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Dendritic Cell-specific Intercellular Adhesion Molecule 3-grabbing Non-integrin (DC-SIGN)-mediated Enhancement of Dengue Virus Infection Is Independent of DC-SIGN Internalization Signals*

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Dengue virus (DV) is a mosquito-borne flavivirus that causes hemorrhagic fever in humans. In the natural infection, DV is introduced into human skin by an infected mosquito vector where it is believed to target immature dendritic cells (DCs) and Langerhans cells (LCs). We found that DV productively infects DCs but not LCs. We show here that the interactions between DV E protein, the sole mannosylated glycoprotein present on DV particles, and the C-type lectin dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) are essential for DV infection of DCs. Binding of mannosylated *N*-glycans on DV E protein to DC-SIGN triggers a rapid and efficient internalization of the viral glycoprotein. However, we observed that endocytosis-defective DC-SIGN molecules allow efficient DV replication, indicating that DC-SIGN endocytosis is dispensable for the internalization step in DV entry. Together, these results argue in favor of a mechanism by which DC-SIGN enhances DV entry and infection *in cis*. We propose that DC-SIGN concentrates mosquito-derived DV particles at the cell surface to allow efficient interaction with an as yet unidentified entry factor that is ultimately responsible for DV internalization and pH-dependent fusion into DCs.

Dengue virus (DV)[1] is an arthropod-borne flavivirus that belongs to the *Flaviviridae* family (1). The four serotypes of DV (DV-1 to DV-4) are transmitted to humans by the mosquito vector *Aedes* aegypti (1, 2). DV infection results in a spectrum of illnesses, ranging from a flu-like disease (dengue fever) to dengue hemorrhagic fever that can progress to dengue shock syndrome and death (1).

DV is a lipid-enveloped virus with a single-stranded, positive sense RNA genome, which replicates in the cytoplasm of infected cells (2, 3). The 11-kb viral RNA encodes for a large polyprotein precursor, which is processed by both host and viral proteases to yield the non-structural proteins NS1 to NS5 and three structural proteins: C (core), prM (the intracellular precursor of the M protein), and E (envelope) glycoprotein (2, 3). The E protein is assumed to bind cellular receptors that direct DV particles to the endocytic pathway. The acidic environment in the endosome is believed to trigger major conformational changes in the E protein, which induce fusion of the viral and host cell membranes, resulting in entry of the virion into the cytoplasm (3).

In the natural infection, DV is introduced into human skin by an infected mosquito during a blood meal (2). Immature dendritic cells (DCs) and Langerhans cells (LCs), which are normally resident in the skin, have been shown to be infected by DV and are believed to be the first cells targeted by the virus (4). We have previously analyzed the interactions between DV and human DCs to identify cellular factors important for virus entry. We and others (5, 6) have identified DC-SIGN (CD209) as an essential molecule for productive DV infection of immature DCs, since anti-DC-SIGN antibodies or soluble DC-SIGN molecules strongly inhibit DV infection.

DC-SIGN is a tetrameric C-type (calcium-dependent) lectin that binds, through its C-terminal carbohydrate recognition domain, high mannose *N*-linked glycans present on the surface of several viral glycoproteins such as human immunodeficiency virus (HIV) gp120 and hepatitis C virus (HCV) E2 (7–9). The DV E protein, which is the only glycoprotein exposed on the surface of mature DV virions, is responsible for attachment to the host cell surface and plays an important role in viral entry (3, 10). Furthermore, carbohydrate residues present on the E glycoprotein have been reported to be important for virus binding to host cells (11). In contrast to other viral glycoproteins that bind DC-SIGN and are highly glycosylated, DV E protein has only two potential *N*-linked glycosylation sites at Asn-67 and Asn-153, which are differentially used by the four DV serotypes (12). The molecular interactions of E protein with DC-SIGN, believed to be a key element for DV entry into DCs,

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[1] The abbreviations used are: DV, dengue virus; Ab, antibody; BHK, baby hamster kidney; BSA, bovine serum albumin; DC, dendritic cell; DC-SIGN, dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin; DMJ, 1-deoxymannojirimycin hydrochloride; EndoH, endoglycosidase H; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; FCS, fetal calf serum; GM-CSF, granulocyte macrophage colony-stimulating factor; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HMAF, hyperimmune mouse ascites fluids; L-SIGN, liver cell-specific intercellular adhesion molecule 3-grabbing non-integrin; LC, Langerhans cell; mAb, monoclonal antibody; m.o.i., multiplicity of infection; PBS, phosphate-buffered saline; PE, phycoerythrin; PNGase F, peptide:*N*-glycosydase F; sE, DV-soluble E protein; SFV, Semliki forest virus; WT, wild type.

are currently poorly understood. Following binding to DC-SIGN, DV is believed to be internalized and targeted to an acidic endosomal compartment where membrane fusion occurs (3). Indeed, treatment of DC-SIGN-expressing cells with bafilomycin A1 or chloroquine, which raise the pH within endosomes, inhibits DV infection (6). Although DC-SIGN is an endocytic receptor that has been shown to internalize several pathogens (9, 13–15), it is currently unknown whether DC-SIGN is directly responsible for targeting DV to endosomes or whether it merely increases the local concentration of virions in the proximity of a true entry receptor.

In this study, we have examined the role of DC-SIGN in the process of DV entry into DCs. Our results formally identify the E protein as the DV determinant responsible for attachment to DC-SIGN and suggest a critical role for high mannose *N*-glycans in interactions between DV E protein and DC-SIGN. We provide strong evidence that DC-SIGN-mediated DV endocytosis is dispensable for DV infection of target cells and hence propose that DC-SIGN acts as a critical DV attachment factor that may facilitate subsequent interaction of viral particles with an as yet unidentified cellular receptor, which leads to DV entry.

EXPERIMENTAL PROCEDURES

Construction of DV-1 sE and DC-SIGN Mutants—The pIND-prM/E plasmid codes for the mature form of DV-1 (strain FGA/NA d1d (French Guiana)) prM and E envelope proteins (16). It was used as a template to generate recombinant Semliki forest virus (SFV) encoding for the DV prM and soluble form of E protein (sE). The sequence coding for prM protein and the ectodomain of DV-1 E envelope protein (amino acids 101–673 in the DV polyprotein) was amplified by PCR (*Pwo* DNA polymerase, Roche Applied Science) using the sense primer 5′-ATATTGGATCCGCGCGCATGTCCGTGACCATGCTCCTC-3′ and the antisense primer 5′-ATATTATGCATGCGGCCGCTAGGCGGGGGCCACCTGGGAGGTCTCGGTGCCCTTGAACCAGCTTAGTTT-3′. The antisense primer encodes for the 1D4 tag peptide sequence. The amplified sequence coding for E was digested with BssHII and NsiI and introduced into corresponding sites of the pSFV2 vector (17).

The DC-SIGN wild type (WT) pcDNA3 vector was described previously (18). DC-SIGN LL/AA, DC-SIGN EEE/AAA, and DC-SIGN Y/A were generated by site-directed mutagenesis (Quikchange kit, Stratagene) and inserted into pcDNA3 vector. TRIP ΔU3-cytomegalovirus vectors (19) encoding DC-SIGN cDNAs were generated by replacing the BamHI-XhoI fragment encoding enhanced green fluorescent protein with a PCR-generated BamHI-SalI fragment encoding either DC-SIGN WT, DC-SIGN LL/AA, DC-SIGN EEE/AAA, DC-SIGN Y/A, or DC-SIGN ΔN (lacking the last 35 amino acids of the cytoplasmic domain of DC-SIGN) nucleotide sequences. All constructs were sequenced using the dideoxy method.

Antibodies—Phycoerythrin (PE)-conjugated anti-human DC-SIGN mouse monoclonal antibody (mAb) clone FAB161P was purchased from R&D Systems. PE-conjugated anti-human Langerin (DC-GM4) and anti-CD83 (HB15a) mAbs were purchased from Pharmingen. Fluorescein isothiocyanate (FITC)-conjugated mAb anti-CD1a (H1149) and PE-conjugated mAb anti-CD86 (IT2.2) were purchased from Immunotech. The anti-DC-SIGN mAbs clone 1B10 (IgG2a, κ) and 8A5 (IgG2a, κ) have been developed in our laboratory and described previously (18, 20, 21). Intracellular DV antigens were stained with anti-DV-specific hyperimmune mouse ascites fluids (HMAF-1 to -4) or anti-DV-1 NS_1mAb ($13A_1$, a gift from M. K. Gentry and E. A. Henchal) (25). The secondary antibody used was a PE-conjugated goat anti-mouse FAb (R0480, Dako). mAb 1D4 is directed against the C9 tag (TETSQVAPA) peptide sequence and was described previously (22). As controls, mouse isotypic Ab IgG2a and IgG2b were used.

