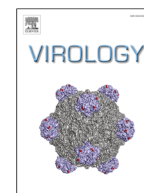
Virology 488 (2016) 108–119

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro



# DC-SIGN as an attachment factor mediates Japanese encephalitis virus infection of human dendritic cells via interaction with a single high-mannose residue of viral E glycoprotein

Ping Wang[a,b], Kai Hu[a], Sukun Luo[a,b], Mudan Zhang[a,b], Xu Deng[a,b], Chang Li[a,b], Wei Jin[a,b], Bodan Hu[a,b], Siyi He[a,b], Mei Li[a,b], Tao Du[a], Gengfu Xiao[a], Bo Zhang[a], Yalan Liu[a], Qinxue Hu[a,c,*]

[a] State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China
[b] University of Chinese Academy of Sciences, Beijing 100049, China
[c] Institute for Infection and Immunity, St George's University of London, London SW17 0RE, UK

ARTICLE INFO

*Article history:*
Received 10 August 2015
Returned to author for revisions
6 November 2015
Accepted 9 November 2015
Available online 26 November 2015

*Keywords:*
Japanese encephalitis virus
Dendritic cell
Attachment factor
DC-SIGN
Glycoprotein
Internalization

ABSTRACT

The skin-resident dendritic cells (DCs) are thought to be the first defender to encounter incoming viruses and likely play a role in Japanese encephalitis virus (JEV) early infection. In the current study, following the demonstration of JEV productive infection in DCs, we revealed that the interaction between JEV envelope glycoprotein (E glycoprotein) and DC-SIGN was important for such infection as evidenced by antibody neutralization and siRNA knockdown experiments. Moreover, the high-mannose *N*-linked glycan at N154 of E glycoprotein was shown to be crucial for JEV binding to DC-SIGN and subsequent internalization, while mutation of DC-SIGN internalization motif did not affect JEV uptake and internalization. These data together suggest that DC-SIGN functions as an attachment factor rather than an entry receptor for JEV. Our findings highlight the potential significance of DC-SIGN in JEV early infection, providing a basis for further understanding how JEV exploits DC-SIGN to gain access to dendritic cells.
© 2015 Elsevier Inc. All rights reserved.

## Introduction

Japanese encephalitis virus (JEV) is an enveloped single-stranded positive-sense RNA virus belonging to the genus *Flavivirus*. JEV is arthropod-borne and maintained in a zoonotic cycle between mosquitoes and pigs or wild birds, and could also be transmitted to humans and horses (Lopez et al., 2015; van den Hurk et al., 2009). According to statistics from the World Health Organization, despite the existence of JEV vaccines, Japanese encephalitis (JE) is still the main cause of viral encephalitis in many countries of Asia with nearly 68,000 clinical cases every year. There is no cure for the disease. Currently, treatment is focused on relieving severe clinical signs by supporting the patients to overcome the infection. Although symptomatic JE is rare, the case-fatality rate can be as high as 30%. Permanent neurological or psychiatric sequelae can occur in 30–50% of those with encephalitis (Lopez et al., 2015). It is believed that a more comprehensive understanding of JEV early infection may offer approaches for the development of novel antiviral drugs and other intervention measures.

The entry of JEV into target cells is mediated by the interactions between the viral envelope glycoprotein E and cellular receptor(s). JEV can replicate in various types of primary cells and immortalized cell lines from a wide variety of avian, mammalian, amphibian and insect species, suggesting that multiple receptors are likely to be involved in JEV entry (Pierson and Kielian, 2013). To date, a number of cell molecules have been proposed as JEV candidate receptors in different cell types. For instance, heparan sulfate and low-density lipoprotein receptors, as low-affinity attachment factors that concentrate the viral particles on the cell surface, can be utilized by several flaviviruses including JEV (Albecka et al., 2012; Chen et al., 1997; Chien et al., 2008; Jiang et al., 2012; Lee et al., 2006). Integrin αVβ3 has been shown to function as a primary receptor on mammalian cells for West Nile virus and JEV (Chu and Ng, 2004b); however, other studies indicate that αVβ3 is not required for viral entry in some cellular context (Medigeshi et al., 2008). HSP70 has also been suggested to serve as the putative receptor for JEV in mouse neuroblastoma cells (Das et al., 2009; Zhu et al., 2012). Nevertheless, receptor(s) that JEV utilizes to entry and/or attach to target cells remains inconclusive.

* Corresponding author at: State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China. Fax: +86 2787199992.
*E-mail address:* qhu@wh.iov.cn (Q. Hu).

http://dx.doi.org/10.1016/j.virol.2015.11.006
0042-6822/© 2015 Elsevier Inc. All rights reserved.

Dendritic cells (DCs) are professional antigen presenting cells that can capture microorganisms in the peripheral mucosal tissues and then migrate to secondary lymphoid organs, where they present microorganisms in antigenic form to resting T cells and thus initiate adaptive immune responses (Malissen et al., 2014). Recent studies suggest that DCs, which are normal residents of the skin, represent an early target for flaviviruses. Infected DCs migrate to draining lymph nodes, where efficient viral replication takes place and subsequently leads to viremic stage with virus entry of circulation and internal organs (Diamond et al., 2003; Pierson and Kielian, 2013), suggesting the potential importance of DCs in JEV primary infection and transmission. Understanding the significance of DCs in JEV early infection is critical not only for elucidating viral pathogenesis but also for the development of effective prevention strategies.

DC-SIGN is one of the best-studied C-type lectin receptors expressed on DCs, and its involvement in the capture and transfer of HIV-1 has been intensively investigated (Arrighi et al., 2004; Geijtenbeek et al., 2000a). DC-SIGN has also been suggested to mediate the infection of DCs by Dengue virus and West Nile virus (Davis et al., 2006; Miller et al., 2008; Navarro-Sanchez et al., 2003). More recently, Shimojima et al. (2014) reported that ectopic expression of DC-SIGN resulted in a moderately enhanced JEV infection in a lymphoid cell line Daudi cells. However, how DC-SIGN promotes JEV infection has yet to be defined. More importantly, whether DC-SIGN mediates JEV infection of primary DCs that endogenously expressing DC-SIGN remains to be fully addressed.

In this study, we have demonstrated that DC-SIGN functions as an attachment factor to facilitate JEV infection of DCs and transfectants. DCs or DC-SIGN-expressing cells capture JEV via the interaction between DC-SIGN and the high-mannose *N*-linked glycan at N154 of JEV E glycoprotein, which could be blocked by DC-SIGN-specific neutralizing antibodies or inhibitors. Binding of JEV to DC-SIGN leads to rapid and effective virus internalization in a DC-SIGN endocytic activity-independent manner. Our findings suggest that DC-SIGN acts as an important JEV attachment factor that may facilitate subsequent interaction of viral particles with an as yet unidentified cellular receptor which leads to JEV entry into DCs.

## Results

### *DCs support productive JEV infection*

Considering the potential significance of DCs in initiating flavivirus infection (Barba-Spaeth et al., 2005; Davis et al., 2006; Jessie et al., 2004; Lozach et al., 2005), we generated primary DCs from human peripheral blood mononuclear cells (PBMCs) and investigated whether these cells could support JEV infection. DCs were exposed to different amounts of JEV (MOI of 0.001, 0.1 and

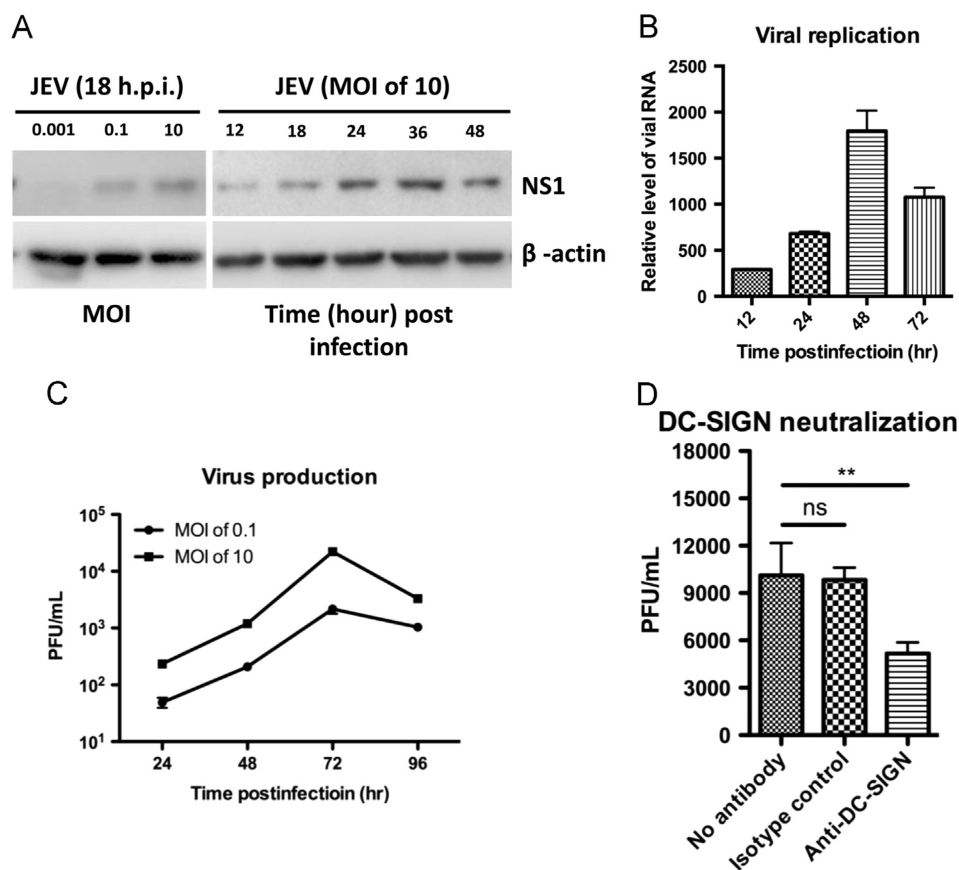



**Fig. 1.** DC-SIGN mediates JEV infection of DCs. (A) JEV replication in DCs. Monocyte-derived DCs were infected with JEV at different MOIs for 18 h or at a MOI of 10 for different periods of time. The intracellular expression of viral NS1 protein was detected by western blotting. One representative experiment out of three is shown. (B) Viral RNA levels in JEV-infected DCs. JEV-infected DCs (MOI of 10) were harvested on the indicated hours post infection (h.p.i.) and the total RNA was extracted and used to quantify JEV RNA by real-time PCR. Data are expressed as JEV RNA levels relative to those of uninfected controls and normalized to GAPDH levels. Data shown are mean ± SD of three independent experiments. (C) Production of progeny virions from DCs. Supernatants of cells infected with JEV (MOI of 0.1 and 10) were harvested at different time points and tittered in BHK-21 cells. Data shown are mean ± SD of three independent experiments. (D) Neutralization of DC-SIGN reduces JEV infection in DCs. DCs were infected with JEV (MOI of 1) in the presence of the anti-DC-SIGN mAb161 or an isotype control IgG2b (20 μg/ml). Infection was quantified by titration of virus production 3 days later. Data shown are mean ± SD of three independent experiments. ** $p < 0.01$. ns, not significant.

