in the dendritic cell-specific receptor DC-SIGN for intercellular adhesion molecule 3 and HIV-1. *J. Biol. Chem.* **277**:11314-11320.
 42. Garcia-Vallejo, J.J., van het Hof, B., Robben, J., Van Wijk, J.A., van Die, I., Joziassse, D.H., and van Dijk W. 2004. Approach for defining endogenous reference genes in gene expression experiments. *Anal. Biochem.* **329**:293-299.