Cells—HeLa and HEK-293T were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. Raji cells were cultured in RPMI 1640 supplemented with 10% FCS and 1% penicillin/streptomycin. Cells expressing DC-SIGN or its mutants were generated by transduction with the retroviral pTRIP ΔU3 vector expressing DC-SIGN as previously described (19). Raji cells with different levels of DC-SIGN expression were obtained by transduction of Raji cells by pTRIP ΔU3 DC-SIGN WT vector at different multiplicities of infection (m.o.i.) as described previously (23). Transduced cells were then sorted by fluorescent activated cell sorting (FACS).

Dendritic and Langerhans Cell Generation—DCs were generated as described previously (19). Briefly, human peripheral blood mononuclear cells were isolated from healthy donors by density gradient centrifugation through Ficoll-Paque Plus (Amersham Biosciences). Monocytes were negatively selected with magnetic beads coated with a mixture of antibodies (Miltenyi Biotec) and seeded at 10^6 cells·ml^{-1} in RPMI 1640 supplemented with 10% FCS, 1% penicillin/streptomycin, 50 ng·ml^{-1} recombinant human interleukin-4 (PeproTech), and 100 ng·ml^{-1} recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF, Centaur) for 6 days. LCs derived from human umbilical cord blood were prepared as described previously (24). Briefly, CD34+ cells were cultured in RPMI 1640 supplemented with 10% FCS, 1% penicillin/streptomycin, stem cell factor (25 ng·ml^{-1}, R&D Systems), GM-CSF (100 ng·ml^{-1}, Centaur), and tumor necrosis factor-α (2.5 ng·ml^{-1}, R&D Systems). At day 7, cells were washed and cultured for 6 additional days in RPMI 1640 supplemented with 10% FCS, 1% penicillin/streptomycin, GM-CSF, and 1 ng·ml^{-1} transforming growth factor-β1 (R&D Systems).

Viruses Stocks and DV Infection—DV-1 strain FGA/NA d1d, DV-2 strain JAM (Jamaica), DV-3 isolate PaH881 (Thailand), and DV-4 isolate 63632 (Burma) were propagated in mosquito (*Aedes* pseudoscutellaris) AP61 cell monolayers. All isolates had undergone limited passage in AP61 cells. Purification on sucrose gradients and virus titration on AP61 cells by focus immunodetection assay were performed as described elsewhere (25).

For infection, 3×10^5 cells were exposed to DV for 2 h at 37 °C at varying m.o.i. (0.1–10) in FSC-free RPMI 1640 supplemented with 0.2% BSA, 1% penicillin/streptomycin, pH 7.5. Cells were subsequently washed with complete RPMI 1640 to remove excess virus and incubated at 37 °C. The cells were harvested 40 h later. For inhibition assays, cells were preincubated with inhibitors for 30 min at 37 °C before addition of DV preparations containing antibodies (1B10 and isotype control IgG; 20 μg·ml^{-1}). Infection studies with the pH-interfering agent bafilomycin A1 (Sigma) were performed as described previously (6).

Flow Cytometry Assays—Intracellular viral antigens were stained with anti-NS1 specific Ab or anti-DV HMAF-1 to -4. Infected cells were fixed and permeabilized by cytofix and cytoperm buffers according to manufacturer's instructions (Pharmingen). Permeabilized cells were incubated in cytoperm buffer containing primary Ab (1/200) for 1 h at 4 °C followed by washing twice with cytoperm buffer. Cells were then incubated in cytoperm buffer complemented with the secondary PE-conjugated goat anti-mouse Ab (R0480) (1/1000) for 30 min at 4 °C. Cells were washed twice with the cytoperm buffer prior to FACS analysis.

Plasma membrane antigens were detected by FACS using PE-conjugated anti-DC-SIGN (FAB161P), anti-Langerin (DCGM4), anti-CD83 (HB15a), anti-CD86 (IT2.2) mAb, and with FITC-conjugated anti-CD1a (H1149) mAb. Cells were washed in FCS-free Dulbecco's modified Eagle's medium and resuspended in FACS analysis buffer (1% bovine serum albumin, 0.2% γ-globulin, 0.1% sodium azide (all from Sigma)) followed by incubation with primary Ab at a 1/100-dilution (PE-conjugated Ab) or a 1/20-dilution (FITC-conjugated Ab) for 30 min at 4 °C. Cells were washed and fixed with paraformaldehyde (3.2%) prior to FACS analysis (BD Biosciences and data processing with CellQuest software (BD Biosciences)).

^{35}S-Radiolabeled Recombinant Proteins—Expression, deglycosylation, and analysis of proteins were described previously (8). Briefly, ^{35}S-radiolabeled DV sE was produced in SFV vector-infected baby hamster kidney (BHK) cells in the presence of α-mannosidase inhibitors DMJ (Calbiochem) and swainsonine (Sigma) (8). Glycosylation patterns were characterized by treatment with endoglycosidase H (EndoH; 2 milliunits, Roche Applied Science) or peptide:*N*-glycosidase F (PNGAse F; 1000 units, New England Biolabs) (8). Proteins were analyzed either by Western blotting (1D4; 2 μg·ml^{-1}) or immunoprecipitation (1D4; 20 μg·ml^{-1} or HMAF-1 at a 1/50 dilution) (8).

Soluble Glycoprotein Binding and Internalization Assays—5×10^5 cells were incubated in 100 μl of serum-free RPMI 1640 containing 1 mM $CaCl_2$, 2 mM $MgCl_2$, and ^{35}S-labeled soluble glycoproteins (50 nM) for 2 h at 4 °C, to inhibit endocytosis pathways. Unbound glycoprotein was removed by two washes with serum-free RPMI 1640. For inhibition assays, cells were preincubated with inhibitors in FACS buffer for 15 min at 4 °C before addition of labeled envelope protein preparations containing inhibitor. Inhibitors were used at 20 μg·ml^{-1} mannan (Sigma), 1B10, isotype control IgG, or 5 mM EGTA (Sigma) final concentration. For internalization assays, washed cells having captured ^{35}S-labeled soluble proteins were incubated for the indicated times either at 4 or at 37 °C to initiate endocytosis. Half the cells incubated at both 4 and 37 °C were treated with EDTA (25 mM, Sigma) at 4 °C for 10 min to remove cell surface bound ^{35}S-labeled soluble glycoprotein, thus

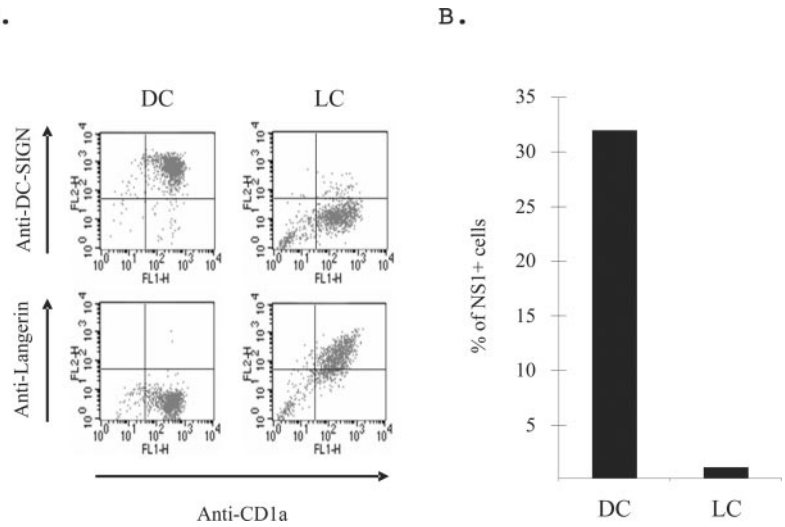

FIG. 1. **Monocyte-derived DCs, but not LCs, are infected by DV.** *A*, *in vitro* derived DCs and LCs were generated from monocytes and CD34+ progenitor cells, respectively. Cell surface expression of CD1a, DC-SIGN, and Langerin was evaluated by FACS analysis using specific mAb. *B*, DCs and LCs were infected with DV-1 at a m.o.i. of 10. Intracellular expression of the NS1 non-structural antigen, which is produced only during active DV replication, was detected using an anti-NS1 mAb followed by a PE-conjugated secondary Ab. Viral infection was quantified by FACS analysis and expressed as the percentage of NS1-positive cells. Data are representative of three independent experiments. Values are given as the mean of triplicates ± S.E. (not visible on the graph).

allowing quantification of internalized protein. The remainder of the cells were mock-treated with RPMI 1640 at 4 °C for 10 min to evaluate the total bound ^{35}S-labeled glycoprotein. All cells were then washed twice in serum-free RPMI 1640 to eliminate background radioactivity. Cell pellets were resuspended in serum-free RPMI 1640 prior to addition of optiphase supermix solution (Wallac) and activity was counted in a 1450 Microbeta Trilux γ counter (Wallac).