10, respectively) for indicated times and the intracellular expression of NS1 was determined. Flavivirus NS1 protein is a nonstructural glycoprotein that is not incorporated into virion but only produced in infected cells. As shown in Fig. 1A, newly synthesized JEV NS1 was observed in JEV-pulsed DCs, indicating that DCs are susceptible to JEV infection. Viral RNA synthesis increased over time and peaked at 48 h post-infection (Fig. 1B), implying that the level of viral RNA was associated with viral replication rather than the amount of input viral particles. Titration of cell-free supernatants, collected from challenged DCs, showed that a large amount of infectious JEV progenies was released within 72 h post-infection (Fig. 1C). Nevertheless, despite a productive JEV infection in DCs, the yield of infectious JEV particles from DCs was much lower than that from other JEV susceptible cell lines (Fig. S1A), reflecting the complexity of JEV infection in primary cells.

### *DC-SIGN mediates JEV infection*

DC-SIGN is highly expressed on DCs (Geijtenbeek et al., 2000b) and involved in the attachment of several viruses to DCs including HIV-1 (Pohlmann et al., 2001), Ebola (Alvarez et al., 2002) and Dengue virus (Lozach et al., 2005; Navarro-Sanchez et al., 2003). To examine the role of DC-SIGN in JEV infection of DCs, we first confirmed the surface expression of DC-SIGN on DCs (Fig. S1B). DCs were then pulsed with JEV in the presence of a neutralizing monoclonal Ab (mAb161; clone 120507) against the carbohydrate recognition domain (CRD) of DC-SIGN. We found that mAb161 inhibited JEV infection in a dose-dependent manner (Fig. S1C), and the inhibition peaked at a concentration of 25 μg/ml (50–60% of inhibition) (Fig. 1D; Fig. S1C), indicating that DC-SIGN plays a specific role in JEV infection of DCs. Another neutralizing antibody (clone 120526) against DC-SIGN CRD demonstrated a similar inhibitory effect as did mAb161, whereas the antibody H200, which targets the neck region of DC-SIGN, had no effect on JEV infection (Fig. S1D).

JEV can infect various types of primary cells and immortalized cell lines, making it difficult to find non-permissive cells for the study of potential viral receptors. We initially performed experiments to assess the permissiveness of different cell lines to JEV. After exposure to the same amount of JEV under the same conditions, the lowest level of bound viral particles was detected in Raji cells among several tested cell lines (Fig. S1E), suggesting that Raji cell line is substantially less susceptible to JEV infection. Therefore, Raji cells were used as a semi-permissive cellular model for subsequent experiments. The capacity of DC-SIGN to promote infection was confirmed using Raji cells stably expressing DC-SIGN (Raji-DC-SIGN) (Fig. S1F). Raji or Raji-DC-SIGN cells were infected with JEV at different MOIs and the infection efficiency was determined 36 h post infection (h.p.i.) by western blotting analysis of JEV E protein. As expected, parental Raji cells were not infected with JEV at a MOI of 0.02 or less, and only moderately infected with JEV at a higher MOI of 0.1 (Fig. 2A). In contrast, when DC-SIGN was expressed, the intracellular expression of E glycoprotein and the production of progeny viruses were considerably elevated (Fig. 2A and B). Similarly, infection of HEK-293T and ME180 cells with JEV was also increased when DC-SIGN was expressed (Fig. 2C). To further confirm DC-SIGN-mediated infection, we performed experiments in the presence of EDTA or mannan. EDTA inhibits DC-SIGN by extracting the bound calcium, while mannan is a competitor of insect-derived glycans. As shown in Fig. 2D, JEV infection was significantly reduced in Raji-DC-SIGN cells in the presence of EDTA or mannan.

### *DC-SIGN enhances JEV binding to cells*

We subsequently used fluorescently labeled JEV particles (JEV-AF488 and JEV-AF647) to determine whether DC-SIGN enhanced

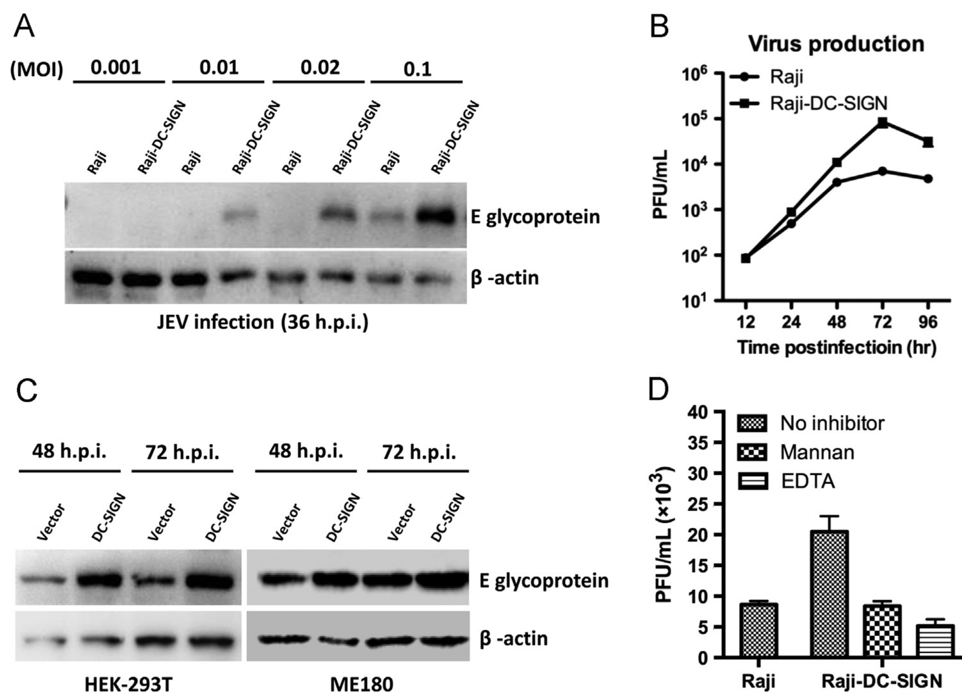



**Fig. 2.** Ectopic expression of DC-SIGN enhances JEV infection. (A) JEV replication in Raji and Raji-DC-SIGN cells. Cells were infected with JEV at various MOIs, and the expression of viral E glycoprotein was detected 36 h later by western blotting. One representative experiment out of three is shown. (B) Production of progeny virions from Raji and Raji-DC-SIGN cells. Supernatants of cells infected with JEV (MOI of 0.1) were harvested at different time points and tittered in BHK-21 cells. Data shown are mean ± SD of three independent experiments. (C) JEV replication in cells transiently expressing DC-SIGN. HEK-293T or ME180 cells transfected with DC-SIGN for 2 days were infected with JEV (MOI of 0.5) and cultured for 48 or 72 h. The intracellular expression of viral proteins was detected by western blotting. One representative experiment out of three is shown. (D) Inhibition of DC-SIGN decreases JEV infection in Raji-DC-SIGN cells. Raji-DC-SIGN cells were infected with JEV (MOI of 1) in the presence of mannan (20 μg/ml) or EDTA (2.5 mM). Infection was determined by titration of virus production 3 days later. Data shown are mean ± SD of three independent experiments.

infection by promoting JEV binding. Infection of JEV-AF488 or JEV-AF647 in DC-SIGN-expressing cells was similar to that of unlabeled JEV (Fig. S1G), indicating that such labeling approach was unlikely to compromise JEV infectivity. Raji and Raji-DC-SIGN cells were exposed to various amounts of JEV-AF488, and then virus-bound cells were determined by FACS analysis. More JEV-AFF488-positive cells and higher corresponding mean fluorescence intensity (MFI) were observed in Raji-DC-SIGN than those in parental Raji cells under the same conditions (Fig. 3A). Moreover, JEV binding to Raji-DC-SIGN cells was suppressed by pretreatment with the inhibitors described above (~80% reduction with mAb161 or EDTA and ~40% with mannan in MFI) (Fig. 3B), suggesting the specificity of DC-SIGN-mediated JEV binding.

We further used DCs to examine the role of DC-SIGN in mediating JEV binding. DCs transfected with DC-SIGN-specific siRNA or control siRNA for 2 days were incubated with JEV-AF488, stained with anti-DC-SIGN Ab and subjected to FACS analysis. The number of virus-bound cells and MFI decreased when DC-SIGN was knocked down (Fig. 3C). In addition, JEV-AF488 particles could be visualized as single spots by confocal microscopy, and the virus binding on DCs was severely compromised when DC-SIGN was knocked down by DC-SIGN siRNA or neutralized by anti-DC-SIGN mAb161 (Fig. 3D). These results suggest an important role of DC-SIGN in DC-mediated JEV binding.

*High-mannose N-linked glycan at N154 of E glycoprotein is responsible for DC-SIGN-mediated JEV binding*

To assess the interaction between DC-SIGN and JEV, CHO cells transiently expressing DC-SIGN were first pulsed with JEV and then fixed and immunostained with anti-JEV E and anti-DC-SIGN antibodies before confocal microscopy. Our results showed that DC-SIGN, in the absence of JEV infection, revealed a punctate expression pattern and was evenly distributed all over the cell surface (Fig. 4A, horizontal middle panel). JEV binding caused enhanced co-clustering of DC-SIGN (red) and virus particles (green) in a large and patchy configuration (yellow), which protruded from the cell surface, indicating that JEV binding may result in DC-SIGN redistribution (Fig. 4A, lower panel). Moreover, virus binding was considerably increased on CHO-DC-SIGN cells than that on parental CHO cells (Fig. 3A, top and lower panels).