Immunofluorescence Microscopy—HeLa cells expressing WT or mutant DC-SIGN (5×10^5 cells) were seeded on coverslips. The following day, cells were incubated with the anti-DC-SIGN mAb 8A5 (20 $\mu g \cdot ml^{-1}$ diluted in PBS containing 0.5% BSA) for 1 h at 4 °C. Cells were washed three times with ice cold PBS to remove unbound Ab and shifted to 37 °C for 30 min to allow DC-SIGN endocytosis. Cells were fixed with 3.2% paraformaldehyde for 15 min, washed twice in PBS, and treated with PBS 0.2 M glycine for 10 min. Cells were then incubated with 500 μl of PBS containing 0.05% saponin and 0.2% BSA for 30 min. To visualize DC-SIGN-bound 8A5, cells were incubated with FITC-coupled goat anti-mouse IgG (1/150, Vector Laboratories). Cells were washed and mounted in Vectashield medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories). Fluorescence was imaged on Zeiss microscope equipped with the Apotome Systems and using a Plan Apochromat ×63/1.4 oil immersion objective.

RESULTS

DCs, but Not LCs, Are Productively Infected by DV—To investigate the capacity of DV to infect immature DCs and LCs, we generated monocyte-derived DCs as well as LCs from CD14+ blood monocytes and CD34+ hematopoeitic progenitors, respectively, and compared their ability to support DV infection. To determine whether each cell type displays the characteristic phenotype of DCs and LCs, cells were stained for expression of CD1a, DC-SIGN, and Langerin, a C-type lectin selectively expressed by LCs and involved in the formation of a LC-specific organelle, the Birbeck granule (26–28). Monocyte-derived DCs express, as expected, high levels of DC-SIGN and CD1a (Fig. 1*A*). In contrast, they do not express the C-type lectin Langerin, which is present on more than 90% of the CD1a+ LCs. As previously described for epidermal LCs (28), we found that *in vitro* differentiated LCs do not express DC-SIGN (Fig. 1*A*). Immature DCs and LCs were infected with the DV-1 strain FGA/NA d1d, produced in *Aedes* AP61 cells. Viral replication was quantified at 40 h post-infection by FACS analysis using antibodies specific for NS1 protein, a non-structural protein produced only during active DV infection. As shown in Fig. 1*B*, more than 30% of monocyte-derived DCs were productively infected by FGA/NA d1d DV-1 strain. In contrast, exposure of LCs to the same DV strain resulted in infection of less than 1% of cells. Similar results were obtained with other DV serotypes (data not shown). These findings indicate that DV productively infects immature DCs and not LCs.

DC-SIGN Mediates Infection of DCs by the Four DV Serotypes—To study the interaction of DV with DCs in more detail, we challenged immature DCs with representative mosquito-derived DV strains from the four serotypes at different m.o.i. Fig. 2*A* shows that DV-1 to DV-4 productively infect human immature DCs. Competition experiments indicated that infection of DCs by DV-1 FGA/NA d1d is completely inhibited by DC-SIGN neutralizing mAb and unaffected by isotype control Ab (Fig. 2*B*). Similar results were obtained with DV serotypes DV-2, DV-3, and DV-4 (data not shown). Titration of cell-free supernatants collected from DCs challenged with the four DV serotypes shows that infected DCs release equal amounts of infectious viral particles. Furthermore, preincubation of DCs with neutralizing anti-DC-SIGN Ab prior to infection abrogates virus production (Fig. 2*C*). Exposure of immature DCs to infectious DV particles has been shown to induce DC maturation (29, 30). To evaluate whether this activation process requires DC-SIGN-mediated viral infection, we challenged immature DCs with DV-1 FGA/NA d1d at an m.o.i. of 5, in the presence or absence of anti-DC-SIGN mAb or isotype control Ab. At 40 h post-infection, cell surface expression of CD83 and CD86, two DC maturation markers, was evaluated by flow cytometry (Fig. 2*D*). Consistent with previous studies, challenge of immature DCs with DV resulted in a robust up-regulation of CD83 and CD86 cell surface expression. As a control, expression levels of CD1a were similar in mock-infected or cells infected with DV-1. We found that mean surface levels of HLA-DR were also up-regulated in DV-infected DCs, whereas DC-SIGN expression was slightly decreased (data not shown). Compared with isotype control Ab, preincubation of cells with neutralizing anti-DC-SIGN Ab significantly inhibited DV-mediated DC maturation as indicated by the absence of CD83 and CD86 up-regulation in infected cells (Fig. 2*D*). Similar results were obtained with the other DV serotypes (data not shown). In control experiments, incubation of immature DCs with UV-inactivated DV did not induce DC maturation, suggesting that this process is dependent on viral replication rather than capture of DV particles by DCs (data not shown). Overall, these data indicate that DC-SIGN is a critical factor for productive infection of DCs by all DV serotypes and consequently for DC maturation.

DC-SIGN Expression Renders Poorly Susceptible Cells Infectable by DV—We investigated the capacity of DC-SIGN to promote DV entry and replication when expressed in several human cell lines. Raji, HeLa, and HEK-293T cells, which lack endogenous DC-SIGN expression, were transduced with vesic-