It has been well documented that DC-SIGN binds high mannose *N*-linked glycans through its C-terminal carbohydrate recognition domain (CRD) (van Kooyk and Geijtenbeek, 2003). We therefore analyzed the glycosylation patterns of JEV E glycoprotein. E glycoprotein was treated with endoglycosidase H (Endo H) or Peptide: N-glycosidase F (PNGase F) before SDS-PAGE and western blotting analyses. Endo H only digests high mannose-type glycans, while PNGase F cleaves both high mannose- and complex-type glycans. In our study, treatment with Endo H or PNGase F had the same effect on the migration of E protein (Fig. 4B), suggesting that *N*-linked glycans on E glycoprotein are highly likely to be high mannose-type. We next examined the potential *N*-linked glycosylation sites on E protein by analyzing sequence motifs N-X-S/T and N-X-C. The JEV E glycoprotein possesses two potential *N*-linked glycosylation sites, N103-G104-C105 and N154-T155-S156. After Endo H or PNGase F treatment, E protein with deglycosylation at N154 (E-N154Q) but not at N103 (E-N103Q) revealed the same migration pattern (Fig. 4B), suggesting a single high-mannose *N*-linked glycan at N154. In the cell–cell fusion system, the fusion between deglycosylation mutant N154 (E-S154A)-expressing effector cells and DC-SIGN-expressing target cells was severely impaired. On the contrary, the glycan-retained mutant E-S156T showed similar fusion activity as the wild-type E protein (Fig. S2A and B). In addition, the fusion efficiency was decreased by inhibitors that targeting the interaction between DC-SIGN and high-mannose glycans (Fig. S2C). These results collectively demonstrate that high-mannose *N*-linked glycan at N154 is important in mediating the interaction between JEV E protein and DC-SIGN.

To further understand the importance of high-mannose *N*-linked glycan in DC-SIGN-JEV interaction, JEV produced in C6/36 cells was treated with Endo H under reducing conditions before exposed to Raji-DC-SIGN cells. Our results revealed that, although glycan removal was incomplete (Fig. S2D), Endo H treatment severely compromised the binding of C6/36 cell-derived JEV to Raji-DC-SIGN (Fig. 4D and E) but did not significantly affect its infectivity in the highly permissive BHK-21 cells (Fig. 4C), implying a close association of the high-mannose *N*-linked glycan on E protein with DC-SIGN binding. This was further confirmed by using an infectious JEV clone bearing a deglycosylation mutation at N154 (JEV-N154A) (Liu et al., 2015). Compared to wild type JEV, JEV-N154A showed compromised infection in Raji-DC-SIGN cells and was not sensitive to neutralizing antibody against DC-SIGN (Fig. S2E).

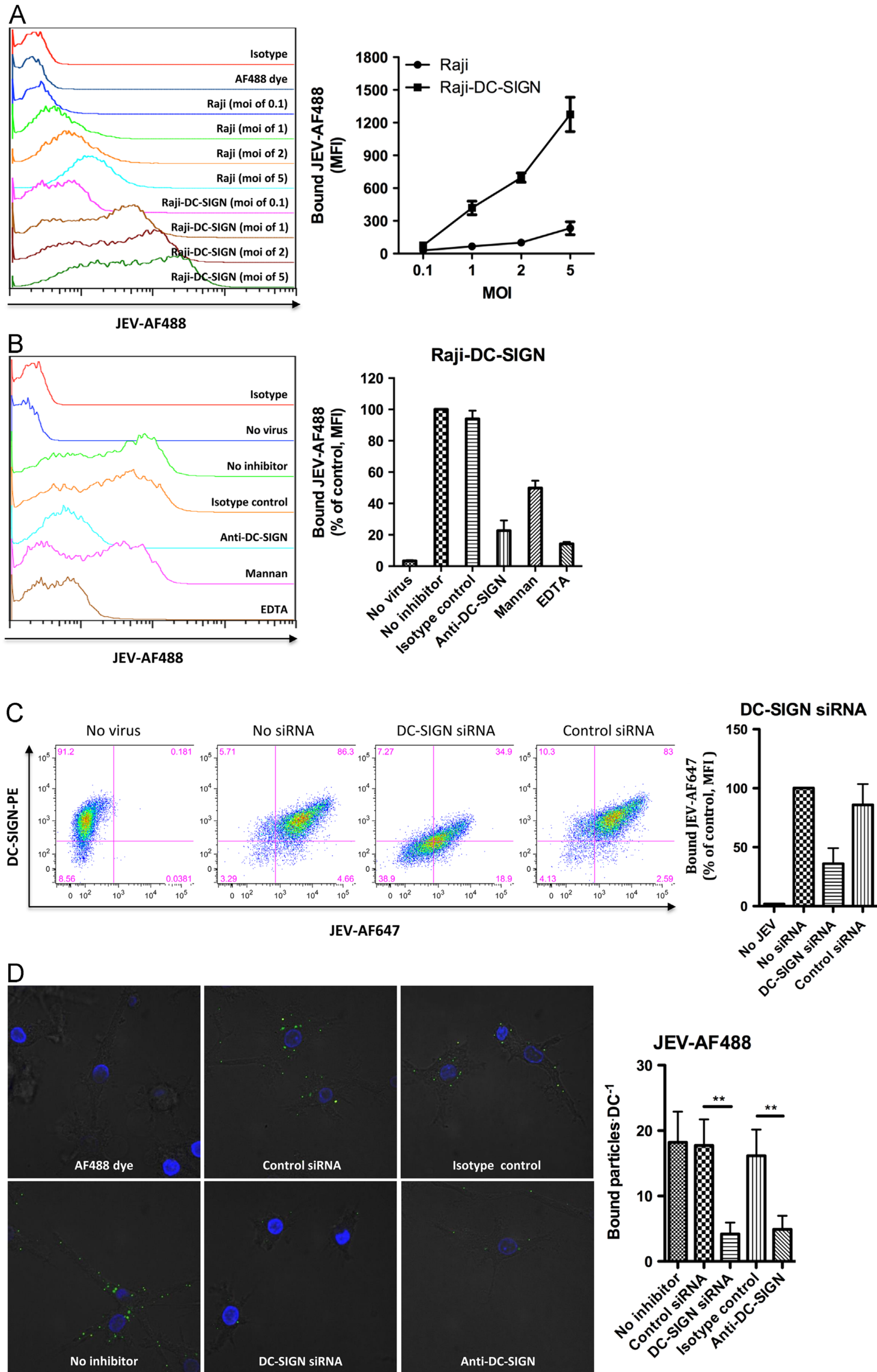
*JEV internalization is independent of DC-SIGN endocytic activity*

The cytosolic tail of DC-SIGN carries several sequence motifs that are believed to be important for signaling, endocytosis and intracellular trafficking. The classical LL motif present in the cytosolic tail has been shown to be critical for DC-SIGN endocytosis (Engering et al., 2002; Figdor et al., 2002; van Kooyk and Geijtenbeek, 2003). To test whether DC-SIGN facilitates JEV internalization, we generated DC-SIGN LL/AA mutant with the LL sequence being substituted with two alanines. Following detection of intracellular and cell-surface expression, we verified that DC-SIGN LL/AA was expressed at the same level as the wild type DC-SIGN (Fig. 5A and B).

To confirm that DC-SIGN LL/AA was devoid of endocytic activity, we performed Ab-mediated DC-SIGN endocytosis in HeLa cells. Cells were incubated with the anti-DC-SIGN mAb161 for 1 h at 4 °C, washed extensively and then shifted to 37 °C to initiate DC-SIGN internalization. At 4 °C, DC-SIGN and DC-SIGN LL/AA were mainly detected at the cell surface. When at 37 °C, a dramatic redistribution of staining in DC-SIGN was observed, indicating that DC-SIGN was efficiently internalized in HeLa cells upon mAb161 binding (Fig. 5C, upper panel). In contrast, the majority of DC-SIGN LL/AA remained mainly on the surface of HeLa cells after mAb161 binding (Fig. 5C, lower panel). These data indicate that DC-SIGN LL/AA can efficiently bind antigens but is incapable of internalization.

Subsequently, HeLa cells expressing wild type DC-SIGN or DC-SIGN LL/AA were incubated with JEV-AF647 on ice. After extensive washes with cold PBS, cells were rapidly shifted to 37 °C for the times indicated to allow internalization. Cells were then mock treated or treated with trypsin to remove the cell-surface bound viruses. FACS data showed that, without trypsin treatment, virus binding was equally efficient in DC-SIGN LL/AA and DC-SIGN expressing cells (Fig. 5D), indicating that the endocytosis motif of DC-SIGN is dispensable for antigen binding. Without shifting to 37 °C, trypsin treatment resulted in an abrogation of JEV-AF647 binding. After incubation for 5 min at 37 °C, approximately 20% of JEV-AF647 particles was protected from trypsin treatment. The internalization of JEV-AF647 increased over time, and most particles were internalized within 30 min. Of importance, there was no difference in JEV internalization between DC-SIGN and DC-SIGNA LL/AA (Fig. 5D). Interestingly, the kinetic of virus uptake into DCs appeared to be slightly faster with most particles being internalized within 15 min (Fig. S3).