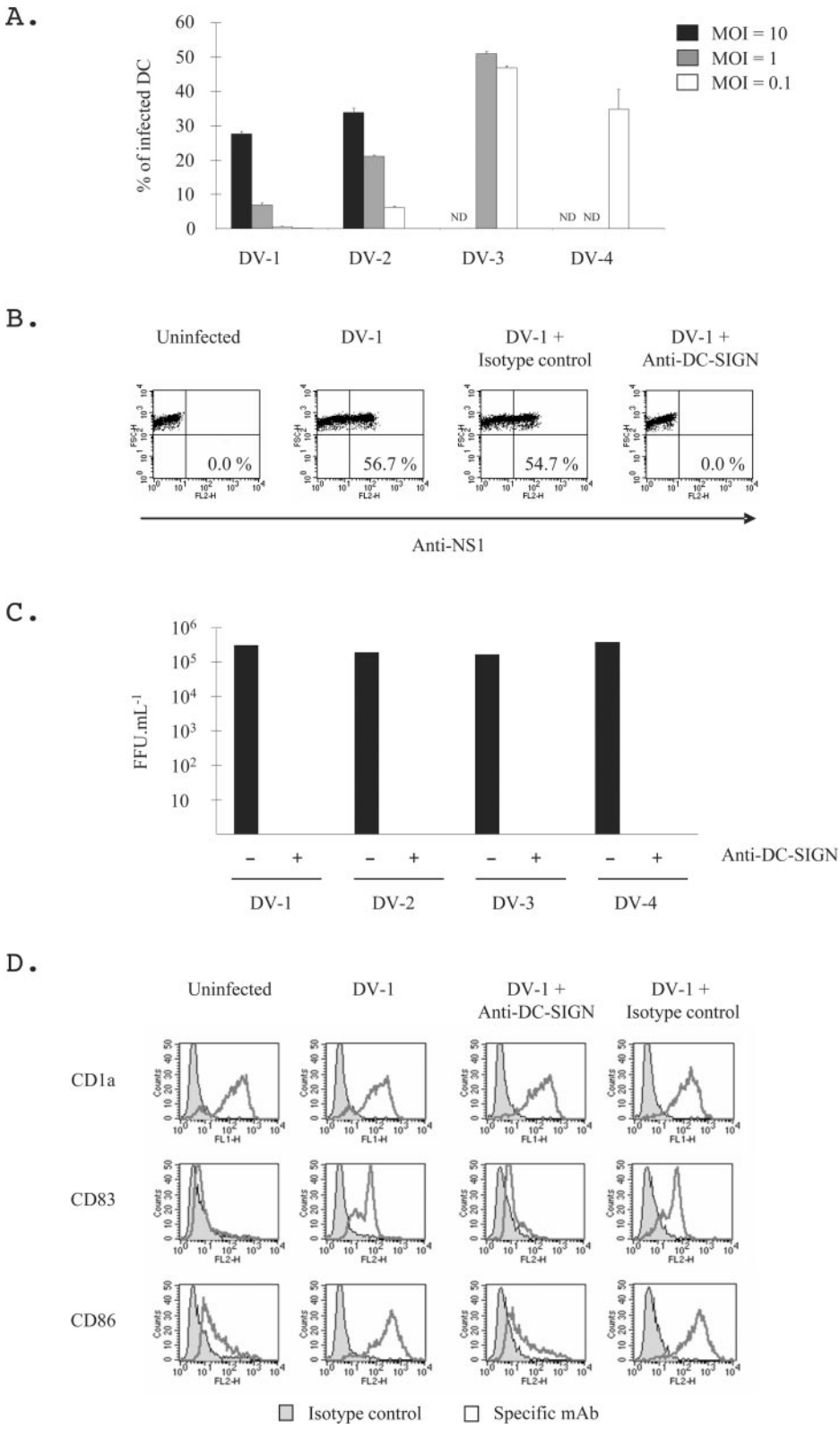

FIG. 2. **DC-SIGN is critical for productive infection of DCs by the four DV serotypes and for DV-induced DC maturation.** *A*, monocyte-derived DCs were infected with representative mosquito-derived DV strains at m.o.i. values of 10, 1, and 0.1. Infected cells were permeabilized and stained with anti-DV antigen HMAF-1 to -4. Viral infection was quantified by FACS as described above (*ND*, undetermined). *B*, anti-DC-SIGN mAb inhibits DV-1 infection in DCs. DCs were infected with DV-1 at a m.o.i. of 10 in the presence of the anti-DC-SIGN mAb1B10 (20 $\mu g \cdot ml^{-1}$) or a control IgG2a (20 $\mu g \cdot ml^{-1}$). Infected cells were stained for the NS1 and viral infection was evaluated by FACS analysis as described above. *C*, anti-DC-SIGN mAb strongly inhibits production of progeny virions in DCs. Cells were infected with DV-1 to DV-4 at a m.o.i. of 1 for 2 h in the presence or absence of anti-DC-SIGN mAb (20 $\mu g \cdot ml^{-1}$). Cells were washed several times and incubated at 37 °C for 24 h. Viral titer in supernatants was expressed as focus-forming units per ml in AP61 cells. *D*, DV-1 infection induces maturation of DCs in a DC-SIGN dependent-manner. DCs were infected with DV-1 (m.o.i. of 5) in the presence or absence of anti-DC-SIGN neutralizing mAb. The up-regulation of CD83 and CD86 was measured by FACS. The data presented are representative of three different experiments.

ular stomatitis virus G glycoprotein-pseudotyped TRIP ΔU3 vector particles, encoding human WT DC-SIGN. Cells were stained with anti-DC-SIGN mAb and cell-sorted for a high level of DC-SIGN expression (Fig. 3*A*). We found that parental HEK-293T, Raji, and HeLa cells are poorly susceptible to DV infection. In marked contrast, when DC-SIGN was exogenously expressed in these cell lines, efficient viral replication occurred (Fig. 3*B*). Indeed, 80% of DC-SIGN-expressing Raji and HEK-293T and 50% of HeLa-DC-SIGN were positive for NS1 expression. Similar results were obtained using other human and murine cell lines expressing DC-SIGN, such as Jurkat or 3T3 (data not shown). Raji-DC-SIGN and HEK-293T-DC-SIGN cells, which express higher levels of DC-SIGN than their HeLa cell counterpart, were found to be more susceptible to DV-1. This suggests that DV infection efficiency is dependent upon DC-SIGN cell surface expression (Fig. 3*B*). Consistent with this, we generated Raji cells expressing DC-SIGN at different levels (Fig. 3*C*) and showed that DV infection is enhanced in

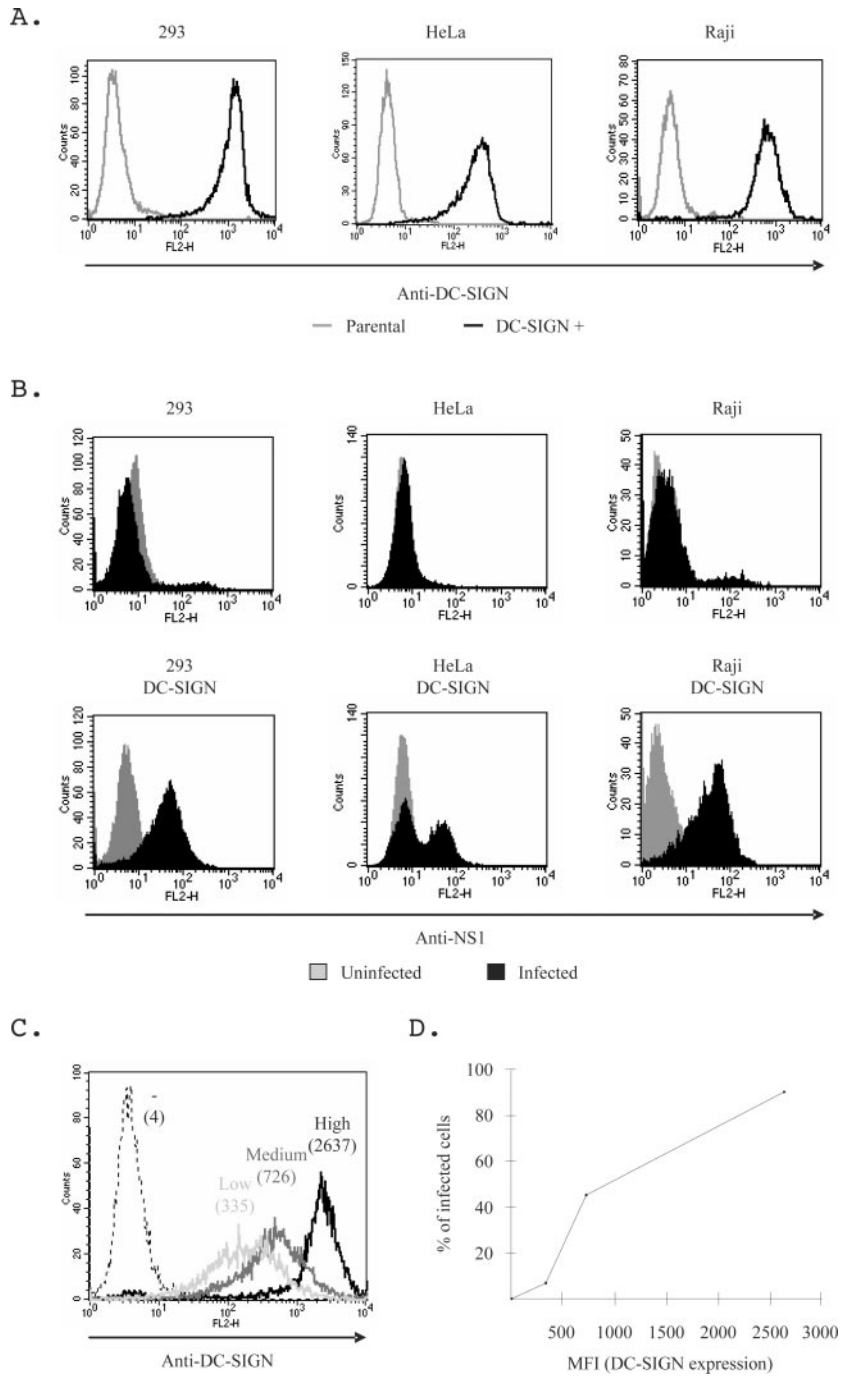

FIG. 3. **Ectopic expression of DC-SIGN enhances DV infection.** *A*, HEK-293T, HeLa, and Raji cells were inoculated with TRIP ΔU3-DC-SIGN WT vector particles. DC-SIGN expression was measured by flow cytometry analysis using PE-conjugated anti-DC-SIGN mAb. *B*, parental and DC-SIGN-expressing HEK-293T, HeLa, and Raji cells were infected with DV-1 at a m.o.i. of 10. Cells were stained for the NS1 non-structural antigen, and viral infection was quantified by FACS. *C*, Raji cells expressing different levels of DC-SIGN were generated by retroviral transduction and sorting for DC-SIGN expression. Cells were than stained with a PE-anti-DC-SIGN mAb and analyzed by FACS. The mean fluorescence intensities (*MFI*) are indicated in parentheses. *D*, parental and Raji-DC-SIGN high, medium, and low were infected by DV-1 (m.o.i. of 10) and stained for the NS1 non-structural antigen. DV infection correlates with DC-SIGN expression. Values are given as the mean of triplicates ± S.E. (not visible on the graph). The data are representative of three independent experiments.

correlation with DC-SIGN expression (Fig. 3*D*). We observed that high DC-SIGN expression levels are required for optimal viral entry and infection (Fig. 3*C*). Taken together, these results indicate that ectopic expression of DC-SIGN renders many cell lines infectable by DV, thus mimicking the role of DC-SIGN in DV entry into immature DCs.