To further verify that the endocytic activity of DC-SIGN is not required for JEV internalization, we examined JEV-pulsed DCs by


A
Isotype
AF488 dye
Raji (moi of 0.1)
Raji (moi of 1)
Raji (moi of 2)
Raji (moi of 5)
Raji-DC-SIGN (moi of 0.1)
Raji-DC-SIGN (moi of 1)
Raji-DC-SIGN (moi of 2)
Raji-DC-SIGN (moi of 5)
JEV-AF488
Raji
Raji-DC-SIGN
Bound JEV-AF488 (MFI)
MOI
B
Isotype
No virus
No inhibitor
Isotype control
Anti-DC-SIGN
Mannan
EDTA
JEV-AF488
Raji-DC-SIGN
Bound JEV-AF488 (% of control, MFI)
No virus
No inhibitor
Isotype control
Anti-DC-SIGN
Mannan
EDTA
C
No virus
No siRNA
DC-SIGN siRNA
Control siRNA
DC-SIGN-PE
JEV-AF647
DC-SIGN siRNA
Bound JEV-AF647 (% of control, MFI)
No JEV
No siRNA
DC-SIGN siRNA
Control siRNA
D
AF488 dye
Control siRNA
Isotype control
No inhibitor
DC-SIGN siRNA
Anti-DC-SIGN
JEV-AF488
Bound particles·DC-1
No inhibitor
Control siRNA
DC-SIGN siRNA
Isotype control
Anti-DC-SIGN

fluorescence confocal microscopy. DCs were pulsed with JEV-AF647 on ice and then shifted to 37 °C for indicated times. Cells were then fixed, permeabilized and stained for DC-SIGN, early endosomal marker EEA-1 or lysosomal/late endosomal marker LAMP-1. After a short incubation of 5–15 min at 37 °C, colocalization of JEV-AF647 was observed with EEA-1-positive early endosomes but not with DC-SIGN (which was mainly distributed on the cell surface) (Fig. 5E), suggesting that unknown factors rather than DC-SIGN mediated internalization and intracellular routing of JEV. Of interest, even after 2 h, JEV-AF647 was still present primarily in EEA-1-positive early endosomes instead of LAMP-1-positive vesicles (Fig. 5F). These results together indicate that JEV entry into DC-SIGN-expressing cells is independent of the endocytic activity of DC-SIGN.

## Discussion

Dendritic cells (DCs) in the skin and mucosal tissues form a first-line defense against viruses and other pathogens (Knight and Patterson, 1997). In this study, we demonstrate that DCs support JEV productive infection. DC-SIGN-mediated JEV binding is required for JEV infection of DCs and transfectants. Moreover, binding of JEV to DC-SIGN leads to rapid and effective virus internalization independent of DC-SIGN endocytic activity, indicating that DC-SIGN functions as an attachment factor rather than an entry receptor for JEV infection. In addition, the high-mannose *N*-linked glycan at N154 of E glycoprotein is essential for JEV binding to DC-SIGN.

Attachment factors that promote virus binding to target cells are not essential for viral entry but increase the chance of interactions between viral glycoproteins and cellular entry receptor(s), and consequently enhance viral infection. DC-SIGN is one of the most extensively characterized viral attachment factor abundantly expressed on DCs, and has been shown to enhance the capture of a number of viral pathogens, including HIV-1, Ebola virus, cytomegalovirus and Dengue virus (Alvarez et al., 2002; Geijtenbeek et al., 2000a; Halary et al., 2002; Navarro-Sanchez et al., 2003). In this study, we have demonstrated that DC-SIGN augmented JEV binding to primary DCs which was significantly impaired but not totally blocked when DC-SIGN was neutralized or knocked down, implying that DC-SIGN likely plays an important but not exclusive role in mediating JEV infection of DCs. Moreover, JEV E protein bound to DC-SIGN in a $Ca^{2+}$-dependent manner and the binding could be inhibited by mannan, indicating a carbohydrate-C type lectin specific interaction. Treatment of JEV particles with Endo H significantly reduced JEV infection of DC-SIGN-expressing cells, suggesting that the high mannose glycans present on the surface of mosquito-derived virions are essential for the interaction of JEV with DC-SIGN. To this end, a single high-mannose *N*-linked glycosylation site at N154 was identified in JEV E protein, which was sensitive to Endo H treatment and carbohydrate-binding agent CV-N, and this glycan accounted for the binding capability of JEV to DC-SIGN. The significance of glycosylation at N154 in DC-SIGN usage was further confirmed in the context of viral infection using an infectious JEV clone carrying N154 deglycosylation mutation. These results were consistent with other studies that DC-SIGN mainly recognizes high-mannose *N*-linked glycans on viral envelope glycoproteins (Guo et al., 2004) and deletion of *N*-glycosylation sites on E glycoprotein of Dengue virus resulted in compromised infectivity in DCs and DC-SIGN-expressing cell lines (Alen et al., 2012).

By analogy to other flaviviruses, JEV is supposed to gain entry into target cells via receptor-mediated endocytosis (Pierson and Kielian, 2013). Indeed, we found that binding of JEV to DC-SIGN triggered a rapid and efficient internalization of viral particles, suggesting the involvement of endocytic pathway in DC-SIGN-mediated JEV infection. However, these results did not distinguish whether DC-SIGN acts as cell surface attachment factor or as an authentic entry receptor that mediates virus internalization. DC-SIGN contains three motifs in its cytoplasmic tail that are believed to be involved in internalization or endocytic trafficking, including two classically defined putative internalization motifs, a dileucine motif (LL) and a tyrosine-based motif (YSKL), and a triacidic (EEE) cluster (Engering et al., 2002; van Kooyk and Geijtenbeek, 2003). Substitution of the LL motif with alanines abolished DC-SIGN endocytosis induced by mAb161, indicating that LL motif is essential to mediate DC-SIGN endocytosis. We observed that cells expressing endocytosis-defective DC-SIGN mediated JEV binding and entry with an efficiency similar to that of cells expressing wild type DC-SIGN, and most of JEV particles were no longer colocalized with DC-SIGN after viral internalization, indicating that JEV entry into DC-SIGN-expressing cells is not dependent on the DC-SIGN endocytic activity. Our findings are in agreement with an earlier study suggesting that DC-SIGN-mediated enhancement of Dengue virus infection is independent of DC-SIGN internalization signals (Lozach et al., 2005). Our data together suggest that DC-SIGN plays an important if not exclusive role as a cell surface attachment factor for JEV. Although JEV internalization could be independent of DC-SIGN endocytic activity in HeLa cells, these results do not rule out the possibility that a proportion of JEV entered DCs directly through DC-SIGN endocytosis or JEV benefited from the endocytic activity of DC-SIGN for a rapid internalization. It is also probable that DC-SIGN-bound JEV particles could be internalized by alternative mechanisms as described previously for DC-SIGN-mediated entry of Mycobacterium tuberculosis (Geijtenbeek et al., 2003; Tailleux et al., 2003). Collectively, our findings imply the existence of a specific receptor rather than DC-SIGN required for JEV internalization. Several molecules such as HSP70, heparan sulfate and low-density lipoprotein receptors have been proposed to participate in JEV entry into target cells but their role as authentic cellular receptors for JEV is doubted (Chien et al., 2008; Das et al., 2009; Liu et al., 2004; Zhu et al., 2012). We propose that DC-SIGN as the attachment factor captures virus on the cell surface and cooperates with an unidentified cellular entry receptor to permit infection of skin DCs during the mosquito's blood meal.

**Fig. 3.** DC-SIGN promotes JEV binding to cells. (A) Binding of JEV-AF488 to Raji and Raji-DC-SIGN cells. Various amounts of fluorescent JEV particles (JEV-AF488) were incubated with Raji or Raji-DC-SIGN cells for 1 h on ice and washed extensively before fixation and FACS analysis. One representative experiment out of three is shown (left). The MFI values of JEV-AF488 from three independent experiments are shown as mean ± SD (right). (B) Inhibition of DC-SIGN decreases JEV-AF48 binding to Raji-DC-SIGN cells. Raji-DC-SIGN cells were pre-treated with anti-DC-SIGN antibody (mAb161, 25 μg/ml), mannan (20 μg/ml) and EDTA (2.5 mM), respectively, before pulsed with JEV-AF488 on ice in the presence of the inhibitors. One representative experiment out of three is shown (left). The MFI values of JEV-AF488 from three independent experiments are shown as mean ± SD (right). Binding to Raji-DC-SIGN cells in the absence of inhibitors was set to 100%. (C) DC-SIGN knockdown reduces JEV-AF488 binding to DCs. DCs were transfected with DC-SIGN siRNA or control siRNA for 36 h before pulsed with JEV-AF488 (MOI of 10). DCs without transfection were used as the control. One representative experiment out of three is shown (left). The MFI of bound viruses was measured and normalized to that without transfection. Data shown are mean ± SD of three independent experiments (right). (D) DC-SIGN neutralizing antibody mAb161 reduces JEV-AF488 binding to DCs. JEV-AF488 bound to DCs transfected with DC-SIGN siRNA or in the presence of anti-DC-SIGN mAb161 (25 μg/ml) on ice before imaging by confocal microscopy. Green spots were cell-associated virus particles (JEV-AF488) seen in one focal plane. Nuclei were stained with Hoechst (blue). Bound particles per DC in the absence ($n$=41) or presence of the anti-DC-SIGN mAb161 ($n$=37), isotype control ($n$=39), control siRNA ($n$=38) or DC-SIGN siRNA ($n$=45), were counted in ten independent fields. ** $p<0.01$. ns, not significant.)

Flaviviruses are internalized via pre-formed clathrin-coated pits that bud into the cytosol and deliver their cargo to endocytic pathways, followed by degradation of viral particles and leaking of viral RNA (Pierson and Kielian, 2013). Studies on Dengue and West Nile viruses have demonstrated that the virus moves from early endosomes to late endosomes before viral RNA is released (Chu et al., 2006; Chu and Ng, 2004a; Mosso et al., 2008; van der Schaar et al., 2008). In contrast, Krishnan et al's study based on RNA interference showed that the uncoating of Dengue and West Nile viruses occurs at the level of early endosomes (Krishnan et al., 2007). A study on JEV suggested that the infection process in both fibroblasts and neuronal cells requires acid-dependent fusion at the level of early endosomes (Kalia et al., 2013). In our study, we found that the number of JEV particles in early endosomes was not significantly decreased throughout the experiment, implying that JEV uncoating may not occurs in early endosomes of DCs. It is likely that the endocytic route of JEV is ligand and cell type dependent.