Soluble DV E Protein Interaction with DC-SIGN—DV E protein has two conserved *N*-linked glycosylation sites at Asn-67 and Asn-153 that are believed to mediate DV binding to DC-SIGN (5, 6, 12). To study the interaction of E protein with DC-SIGN, the coding sequence for PrM and the extracellular domain of E (sE), corresponding to amino acid 101–673 of polyprotein of DV-1 isolate FGA/NA d1d (fused to C9 bovine rhodopsin peptide at the C terminus), was cloned into a SFV expression vector. To produce sE protein, BHK cells were infected with SFV encoding the PrM-sE precursor protein and then radiolabeled with [^{35}S]methionine/cysteine. SDS-PAGE analysis shows that sE protein is the major protein secreted by SFV-infected cells, as confirmed by immunoprecipitation with anti-C9 or HMAF-1 Ab (Fig. 4*A*). As the natural transmission vector of DV is the *Aedes* aegypti insect, the E protein glycosylation at the surface of virions transmitted to humans contains only mannose residues (2, 31). To mimic this mannose glycosylation pattern, we treated SFV-infected BHK cells with α-mannosidase inhibitors DMJ and swainsonine. The resulting protein, sEman, acquires only mannose *N*-glycans as demonstrated by sensitivity to EndoH digestion (Fig. 4*B*). In contrast, sE synthesized in untreated cells acquired complex glycosylation and was completely EndoH-resistant (Fig. 4*B*). However, both sEman and sE were sensitive to PNGase F digestion, which cleaves both complex and mannose *N*-glycans at the Asn-GlcNac bond, resulting in a deglycosylated E protein. We

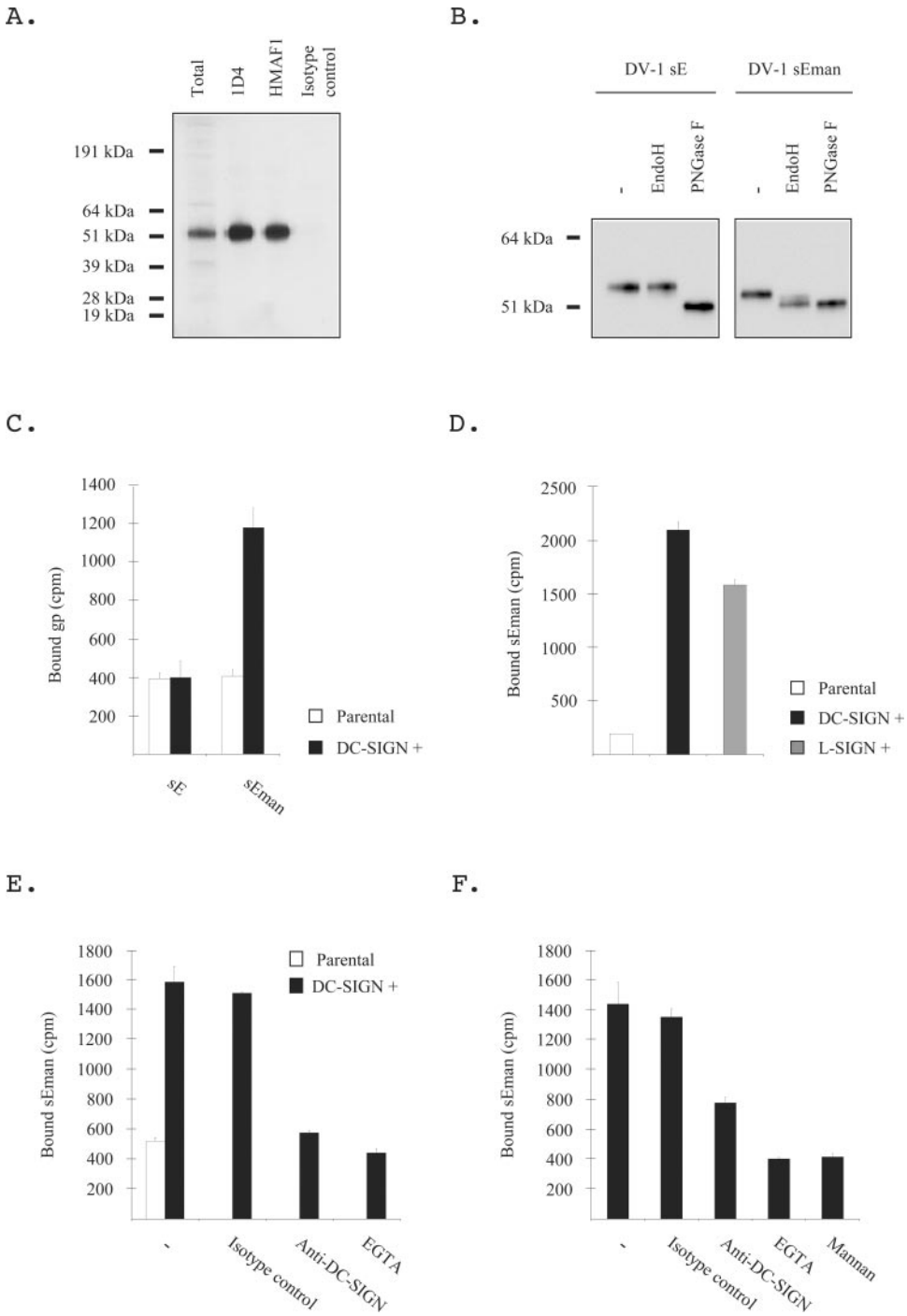

FIG. 4. **E glycoprotein binding to DC-SIGN requires mannosylated *N*-glycans.** *A*, BHK cells were infected with SFV encoding for DV-1 prM and sE and then radiolabeled with [^{35}S]methionine/cysteine. Total secreted proteins were immunoprecipitated with an anti-C9 epitope mAb (1D4), an anti-DV-1 antigen antibody (HMAF-1), or an isotype control and analyzed by SDS-PAGE. *B*, DV-1 sE was produced in BHK cells in the presence or absence of mannosidase inhibitors (1 mM DMJ and 5 μM swainsonine) (sEman or sE, respectively). Secreted E proteins were subjected to digestion with EndoH or PNGase F and analyzed by Western blot using anti-C9 antibody (1D4). *C*, sEman, but not sE, binds to DC-SIGN. ^{35}S-Labeled sE (50 nM) and sEman (50 nM) were incubated for 2 h at 4 °C with HeLa and HeLa-DC-SIGN. Cells were washed three times before measuring cell-associated radioactivity. *D*, ^{35}S-labeled sEman (50 nM) was incubated for 2 h at 4 °C with Raji, Raji-DC-SIGN, or Raji-L-SIGN, and washed three times before counting of cell-associated radioactivity. *E*, specificity of sEman binding to DC-SIGN. HeLa-DC-SIGN cells were incubated with sEman (50 nM) in the presence of known DC-SIGN inhibitors such as EGTA (5 mM) and mAb 1B10 (20 $\mu g \cdot ml^{-1}$) or an isotype control (20 $\mu g \cdot ml^{-1}$). Bound radioactivity was quantified as described above. *F*, DC-SIGN mediates sEman binding to dendritic cells. sEman (50 nM) was incubated with DCs in the presence of mannan (20 $\mu g \cdot ml^{-1}$), EGTA (5 mM), and mAb 1B10 (20 $\mu g \cdot ml^{-1}$) or isotypic control (20 $\mu g \cdot ml^{-1}$). Cells were washed, and cell-associated radioactivity was counted. The data are representative of three independent experiments. Values are given as the mean of triplicates ± S.E.

found that ^{35}S-labeled sEman proteins bind to Raji cells expressing either DC-SIGN or its homologue L-SIGN but not to parental cells (Fig. 4*D*). In contrast to sEman, we observed that sE, which contains complex glycosylation, was unable to bind to DC-SIGN-expressing cells, indicating that high mannose *N*-glycans on the DV E protein are crucial for interactions with DC-SIGN (Fig. 4*C*). Attachment of sEman proteins to DC-SIGN-expressing cells is inhibited by neutralizing anti-DC-