Tracking studies of fluorescently labeled particles in live cells indicate that cargo delivered from cell surface such as transferrin or epidermal growth factor typically reaches early endosomes in less than 2 min after internalization, late endosomes in the perinuclear region after 10–12 min, and the lysosomes within 30–60 min (Griffiths et al., 1989; Lakadamyali et al., 2006; Mercer and

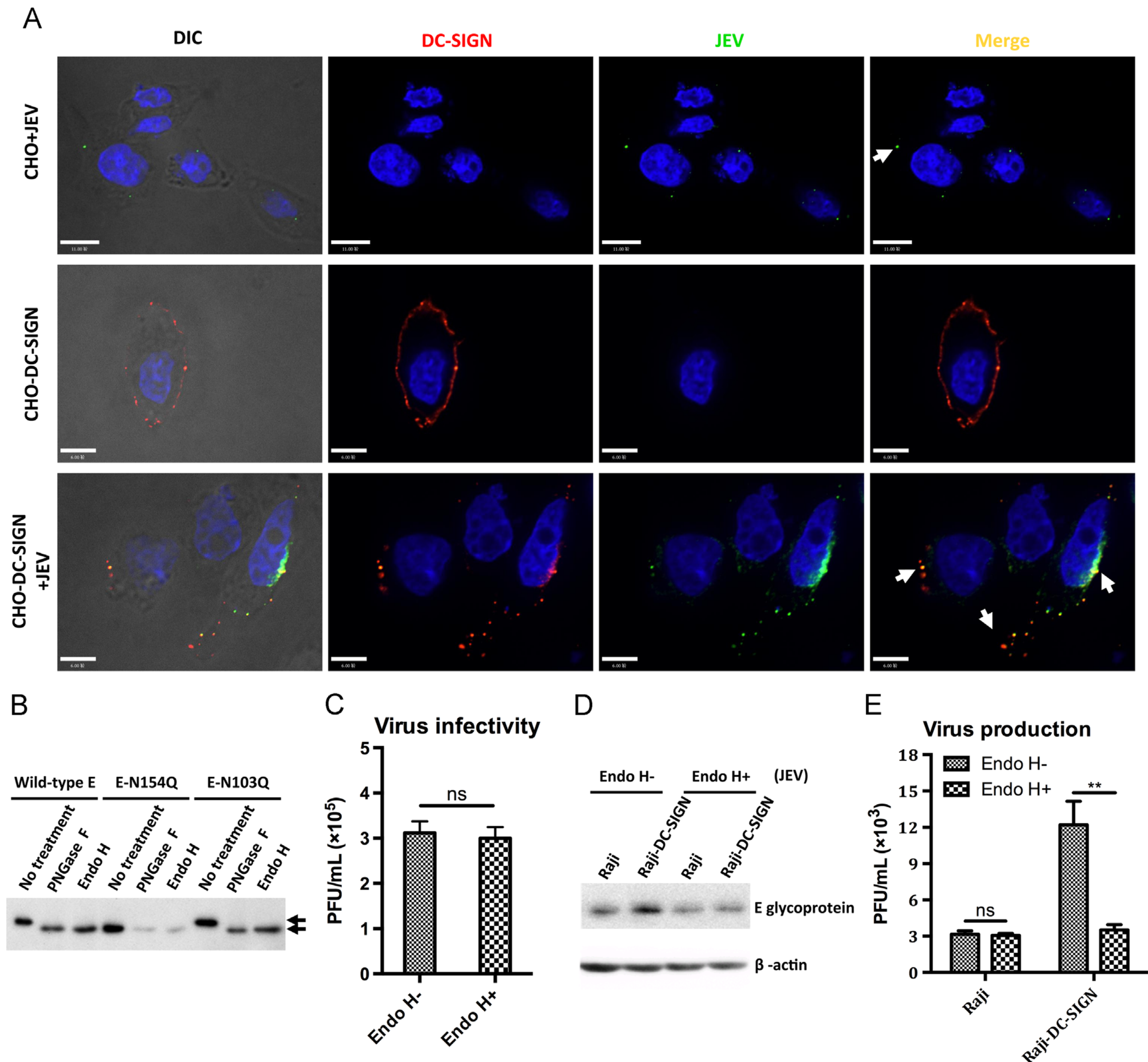



**Fig. 4.** High-mannose *N*-linked glycan at N154 of E glycoprotein is responsible for the binding of JEV to DC-SIGN. (A) Confocal microscopy of JEV bound to DC-SIGN-expressing cells. DC-SIGN transfectants (CHO-DC-SIGN) and parental CHO cells were incubated with JEV (MOI of 5), respectively, for 2 h on ice and then washed extensively. Cells were fixed and stained for E glycoprotein (green) and DC-SIGN (red) followed by analyses under fluorescence (488/640 nm) and differential interference contrast (DIC) confocal microscope. Arrows denote the overlapping domains of expression (yellow). As a control, CHO-DC-SIGN cells without JEV incubation were performed as described above. Scale bar represents 11 μm. (B) Glycosylation pattern of E glycoprotein. Wild type and mutated E glycoproteins were digested with PNGase F or Endo H (2000 units) under reducing conditions and analyzed by western blotting with polyclonal Abs against JEV E glycoprotein. Arrows denote protein bands of interest. (C) Endo H treatment under nondenaturing conditions has no effect on JEV infectivity. C6/36-derived JEV was treated with Endo H (5000 units) under nondenaturing conditions and assessed for infectivity in BHK-21 cells. Data shown are mean ± SD of three independent experiments with each condition performed in duplicate. ** $p < 0.01$. ns, not significant. (D,E) Endo H treatment reduces the capability of JEV in infecting Raji-DC-SIGN cells. Raji or Raji-DC-SIGN cells were infected with Endo H-pre-treated JEV as described in (C), and the expression of viral proteins was detected 2 d later by western blotting (D). One representative experiment out of three is shown. The supernatants of cells were collected and tittered in BHK-21 cells. Data shown are mean ± SD of three independent experiments. ** $p < 0.01$. ns, not significant.

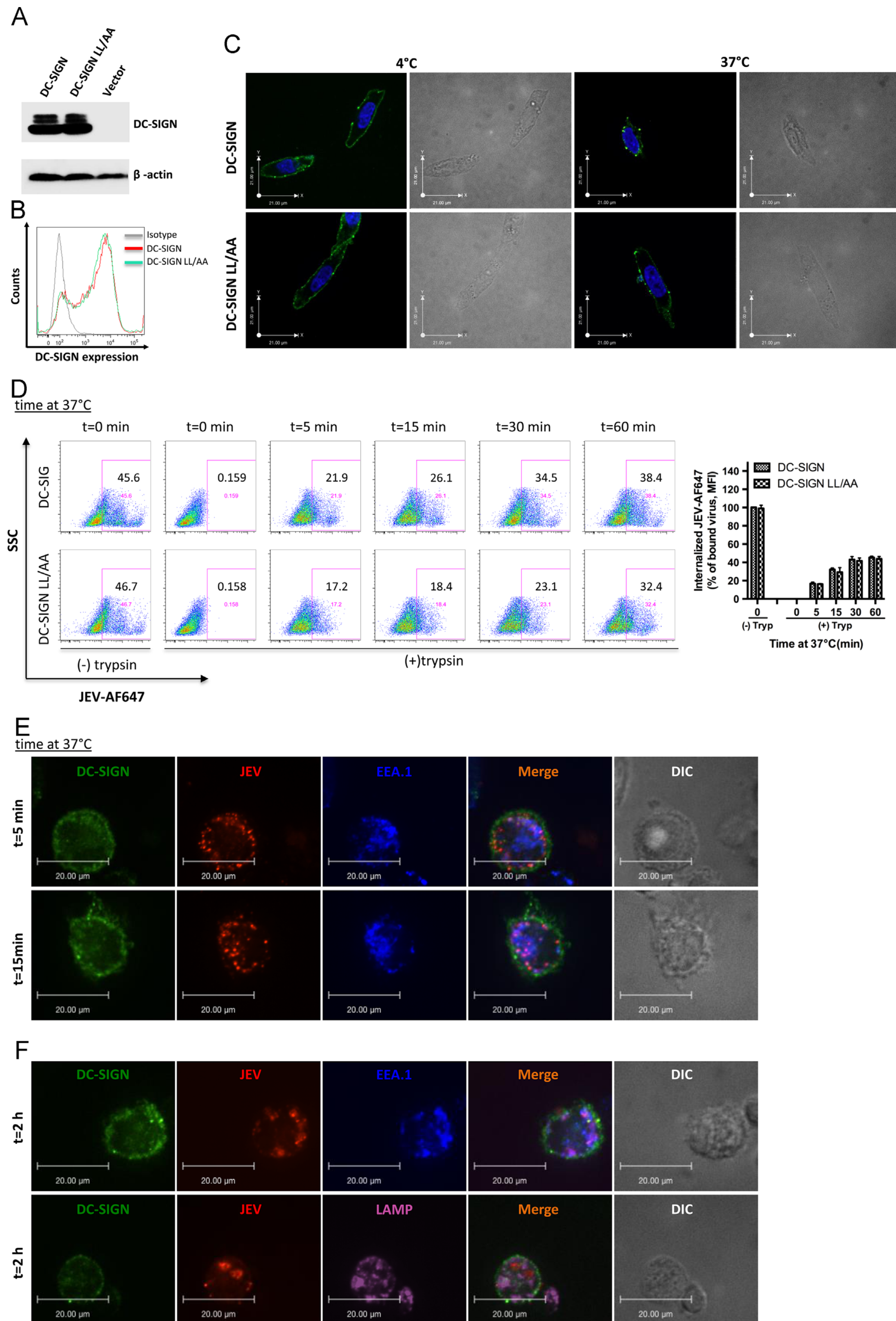



**Fig. 5.** JEV internalization does not require DC-SIGN endocytosis. (A,B) HeLa cells were transfected with expressing constructs encoding DC-SIGN or DC-SIGN LL/AA followed by analyses of intracellular expression by western blot (A) or surface expression by FACS (B). (C) Subcellular localization of DC-SIGN and DC-SIGN LL/AA in HeLa cells. Anti-DC-SIGN mAb161 was incubated with HeLa cells expressing DC-SIGN or DC-SIGN LL/AA for 1 h at 4 °C. Cells were washed extensively to remove unbound antibody and incubated for 30 min at 4 or 37 °C. mAb161-bound DC-SIGN was detected with a FITC-conjugated secondary Ab. Cell surface and intracellular localization of DC-SIGN was examined by confocal microscopy. Representative images from three independent experiments are shown. (D) JEV-AF647 internalization into HeLa-DC-SIGN or HeLa-DC-SIGN LL/AA cells. HeLa cells expressing DC-SIGN or DC-SIGN LL/AA were incubated with JEV-AF647 (MOI of 10) on ice, washed, and shifted to 37 °C for the times indicated. Cells were then treated with trypsin or mock treated, fixed, and washed extensively followed by FACS analysis. One representative experiment out of three is shown (left). The MFI values of internalized JEV-AF647 from three independent experiments are shown as mean ± SD (right). Binding of JEV-A647 to DC-SIGN-expressing HeLa cells without shifting and trypsin treatment was set to 100%. (E,F) JEV-AF647 internalization into DCs. DCs were incubated with JEV-AF647 (MOI of 20) on ice, washed, and shifted to 37 °C for the times indicated. Cells were then fixed, permeabilized and stained for DC-SIGN, early endosome-specific marker EEA-1 or late endosome-specific marker LAMP-1.