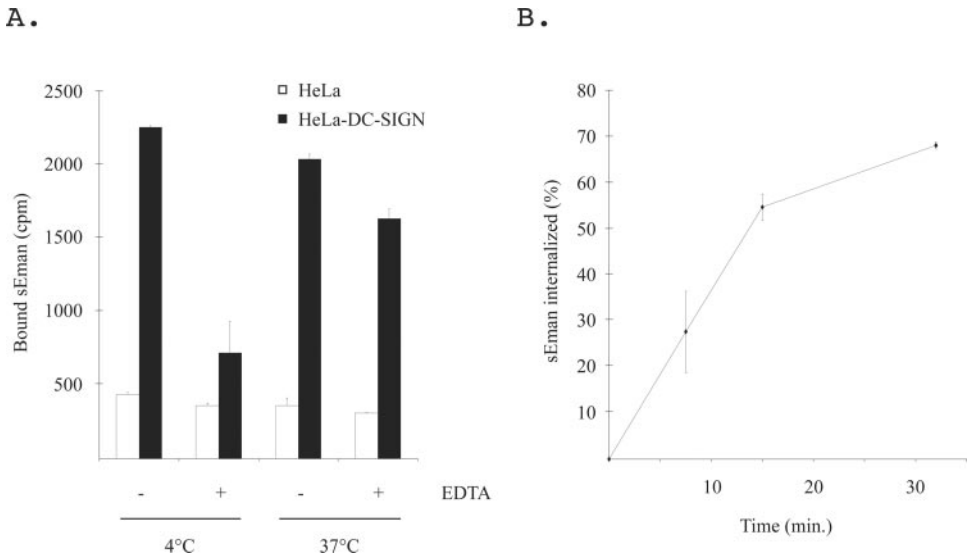

FIG. 5. **DC-SIGN induces internalization of DV-1 sEman.** *A*, ^{35}S-labeled sEman was bound to HeLa or HeLa-DC-SIGN cells for 2 h at 4 °C. Cells were extensively washed to eliminate unbound material and incubated for 30 min either at 4 or 37 °C. Cells were treated with EDTA (50 mM) or mock-treated to distinguish internalized from cell surface bound E glycoprotein. *B*, internalization time course of ^{35}S-labeled sEman in HeLa-DC-SIGN cells. The data are representative of two independent experiments. Values are given as the mean of triplicates ± S.E.

SIGN mAb and the calcium chelator EDTA (Fig. 4*E*). Moreover, we found that primary immature DCs, which constitutively express DC-SIGN, efficiently capture ^{35}S-labeled sEman. This interaction is diminished by anti-DC-SIGN mAb and strongly inhibited by EDTA or mannan (Fig. 4*F*). Together, these results identify DV E as a specific DC-SIGN-binding protein and suggest a critical role for high mannose *N*-glycans in DV E glycoprotein attachment to DC-SIGN.

E Protein Endocytosis into DC-SIGN-expressing Cells—To determine whether E protein is internalized by DC-SIGN, we incubated HeLa-DC-SIGN cells on ice with ^{35}S-labeled sEman. After washing to remove unbound material, cells were maintained on ice or shifted to 37 °C for 30 min. Cells were then treated with PBS containing EDTA for 10 min to distinguish internalized E protein from E protein bound to DC-SIGN at the cell surface. We found that more than 90% of the E protein bound to DC-SIGN was eliminated by EDTA washing when cells were maintained at 4 °C. In contrast, after incubation for 30 min at 37 °C, we observed that a large fraction of sE protein was resistant to EDTA treatment, indicating that DC-SIGN mediates internalization of DV E protein at 37 °C (Fig. 5*A*). Kinetic analysis of E protein endocytosis indicates that DC-SIGN-mediated DV E protein endocytosis is rapid, as more than 50% of E protein is internalized after 15 min incubation at 37 °C (Fig. 5*B*)

Role of DC-SIGN Internalization in DV Entry and Replication—DC-SIGN contains three motifs in its cytoplasmic tail that are believed to be involved in either internalization or endocytic trafficking (9, 26). These include a dileucine motif (LL), a tyrosine-based motif (YSKL), which are both two classically defined putative internalization motifs, and a triacidic (EEE) cluster (9, 28). To study the role of these three motifs in DC-SIGN trafficking and DV internalization, we cloned DC-SIGN mutants containing alanine substitutions either at the LL, YSKL, or EEE motifs into the TRIP ΔU3 vector. HeLa cells were then transduced with vesicular stomatitus virus G glycoprotein-Env pseudotyped-TRIP ΔU3 vector particles, encoding either DC-SIGN WT or mutated molecules. Immunostaining with anti-DC-SIGN mAb indicates that DC-SIGN WT, LL, Y, and EEE proteins were expressed at comparable levels at the cell surface (Fig. 6*A*). Confocal microscopy was used to assess Ab-mediated DC-SIGN endocytosis in HeLa cells. For this, cells were incubated with the anti-DC-SIGN 8A5 mAb for 1 h at 4 °C, washed extensively, and shifted to 37 °C to initiate DC-SIGN internalization. At 4 °C, DC-SIGN WT and mutant receptors were mainly detected at the cell surface. When shifted at 37 °C, we observed a dramatic redistribution of 8A5 staining in DC-SIGN WT, Y, and EEE cells, consistent with an intracellular accumulation of 8A5-bound DC-SIGN. This indicates that WT, Y, and EEE DC-SIGN receptors are efficiently internalized in HeLa cells upon 8A5 mAb binding. In marked contrast, we found that the majority of DC-SIGN-LL receptors remained mainly at the surface of HeLa cells after 8A5 binding. These data suggest that the LL motif is required for efficient DC-SIGN endocytosis and intracellular trafficking.

DC-SIGN WT and mutated proteins bind radiolabeled DV sEman with similar efficiency (Fig. 7*A*). Since DC-SIGN expression correlates strongly with efficient internalization and infection of DV, we investigated whether DV entry in DC-SIGN-expressing cells requires a functional DC-SIGN-coupled endocytic pathway. For this purpose, HeLa cells expressing WT and mutated DC-SIGN molecules were infected with the DV-1 strain FGA/NA d1d virus at varying m.o.i. Fig. 7*B* shows that internalization-defective DC-SIGN-LL molecules enhance DV infection as efficiently as DC-SIGN WT, Y and EEE (Fig. 7*B*.). Similar results were obtained in HEK-293T cell lines expressing DC-SIGN LL (Fig. 7, *C* and *D*). To rule out a potential role of DC-SIGN-mediated signaling in DV infection, we also generated HEK-293T cells expressing a DC-SIGN mutant lacking its entire cytoplasmic tail (DC-SIGN ΔN). Cell surface expression levels of DC-SIGN ΔN were found to be significantly lower than WT or LL DC-SIGN (Fig. 7*C*); however, our infection studies show that DC-SIGN ΔN is still able to enhance DV entry (Fig. 7*D*). Treatment of HeLa cells expressing WT or LL DC-SIGN molecules with the pH-interfering drug bafilomycin A1 induces a dose-dependent inhibition of DV-1 replication (Fig. 8). Together, these data indicate that DC-mediated enhancement of DV entry is a pH-dependent process, which requires internalization of the incoming virus. However, our results show that DC-SIGN endocytosis is dispensable for targeting DV to acidified endosomes. Thus, DV entry into DC-SIGN-expressing cells can be dissociated from DC-SIGN-signaling and internalization pathways.