Helenius, 2008). Studies on Dengue and West Nile viruses showed that the virus could be observed in numerous of early endosomes at 2–5 min after internalization, late endosomes at 10–15 min, and predominantly in lysosomes within 20–60 min (Acosta et al., 2012; Chu and Ng, 2004a; van der Schaar et al., 2008, 2007; Zaitseva et al., 2010). The trafficking process of Dengue and West Nile viruses along the endocytic pathway occurs rapidly and is consistent with the speed of typical trafficking of cellular ligands. In consistence, our study showed that binding of JEV to DC-SIGN induced rapid and efficient internalization of the viruses into DCs. At 5 min after cells were warmed to 37 °C, virus particles were found mainly in EEA-1-positive early endosomes. However, colocalization remained high for JEV and early endosomes by 2 h, indicating that some virus particles remained in early endosomes of DCs for a longer period of time. Kwon et al. demonstrated that the vast of internalized HIV-1 virions into DCs do not enter late endosomes or lysosomes but instead in the early endosomes with stable and retaining infectivity for days (Geijtenbeek et al., 2000a). Another study has shown that Hepatitis C virus targets DC-SIGN to early endosomes of DCs, where the virus particles are protected from degradation for a long period ( > 24 h) (Ludwig et al., 2004). Although we did not observe a colocalization of JEV with DC-SIGN in early endosomes, we cannot completely rule out the possibility that intracellular trafficking of JEV particles may be dependent on the initial virion uptake. Moreover, future studies are warranted to determine whether a delayed degradation of JEV in DCs is associated with viral immune evasion or/and transmission.

In conclusion, we have demonstrated that DC-SIGN functions as an attachment factor to promote JEV binding to DCs via its high affinity for high-mannose glycan on E glycoprotein, while ectopic expression of DC-SIGN enhances JEV infection in cell lines. Binding to DC-SIGN triggers rapid JEV internalization into cells. However, JEV internalization appears to be independent of DC-SIGN endocytic activity with virions persistent in the early endosomes of DCs for a long period. Our findings highlight the potential importance of DC-SIGN in the early stages of JEV infection, although the significance *in vivo* remains to be further elucidated in future animal or clinical studies. Further understanding the roles of DC-SIGN in JEV infection may have important implications for the development of intervention strategies.

## Materials and methods

### *Ethic statement*

All protocols involving human blood samples were reviewed and approved by the Local Research Ethics Committee. Informed written consents from the human subjects were obtained in this study. Animal experiments were approved by the Institutional Animal Care and Use Committee (Hubei Laboratory Animal Science Association, Wuhan, China, Approval ID: S03409100L and S03409110D) and performed in accordance with the guidelines of Hubei Laboratory Animal Science Association.

### *Cells and viruses*

Japanese quail fibrosarcoma cells (QT6), Chinese hamster ovary (CHO) cells, human cervical carcinoma cells (ME180), baby hamster kidney cell line (BHK-21) and human embryonic kidney 293T cells (HEK-293T) were purchased from the American Type Culture Collection (ATCC). All cells were grown in Dulbecco's Modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin–streptomycin, and 2 mM L-glutamine. Human Burkitt's lymphoma cell line Raji was purchased from ATCC and cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin–streptomycin, 2 mM L-glutamine. Stable cell line Raji-DC-SIGN was described previously (Du et al., 2012) and maintained in RPMI-1640 supplemented with 10% FBS, 500 μg/ml G418, 100 U/ml penicillin–streptomycin, and 2 mM L-glutamine. *Aedes albopictus* C6/36 cells (ATCC) were grown in EMEM (ATCC) supplemented with 10% FBS and maintained at 28 °C in the presence of 5% $CO_2$. All other cell lines were grown at 37 °C in the presence of 5% $CO_2$.

Primary DCs were obtained as described previously (Hu et al., 2004; Jin et al., 2014). In brief, human peripheral blood mononuclear cells (PBMCs) were isolated from blood samples collected from healthy adult volunteers by the standard Ficoll–Paque method. For preparation of DCs, monocytes were first isolated from PBMCs by negative selection using human Monocyte Isolation kit II (Miltenyi Biotec). Purified monocytes were then differentiated into DCs in the presence of IL-4 (500 U/ml) and granulocyte-macrophage colony-stimulating factor (GM-CSF, 800 U/ml). DCs were harvested at day 6, analyzed by FACS and used in subsequent assays.

The JEV strain AT31 (Zu et al., 2014) was propagated in C6/36 cells and tittered in BHK-21 cells. JEV infectious clone carrying a deglycosylation mutation at N154 (JEV-N154A) was described previously (Liu et al., 2015). For JEV deglycosylation assay and fluorescent staining, viruses were concentrated and purified by ultracentrifugation. Briefly, 25 ml of virus-containing medium (cleared by centrifugation and filtration) was overlaid on 5 ml of 25% sucrose in Beckman conical tubes and spun at 25,000 rpm for 2 h at 4 °C in Beckman SW28 swingle bucket rotor (Beckman Coulter). The resulting pellet was dissolved in 100 μl/tube of PBS without calcium or magnesium (Invitrogen) for 2 h at 4 °C. Aliquots were stored at −80 °C or proceeded to purification. For purification of JEV, concentrated viruses were overlaid on a continuous sucrose gradient (20–55% w/w in $H_2O$) and spun at 35,000 rpm for 2 h at 4 °C in Beckman SW55 swingle bucket rotor (Beckman Coulter). Labeling of JEV with Alexa Fluor® 488 or Alexa Fluor® 647 Carboxylic Acid (Invitrogen) was performed in HEPES (20 mM) according to the manufacturer's instructions. Labeled viruses were separated from free dye by using a gel filtration column and subsequently banded on a sucrose step gradient as described above. Proper labeling was verified by SDS-PAGE, and the infectivity was monitored by plaque assays. The labeled JEV was designated JEV-AF488 or JEV-AF647.

### *Antibodies, plasmids, siRNAs and chemicals*

Mouse anti-DC-SIGN neutralizing mAb mAb161 (clone 120507), mouse anti-DC-SIGN phycoerythrin (PE)-conjugated mAb (mAb507) and corresponding isotype control antibodies were purchased from R&D Systems. Mouse anti-DC-SIGN neutralizing mAb clone 120526 was from the AIDS Research and Reference Reagent Program of NIH. Rabbit anti-EEA-1 mAb and rabbit anti-LAMP-1 mAb were purchased from Cell Signaling Technology (Beverly, MA). Mouse anti-DC-SIGN mAb (sc-65740), rabbit anti-DC-SIGN polyclonal antibody H200, mouse anti-β-actin and horseradish peroxidase (HRP) conjugated goat-anti-mouse antibodies were from Santa Cruz. Anti-Japanese encephalitis virus NS1 glycoprotein antibody (ab41651) was from Abcam. Alexa Fluor® 488-conjugate goat anti-mouse IgG and Alexa Fluor® 568-conjugate goat anti-rabbit IgG were purchased from Invitrogen Life Technologies. Mouse polyclonal antibody against the domain III of JEV E glycoprotein was generated by immunization of BALB/c mice (BHK ltd. Beijing) with JEV E DIII protein (Zu et al., 2014). Briefly, mice were first immunized subcutaneously with 200 μg of JEV E DIII protein in Freund's complete adjuvant followed by two boost immunizations with the same amount of antigen in Freund's incomplete adjuvant at 3-week intervals. Two weeks after the final immunization, blood samples were collected and sera were pooled and purified with protein A/G (Invitrogen).

Given the importance role of PrM in E folding and JEV maturation (Li et al., 2008; Lorenz et al., 2002), the DNA sequence containing full length PrM and the gene which encodes functional E protein was amplified from JEV infectious cDNA (Li et al., 2014) and cloned into pcDNA3.1 (+), designated wild type E. Deglycosylation E mutants E-N154Q, E-N103Q, E-S156A and glycan-retained mutant E-S156T were generated by site-direct mutagenesis (Aligent Technology) according to the manufacturer's instructions. Cyanovirin (CV-N) (Hu et al., 2010) and construct expressing wild type DC-SIGN (pDC-SIGN) was described previously (Du et al., 2012). DC-SIGN LL/AA, a mutant defective in endocytosis, was generated by site-directed mutagenesis and inserted into pcDNA3.1 vector. DC-SIGN-specific siRNA (sc-43719) and control siRNA were from Santa Cruz and described previously (Jin et al., 2014). EDTA and mannan were from Sigma-Aldrich.

*Western blotting*

Protein extracts, separated by SDS-PAGE and transferred onto PVDF membranes, were probed with antibodies against JEV E (1:2000), NS1 (1:1000), DC-SIGN (1:3000) and β-actin (1:2000, Santa Cruz Biotech), respectively. Proteins of interest were detected with the HRP-conjugated goat anti-mouse IgG antibody (1:5000, Santa Cruz Biotech) followed by the addition of the Pierce ECL Western blotting substrate (Thermo Scientific, Rockford, IL).

*Virus deglycosylation*

Wild-type and mutated E glycoproteins were digested with PNGase F (2000 U, New England Biolabs) or Endo H (2000 U, New England Biolabs) under reducing conditions for 3 h at 37 °C according to the manufacturer's instructions. Native deglycosylated viruses were obtained under nondenaturing conditions where samples were incubated with Endo H (5000 U) for 6 or 12 h at 37 °C without the denaturing step and Nonidet P-40 buffer (New England Biolabs) treatment.

*E glycoprotein-mediated cell–cell fusion*

The interaction between E glycoprotein and DC-SIGN was assayed using a cell–cell fusion assay employing a luciferase reporter gene, as described previously with modification (Hu et al., 2010). Briefly, QT6 effector cells were firstly infected with vTF1.1, a virus encoding T7 polymerase, for 1 h followed by transfection with construct expressing wild type or mutated E protein. QT6 target cells were transfected with pDC-SIGN and construct encoding luciferase under the transcriptional control of T7 promoter. The effector and target cell populations were mixed at 16–18 h post-transfection. Luciferase activity of cell lysates was determined approximately 8 h later (Promega). In some cases, effector cells were pre-treated with inhibitors for 30 min before mixed with target cells.