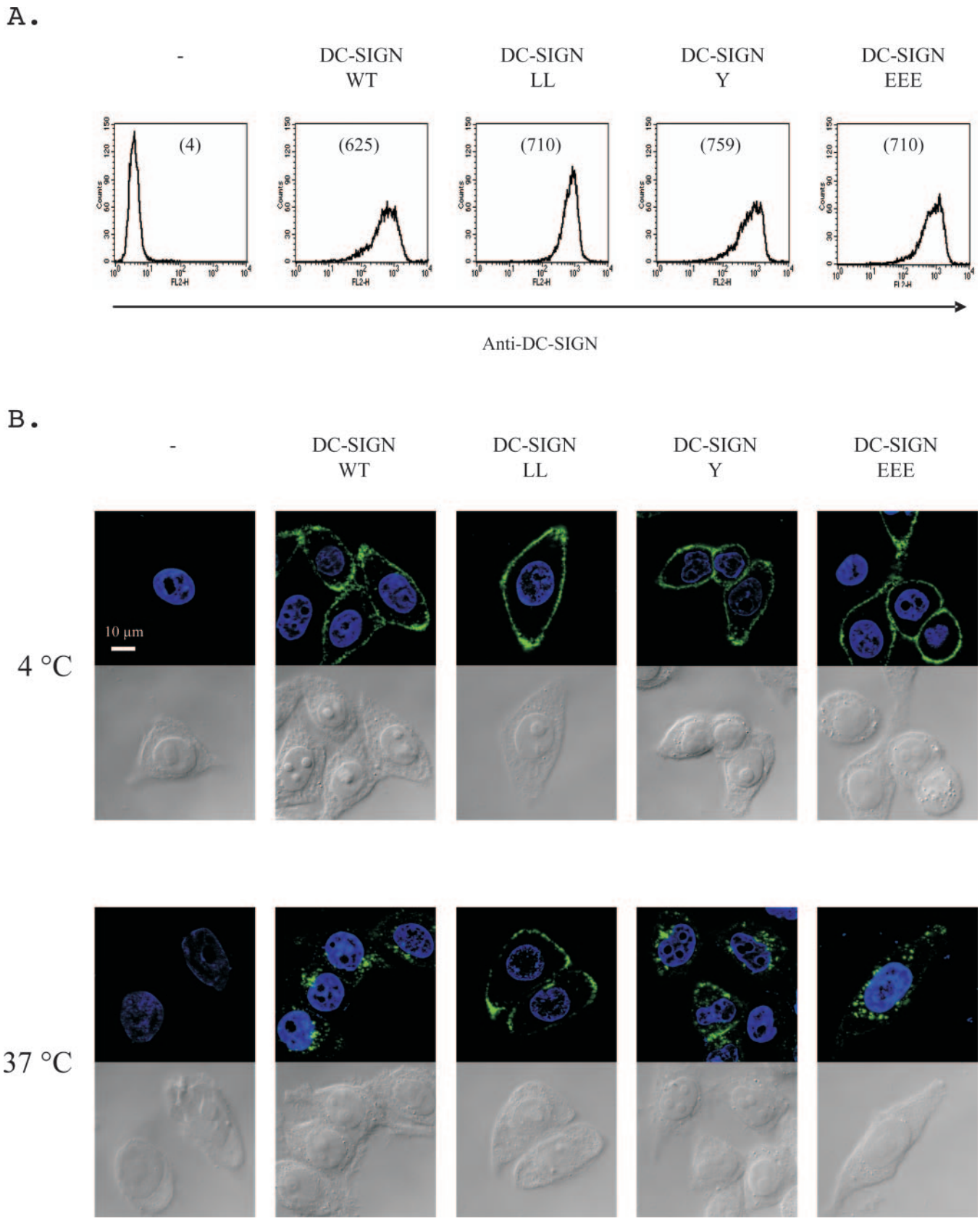

FIG. 6. **Charaterization of WT and mutant DC-SIGN proteins.** *A*, HeLa cells were transduced with a lentiviral vector encoding either for WT or mutated forms of DC-SIGN and stained for DC-SIGN expression. Mean fluorescence intensities are indicated in *parentheses*. *B*, subcellular localization of WT and DC-SIGN mutants in HeLa cells. 8A5 anti-DC-SIGN mAb was incubated with parental HeLa or cells expressing DC-SIGN WT, LL, Y, and EEE proteins for 1 h at 4 °C. Cells were washed to eliminate unbound antibody and incubated for 40 min either at 4 or 37 °C. DC-SIGN-bound 8A5 was detected with a FITC-conjugated secondary Ab. Cell surface and intracellular localization of DC-SIGN was examined by confocal microscopy. The data are representative of three independent experiments.

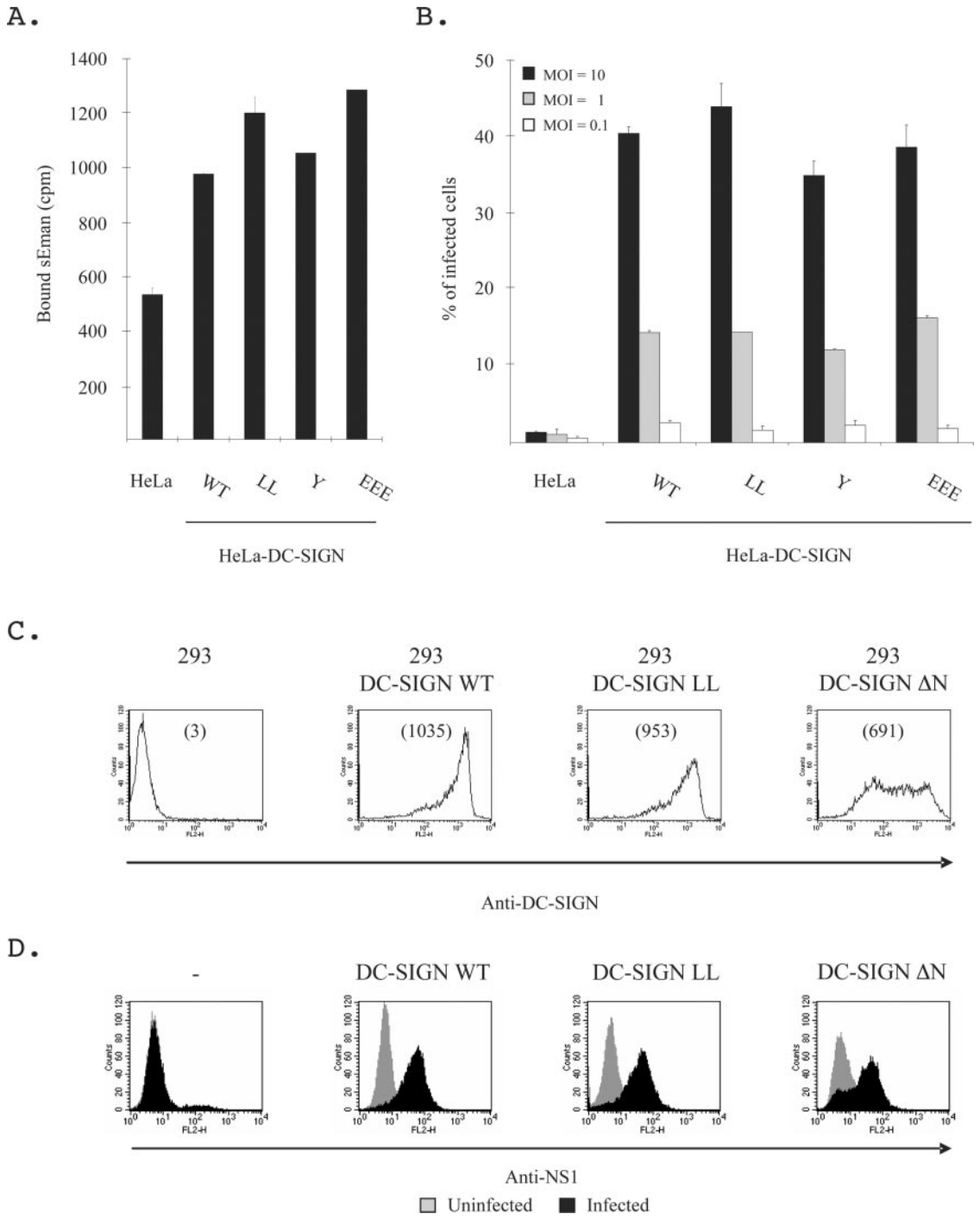

FIG. 7. **DV infection does not require DC-SIGN internalization.** *A*, ^{35}S-labeled sEman was incubated for 2 h at 4 °C with HeLa expressing mutants and WT DC-SIGN. Cells were washed three times before measuring cell-associated radioactivity. *B*, cells were infected with DV-1 at different m.o.i, stained for the NS1 antigen, and analyzed by FACS. Viral infection was expressed as the percentage of NS1-positive cells. *C*, HEK-293T cells were transduced with a lentiviral vector encoding for WT, LL, or ΔN DC-SIGN molecules and stained for DC-SIGN expression. *D*, HEK-293T cells expressing WT, LL, or ΔN DC-SIGN were infected with DV-1 at m.o.i. of 10. Viral infection was evaluated by FACS analysis and expressed as the percentage of NS1-positive cells. Values are given as the mean of triplicates ± S.E. The data are representative of three independent experiments.

DISCUSSION

We and others (5, 6) have previously shown that the C-type lectin DC-SIGN (CD209) is an essential cellular factor for DV infection of immature DCs. The restricted expression of DC-SIGN on skin dermal DCs combined with the ability of this lectin to enhance DV infection suggests that DC-SIGN may be a critical factor allowing the early steps of DV pathogenesis. In this study, we provide new insights into the molecular mechanisms by which DC-SIGN binds DV particles and mediates viral entry.

Different subpopulations of DCs reside in human skin, the site of DV exposure (32). Among skin DCs, immature LCs, a unique DC subset found in the epidermis, are believed to be productively infected by DV and thus proposed to be the first cells targeted by the virus (4). Indeed, CD1a+ LCs in the skin of a human volunteer who had received a live attenuated DV vaccine have been shown to immunostain for DV E protein (4). Furthermore, HLA-DR+ cells emigrating from human skin explants inoculated with DV particles were found to support

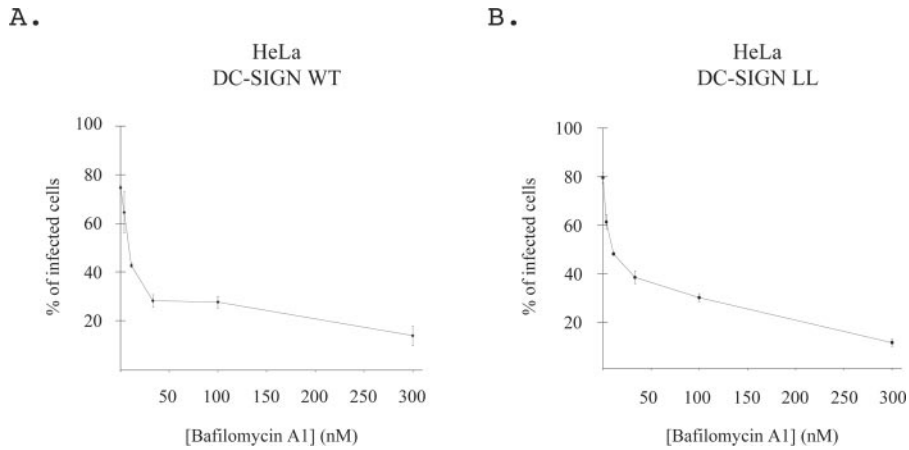

FIG. 8. **DV entry mediated by internalization-defective DC-SIGN mutants requires acidification.** HeLa cells expressing DC-SIGN WT (*A*) or DC-SIGN LL (*B*) were infected with DV-1 at a m.o.i. of 15 in the presence of the pH-interfering drug bafilomycin A1 at varying concentrations (0–300 nM). Treated cells were stained 40 h post-infection for the NS1 and viral infection was quantified by FACS. The data are representative of two independent experiments.

DV replication (4). However, these data do not unequivocally demonstrate that immature LCs replicate DV, since the emigrant cells are a mixture of both epidermal LCs and interstitial DCs. Recent progress in LCs and DCs generation *in vitro*, as well as the identification of new LCs- and DC-specific cell markers (Langerin and DC-SIGN, respectively), now allows more accurate characterization of DV target cells in human skin. DC-SIGN and Langerin are C-type lectins that recognize high mannose oligosaccharides and have both been shown to interact with HIV-1 gp120 (26, 33).[2] Since DV particles produced in insect cells are rich in mannose residues, DV transmitted by infected mosquitoes could potentially use either DC-SIGN or Langerin to enter target cells. However, our results show that, in contrast to monocyte-derived DCs (DC-SIGN-positive, Langerin-negative), *in vitro* differentiated LCs (Langerin-positive, DC-SIGN-negative) are poorly infectable by DV. Although these results do not exclude the possibility that Langerin interacts with DV, our findings argue against a role of LCs as primary targets of DV infection and replication. These results suggest that during DV transmission from mosquito to humans, dermal DC-SIGN+ DCs, rather that intraepithelial LCs, are infected by DV.

DC-SIGN recognizes the envelope glycoproteins of several viruses such as HIV, cytomegalovirus, and HCV (8, 18, 34). In this study, we demonstrate that DV E protein, the sole glycoprotein exposed at the surface of DV particles, binds specifically to DC-SIGN. Our results indicate that the nature of E protein glycosylation is critical for DV attachment to DC-SIGN. We observed that only mannosylated E glycoprotein, and not protein with complex glycosylation, is able to interact with DC-SIGN. In keeping with this finding, we have previously shown that treatment of DV particles with EndoH greatly reduces their ability to infect immature DCs (6). Together, these results strongly suggest that the high mannose glycans present at the surface of mosquito-derived virions are essential for DV interaction with DC-SIGN and viral entry into DCs. Two putative *N*-linked glycosylation sites are found in the DV E protein (12). The Asn-67 site is unique to DV, whereas Asn-153 is common to other flaviviruses. Interestingly, DV-2 and DV-4 E proteins are apparently glycosylated only at the Asn-67 site, while DV-1 and DV-3 E proteins are glycosylated at both Asn-67 and Asn-153 sites (12). Our results show all four DV serotypes require DC-SIGN to productively infect immature DCs, in agreement with other studies (5). This suggests that Asn-67 is sufficient to mediate DV interaction with DC-SIGN and infection of DCs. However, we cannot exclude the possility that Asn-153 contributes to the interaction between DC-SIGN and DV-1 or DV-3 particles. Mutagenesis studies will be required to fully characterize the role of these two *N*-linked glycosylation sites in DV infection.

DC-SIGN is oligomeric at the cell surface (35), and only tetrameric DC-SIGN molecules bind mannosylated *N*-glycans with high affinity (8, 36). This suggests that multiple contacts may occur between DC-SIGN and several high mannose *N*-glycans on viral glycoproteins and that the affinity of such an interaction is a function of the glycosylation level (7, 8, 36–38). In support of this model, we found that the affinity of DC-SIGN for DV E protein, which has only two potential glycosylation sites, appears to be lower than that observed for the heavily glycosylated HIV gp120 and HCV E2 proteins (1.5 and 3 nM, respectively) (8, 39). Indeed, saturation of all binding sites on DC-SIGN-expressing cells was not observed even with 50 nM of sE (data not shown). These observations are in agreement with the conclusions of a recent *in silico* model that predicts a weak interaction between E protein and DC-SIGN (38) but contrast with the high efficiency of DC-SIGN to mediate DV infection. Cryoelectron microscopy of DV particles reveals a highly organized network of densely packed E protein dimers on the virion surface (10, 40, 41). Although we cannot exclude that a low affinity interaction between DC-SIGN and the E glycoprotein is sufficient to mediate efficient DV entry, we propose that the structural organization of *N*-glycans on the surface of viral particles could favor the engagement of multiple E proteins dimers by each tetrameric lectin. This would allow a high avidity interaction between oligomeric DC-SIGN and DV particles, resulting in efficient viral capture and entry.

By analogy to other flaviviruses, DV is assumed to gain entry into target cells by receptor-mediated endocytosis, leading to viral fusion in acidified endosomes (3). In keeping with this, our observations show that DV entry into DC-SIGN-expressing cells is pH-dependent. Our data indicate that binding of DV E protein to DC-SIGN triggers a rapid and efficient internalization of the viral glycoprotein, suggesting the involvement of the endocytic pathway in DC-SIGN-mediated DV infection. However, these observations do not permit to conclude whether DC-SIGN acts as cell surface attachment factor or as an authentic entry receptor that mediates virus internalization. The DC-SIGN cytoplasmic tail carries two putative internalization motifs (a dileucine, LL and a tyrosine-based motifs, *YXX*L) and a triacidic cluster (EEE) that is believed to be involved in intracellular trafficking (9, 28, 42). Replacement of the LL motif by alanines abolishes DC-SIGN endocytosis mediated by 8A5 mAb. In contrast, we found that both the YSKL motif and the triacidic EEE cluster are not involved in DC-SIGN internalization. It is interesting to note that acidic sequences in

[2] P.-Y. Lozach, L. Burleigh, I. Staropoli, E. Navarro-Sanchez, J. Harriague, J.-L. Virelizier, F. A. Rey, P. Desprès, F. Arenzana-Seisdedos, and A. Amara, unpublished data.

other receptors are implicated in the routing of antigens to the major histocompatibility complex class II pathway (43). It remains to be determined whether the triacidic cluster in DC-SIGN has a similar function and is therefore involved in initiation of immune responses against DV. We observed that the endocytosis-defective DC-SIGN molecules (LL and ΔN mutants) permit DV entry with similar efficiency to WT DC-SIGN. These results indicate that DV entry into DC-SIGN-expressing cells may be dissociated from DC-SIGN internalization. Our data suggest that DC-SIGN plays a prominent if not exclusive role as a DV cell surface attachment factor and argue in favor of a mechanism by which DC-SIGN enhances DV entry *in cis*. These results do not formally rule out the possibility that in immature DCs, DV particles bound to DC-SIGN could be internalized by alternative mechanisms such as phagocytosis, as described previously for DC-SIGN-mediated entry of *Mycobacterium* tuberculosis (15, 44). Furthermore, we cannot exclude the possibility that, in parallel to the major *cis*-mechanism described above, a proportion of DV enters immature DCs directly through DC-SIGN endocytosis. Nevertheless, and according to our results, the contribution of this additional entry route would be minor. Collectively, our findings imply the existence of a specific receptor required for DV internalization into DCs. We hypothesize that such a molecule is present at low levels on DCs and mediates DV entry with very poor efficiency in the absence of DC-SIGN. Several molecules such as GRP78/BIP, HSP90, HSP70, heparan sulfate, and LPS/CD14-associated binding proteins have been proposed to participate in DV entry into target cells but their role as authentic cellular receptors for DV remains unclear (45–48). In conclusion, we propose that DC-SIGN cooperates with an unidentified cellular entry receptor to permit infection of skin DCs during the mosquito's blood meal. DV may take advantage of the DC mobility to ensure its dissemination within the host and propagate the infection to other tissues. To determime whether these functions are associated with DC maturation induced by DV infection requires further investigation. The development of an animal model to study the role played by DC-SIGN in DV transmission *in vivo* should permit us to investigate these and other important questions related to DV pathogenesis.

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