*Virus binding and infection*

Cells were incubated with JEV or JEV-AF488 for 2 h on ice at various multiplicity of infection (MOI) in binding buffer (RPMI-1640 pH ~7.4 containing 0.2% FBS, 1 mM $CaCl_2$, and 2 mM $MgCl_2$), followed by washes with cold PBS + 1% BSA to remove unbound viruses. Binding was subsequently quantified by FACS or analyzed by fluorescence microscopy. For microscopy, DCs were stretched on poly-L-lysine-coated cover slips for 1 h at 37 °C before incubation with JEV-AF488. For inhibition assay, cells were pretreated with inhibitors for 30 min before exposure to viruses.

For virus infection assay, cells were incubated with JEV for 2 h at 37 °C and cultured for indicated times. After incubation, intracellular expression of viral protein NS1 or E was detected by western blotting. For inhibition assay, cells were pretreated with inhibitors for 30 min before exposure to viruses.

*DC-SIGN endocytosis assay*

HeLa cells expressing wild type DC-SIGN or DC-SIGN LL/AA ($6 \times 10^5$ cells) were seeded on 3.5 cm diameter glass bottom culture dishes (MatTek Corporation) pre-coated with poly-L-lysine. Cells were incubated with the anti-DC-SIGN mAb161 (20 μg/ml diluted in PBS containing 3% BSA) for 1 h at 4 °C. After three washes with ice cold PBS to remove unbound Abs, cells were shifted to 37 °C for 30 min to allow DC-SIGN endocytosis. Cells were fixed with 3% paraformaldehyde for 15 min, washed and treated with PBS 0.2 M glycine for 10 min. Cells were then incubated with PBS containing 0.02% saponin and 1.5% BSA for 30 min. To visualize mAb161-bound DC-SIGN, cells were incubated with FITC-conjugated goat anti-mouse IgG, then washed and subjected to microscopy imaging.

*JEV internalization assay*

Wild type DC-SIGN or DC-SIGN LL/AA-expressing HeLa cells were incubated in serum-free DMEM containing 1 mM $CaCl_2$, 2 mM $MgCl_2$, and JEV-AF647 (MOI of 10) for 1 h at 4 °C. Unbound viruses were removed by extensive washes with serum-free DMEM. Cells were then suspended in serum-free DMEM, and shifted to 37 °C for various time periods. Cells were mock treated with serum-free DMEM or treated with 0.05% trypsin (Life Technologies) for 5 min. Proteolysis was stopped by adding an equal volume of medium containing 20% FCS. Cells were then subjected to FACS analysis.

DCs were adhered to polylysine-treated glass slides for 45 min in PBS at room temperature. Slides were then incubated with JEV-AF647 (MOI of 20) for 2 h on ice, washed, and then shifted to 37 °C for the indicated times. After incubation, cells were fixed with 4% paraformaldehyde, and permeabilized with PBS containing 0.05% saponin and 0.2% BSA. Cells were then stained with mouse anti-DC-SIGN mAb, rabbit anti-EEA-1 mAb or rabbit anti-LAMP-1 mAb for 1 h on ice followed by staining with Alexa Fluor® 488-conjugate goat anti-mouse IgG or Alexa Fluor® 568-conjugate goat anti-rabbit IgG. After a final wash, cells were subjected to confocal analysis as described elsewhere (Jiang et al., 2014). Briefly, images were obtained by using a 60 × NA 1.4 oil objective (Nikon eclipse Ti inverted microscope) on a spinning-disk confocal microscope (UltraView VoX; PerkinElmer Life and Analytical Sciences). Images were gathered under the laser of 488 nm, 561 nm, 640 nm and differential interference contrast (DIC) mode. Image sequences were processed and analyzed with Volocity software (PerkinElmer).

*Statistical analysis*

Analyses were performed with GraphPad Prism 4.00 software (GraphPad). Values were given as the mean of triplicates ± standard deviation (SD). Statistical analysis was performed using the Student's *t*-test. A $p$ value $< 0.05$ was considered statistically significant.

## Acknowledgments

This work was supported by Ministry of Science and Technology of China Grants 2010CB530100 and 2013ZX10001005-003-

002 (to Q.H.), and National Natural Science Foundation of China Grants 81273250, 81572009 (to Q.H.) and 31570165 (to Y.L.).

## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.11.006.

## References


Acosta, E.G., Castilla, V., Damonte, E.B., 2012. Differential requirements in endocytic trafficking for penetration of dengue virus. PLoS One 7 (9), e44835.

Albecka, A., Belouzard, S., Op de Beeck, A., Descamps, V., Goueslain, L., Bertrand-Michel, J., Terce, F., Duverlie, G., Rouille, Y., Dubuisson, J., 2012. Role of low-density lipoprotein receptor in the hepatitis C virus life cycle. Hepatology 55 (4), 998–1007.

Alen, M.M., Dallmeier, K., Balzarini, J., Neyts, J., Schols, D., 2012. Crucial role of the N-glycans on the viral E-envelope glycoprotein in DC-SIGN-mediated dengue virus infection. Antivir. Res. 96 (3), 280–287.

Alvarez, C.P., Lasala, F., Carrillo, J., Muniz, O., Corbi, A.L., Delgado, R., 2002. C-type lectins DC-SIGN and L-SIGN mediate cellular entry by Ebola virus in cis and in trans. J. Virol. 76 (13), 6841–6844.

Arrighi, J.F., Pion, M., Garcia, E., Escola, J.M., van Kooyk, Y., Geijtenbeek, T.B., Piguet, V., 2004. DC-SIGN-mediated infectious synapse formation enhances X4 HIV-1 transmission from dendritic cells to T cells. J. Exp. Med. 200 (10), 1279–1288.

Barba-Spaeth, G., Longman, R.S., Albert, M.L., Rice, C.M., 2005. Live attenuated yellow fever 17D infects human DCs and allows for presentation of endogenous and recombinant T cell epitopes. J. Exp. Med. 202 (9), 1179–1184.

Chen, Y., Maguire, T., Hileman, R.E., Fromm, J.R., Esko, J.D., Linhardt, R.J., Marks, R.M., 1997. Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. Nat. Med. 3 (8), 866–871.

Chien, Y.J., Chen, W.J., Hsu, W.L., Chiou, S.S., 2008. Bovine lactoferrin inhibits Japanese encephalitis virus by binding to heparan sulfate and receptor for low density lipoprotein. Virology 379 (1), 143–151.

Chu, J.J., Leong, P.W., Ng, M.L., 2006. Analysis of the endocytic pathway mediating the infectious entry of mosquito-borne flavivirus West Nile into aedes albopictus mosquito (C6/36) cells. Virology 349 (2), 463–475.

Chu, J.J., Ng, M.L., 2004a. Infectious entry of West Nile virus occurs through a clathrin-mediated endocytic pathway. J. Virol. 78 (19), 10543–10555.

Chu, J.J., Ng, M.L., 2004b. Interaction of West Nile virus with alpha v beta 3 integrin mediates virus entry into cells. J. Biol. Chem. 279 (52), 54533–54541.

Das, S., Laxminarayana, S.V., Chandra, N., Ravi, V., Desai, A., 2009. Heat shock protein 70 on Neuro2a cells is a putative receptor for Japanese encephalitis virus. Virology 385 (1), 47–57.

Davis, C.W., Nguyen, H.Y., Hanna, S.L., Sanchez, M.D., Doms, R.W., Pierson, T.C., 2006. West Nile virus discriminates between DC-SIGN and DC-SIGNR for cellular attachment and infection. J. Virol. 80 (3), 1290–1301.

Diamond, M.S., Shrestha, B., Mehlhop, E., Sitati, E., Engle, M., 2003. Innate and adaptive immune responses determine protection against disseminated infection by West Nile encephalitis virus. Viral Immunol. 16 (3), 259–278.

Du, T., Hu, K., Yang, J., Jin, J., Li, C., Stieh, D., Griffin, G.E., Shattock, R.J., Hu, Q., 2012. Bifunctional CD4-DC-SIGN fusion proteins demonstrate enhanced avidity to gp120 and inhibit HIV-1 infection and dissemination. Antimicrob. Agents Chemother. 56 (9), 4640–4649.

Engering, A., Geijtenbeek, T.B., van Vliet, S.J., Wijers, M., van Liempt, E., Demaurex, N., Lanzavecchia, A., Fransen, J., Figdor, C.G., Piguet, V., van Kooyk, Y., 2002. The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells. J. Immunol. 168 (5), 2118–2126.

Figdor, C.G., van Kooyk, Y., Adema, G.J., 2002. C-type lectin receptors on dendritic cells and langerhans cells. Nat. Rev. Immunol. 2 (2), 77–84.

Geijtenbeek, T.B., Kwon, D.S., Torensma, R., van Vliet, S.J., van Duijnhoven, G.C., Middel, J., Cornelissen, I.L., Nottet, H.S., KewalRamani, V.N., Littman, D.R., Figdor, C.G., van Kooyk, Y., 2000a. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. Cell 100 (5), 587–597.

Geijtenbeek, T.B., Torensma, R., van Vliet, S.J., van Duijnhoven, G.C., Adema, G.J., van Kooyk, Y., Figdor, C.G., 2000b. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. Cell 100 (5), 575–585.

Geijtenbeek, T.B., Van Vliet, S.J., Koppel, E.A., Sanchez-Hernandez, M., Vandenbroucke-Grauls, C.M., Appelmelk, B., Van Kooyk, Y., 2003. Mycobacteria target DC-SIGN to suppress dendritic cell function. J. Exp. Med. 197 (1), 7–17.

Griffiths, G., Back, R., Marsh, M., 1989. A quantitative analysis of the endocytic pathway in baby hamster kidney cells. J. Cell Biol. 109 (6 Pt 1), 2703–2720.

Guo, Y., Feinberg, H., Conroy, E., Mitchell, D.A., Alvarez, R., Blixt, O., Taylor, M.E., Weis, W.I., Drickamer, K., 2004. Structural basis for distinct ligand-binding and targeting properties of the receptors DC-SIGN and DC-SIGNR. Nat. Struct. Mol. Biol. 11 (7), 591–598.

Halary, F., Amara, A., Lortat-Jacob, H., Messerle, M., Delaunay, T., Houles, C., Fieschi, F., Arenzana-Seisdedos, F., Moreau, J.F., Dechanet-Merville, J., 2002. Human cytomegalovirus binding to DC-SIGN is required for dendritic cell infection and target cell trans-infection. Immunity 17 (5), 653–664.

Hu, Q., Frank, I., Williams, V., Santos, J.J., Watts, P., Griffin, G.E., Moore, J.P., Pope, M., Shattock, R.J., 2004. Blockade of attachment and fusion receptors inhibits HIV-1 infection of human cervical tissue. J. Exp. Med. 199 (8), 1065–1075.

Hu, Q., Huang, X., Shattock, R.J., 2010. C–C chemokine receptor type 5 (CCR5) utilization of transmitted and early founder human immunodeficiency virus type 1 envelopes and sensitivity to small-molecule CCR5 inhibitors. J. Gen. Virol. 91 (Pt 12), 2965–2973.

Jessie, K., Fong, M.Y., Devi, S., Lam, S.K., Wong, K.T., 2004. Localization of dengue virus in naturally infected human tissues, by immunohistochemistry and in situ hybridization. J. Infect. Dis. 189 (8), 1411–1418.

Jiang, H., Wang, P., Li, X., Wang, Q., Deng, Z.B., Zhuang, X., Mu, J., Zhang, L., Wang, B., Yan, J., Miller, D., Zhang, H.G., 2014. Restoration of MiR-17/20a in solid tumor cells enhances the natural killer cell anti-tumor activity by targeting Mekk2. Cancer Immunol. Res. 2 (8), 789–799.

Jiang, J., Cun, W., Wu, X., Shi, Q., Tang, H., Luo, G., 2012. Hepatitis C virus attachment mediated by apolipoprotein E binding to cell surface heparan sulfate. J. Virol. 86 (13), 7256–7267.

Jin, W., Li, C., Du, T., Hu, K., Huang, X., Hu, Q., 2014. DC-SIGN plays a stronger role than DCIR in mediating HIV-1 capture and transfer. Virology 458-459, 83–92.

Kalia, M., Khasa, R., Sharma, M., Nain, M., Vrati, S., 2013. Japanese encephalitis virus infects neuronal cells through a clathrin-independent endocytic mechanism. J. Virol. 87 (1), 148–162.

Knight, S.C., Patterson, S., 1997. Bone marrow-derived dendritic cells, infection with human immunodeficiency virus, and immunopathology. Annu. Rev. Immunol. 15, 593–615.

Krishnan, M.N., Sukumaran, B., Pal, U., Agaisse, H., Murray, J.L., Hodge, T.W., Fikrig, E., 2007. Rab 5 is required for the cellular entry of dengue and West Nile viruses. J. Virol. 81 (9), 4881–4885.

Lakadamyali, M., Rust, M.J., Zhuang, X., 2006. Ligands for clathrin-mediated endocytosis are differentially sorted into distinct populations of early endosomes. Cell 124 (5), 997–1009.

Lee, E., Pavy, M., Young, N., Freeman, C., Lobigs, M., 2006. Antiviral effect of the heparan sulfate mimetic, PI-88, against dengue and encephalitic flaviviruses. Antivir. Res. 69 (1), 31–38.

Li, L., Lok, S.M., Yu, I.M., Zhang, Y., Kuhn, R.J., Chen, J., Rossmann, M.G., 2008. The flavivirus precursor membrane-envelope protein complex: structure and maturation. Science 319 (5871), 1830–1834.

Li, X.D., Li, X.F., Ye, H.Q., Deng, C.L., Ye, Q., Shan, C., Shang, B.D., Xu, L.L., Li, S.H., Cao, S.B., Yuan, Z.M., Shi, P.Y., Qin, C.F., Zhang, B., 2014. Recovery of a chemically synthesized Japanese encephalitis virus reveals two critical adaptive mutations in NS2B and NS4A. J. Gen. Virol. 95 (Pt 4), 806–815.

Liu, H., Chiou, S.S., Chen, W.J., 2004. Differential binding efficiency between the envelope protein of Japanese encephalitis virus variants and heparan sulfate on the cell surface. J. Med. Virol. 72 (4), 618–624.

Liu, H., Liu, Y., Wang, S., Zhang, Y., Zu, X., Zhou, Z., Zhang, B., Xiao, G., 2015. Structure-based mutational analysis of several sites in the E protein: implications for understanding the entry mechanism of Japanese encephalitis virus. J. Virol. 89 (10), 5668–5686.

Lopez, A.L., Aldaba, J.G., Roque Jr., V.G., Tandoc 3rd, A.O., Sy, A.K., Espino, F.E., DeQuiroz-Castro, M., Jee, Y., Ducusin, M.J., Fox, K.K., 2015. Epidemiology of Japanese encephalitis in the Philippines: a systematic review. PLoS Negl. Trop. Dis. 9 (3), e0003630.

Lorenz, I.C., Allison, S.L., Heinz, F.X., Helenius, A., 2002. Folding and dimerization of tick-borne encephalitis virus envelope proteins prM and E in the endoplasmic reticulum. J. Virol. 76 (11), 5480–5491.

Lozach, P.Y., Burleigh, L., Staropoli, I., Navarro-Sanchez, E., Harriague, J., Virelizier, J. L., Rey, F.A., Despres, P., Arenzana-Seisdedos, F., Amara, A., 2005. 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. J. Biol. Chem. 280 (25), 23698–23708.

Ludwig, I.S., Lekkerkerker, A.N., Depla, E., Bosman, F., Musters, R.J., Depraetere, S., van Kooyk, Y., Geijtenbeek, T.B., 2004. Hepatitis C virus targets DC-SIGN and L-SIGN to escape lysosomal degradation. J. Virol. 78 (15), 8322–8332.

Malissen, B., Tamoutounour, S., Henri, S., 2014. The origins and functions of dendritic cells and macrophages in the skin. Nat. Rev. Immunol. 14 (6), 417–428.

Medigeshi, G.R., Hirsch, A.J., Streblow, D.N., Nikolich-Zugich, J., Nelson, J.A., 2008. West Nile virus entry requires cholesterol-rich membrane microdomains and is independent of alphavbeta3 integrin. J. Virol. 82 (11), 5212–5219.

Mercer, J., Helenius, A., 2008. Vaccinia virus uses macropinocytosis and apoptotic mimicry to enter host cells. Science 320 (5875), 531–535.

Miller, J.L., de Wet, B.J., Martinez-Pomares, L., Radcliffe, C.M., Dwek, R.A., Rudd, P.M., Gordon, S., 2008. The mannose receptor mediates dengue virus infection of macrophages. PLoS Pathog. 4 (2), e17.

Mosso, C., Galvan-Mendoza, I.J., Ludert, J.E., del Angel, R.M., 2008. Endocytic pathway followed by dengue virus to infect the mosquito cell line C6/36 HT. Virology 378 (1), 193–199.

Navarro-Sanchez, E., Altmeyer, R., Amara, A., Schwartz, O., Fieschi, F., Virelizier, J.L., Arenzana-Seisdedos, F., Despres, P., 2003. Dendritic-cell-specific ICAM3-grabbing non-integrin is essential for the productive infection of human dendritic cells by mosquito-cell-derived dengue viruses. EMBO Rep. 4 (7), 723–728.

Pierson, T.C., Kielian, M., 2013. Flaviviruses: braking the entering. Curr. Opin. Virol. 3 (1), 3–12.

Pohlmann, S., Leslie, G.J., Edwards, T.G., Macfarlan, T., Reeves, J.D., Hiebenthal-Millow, K., Kirchhoff, F., Baribaud, F., Doms, R.W., 2001. DC-SIGN interactions

with human immunodeficiency virus: virus binding and transfer are dissociable functions. J. Virol. 75 (21), 10523–10526.
Shimojima, M., Takenouchi, A., Shimoda, H., Kimura, N., Maeda, K., 2014. Distinct usage of three C-type lectins by Japanese encephalitis virus: DC-SIGN, DC-SIGNR, and LSECtin. Arch. Virol. 159 (8), 2023–2031.
Tailleux, L., Schwartz, O., Herrmann, J.L., Pivert, E., Jackson, M., Amara, A., Legres, L., Dreher, D., Nicod, L.P., Gluckman, J.C., Lagrange, P.H., Gicquel, B., Neyrolles, O., 2003. DC-SIGN is the major mycobacterium tuberculosis receptor on human dendritic cells. J. Exp. Med. 197 (1), 121–127.
van den Hurk, A.F., Ritchie, S.A., Mackenzie, J.S., 2009. Ecology and geographical expansion of Japanese encephalitis virus. Annu. Rev. Entomol. 54, 17–35.
van der Schaar, H.M., Rust, M.J., Chen, C., van der Ende-Metselaar, H., Wilschut, J., Zhuang, X., Smit, J.M., 2008. Dissecting the cell entry pathway of dengue virus by single-particle tracking in living cells. PLoS Pathog. 4 (12), e1000244.
van der Schaar, H.M., Rust, M.J., Waarts, B.L., van der Ende-Metselaar, H., Kuhn, R.J., Wilschut, J., Zhuang, X., Smit, J.M., 2007. Characterization of the early events in dengue virus cell entry by biochemical assays and single-virus tracking. J. Virol. 81 (21), 12019–12028.
van Kooyk, Y., Geijtenbeek, T.B., 2003. DC-SIGN: escape mechanism for pathogens. Nat. Rev. Immunol. 3 (9), 697–709.
Zaitseva, E., Yang, S.T., Melikov, K., Pourmal, S., Chernomordik, L.V., 2010. Dengue virus ensures its fusion in late endosomes using compartment-specific lipids. PLoS Pathog. 6 (10), e1001131.
Zhu, Y.Z., Cao, M.M., Wang, W.B., Wang, W., Ren, H., Zhao, P., Qi, Z.T., 2012. Association of heat-shock protein 70 with lipid rafts is required for Japanese encephalitis virus infection in Huh7 cells. J. Gen. Virol. 93 (Pt 1), 61–71.
Zu, X., Liu, Y., Wang, S., Jin, R., Zhou, Z., Liu, H., Gong, R., Xiao, G., Wang, W., 2014. Peptide inhibitor of Japanese encephalitis virus infection targeting envelope protein domain III. Antivir. Res. 104, 7–14.