Pakistan Veterinary Journal
ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

DC-SIGN Increases Japanese Encephalitis Virus Infection

Yang Yang, Ali Zohaib, Gong Chen, Jing Ye and Shengbo Cao*

State Key Laboratory of Agricultural Microbiology, Laboratory of Animal Virology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, Hubei, 430070, PR China
*Corresponding author: sbcao@mail.hzau.edu.cn

ARTICLE HISTORY

Received: December 21, 2012
Revised: June 23, 2103
Accepted: June 26, 2013

Key words:
DC-SIGN
DC-SIGNR
Japanese Encephalitis virus

ABSTRACT

Japanese Encephalitis virus (JEV) is a mosquito borne flavivirus that infects macrophages, monocytes and dendritic cells (DCs) during *in vivo* replication. The C-type lectins DC-SIGN and DC-SIGNR have been reported to act as cell attachment factors for diverse array of pathogens. In this study, the effect of these lectins on JEV infection was investigated after the generation of 293T-SIGN (R) cell lines expressing DC-SIGN and DC-SIGNR receptors. It was observed that only DC-SIGN but not the DC-SIGNR can act as a viral attachment factor in case of JEV infection. The infection to cells expressing DC-SIGN was efficiently blocked by anti-DC-SIGN and mannan molecules. It was also found that insect derived JEV has higher affinity for DC-SIGN as compare to the mammalian derived JEV. These results initially suggest that DC-SIGN could act as viral attachment receptors (VAR) for JEV and enhance JEV infection.

To Cite This Article: Yang Y, A Zohaib, G Chen, J Ye and S Cao, 2013. DC-SIGN increases Japanese encephalitis virus infection. Pak Vet J, 33(4): 408-412.

INTRODUCTION

Japanese Encephalitis virus (JEV) is the most common cause of vaccine preventable encephalitis in Asia leading to 10,000 deaths every year. It is a mosquito-borne (+) sense single stranded RNA virus belonging to *Flaviviridae* family. After the bite of an infected mosquito to vertebrate host, virus infects local lymph nodes and begins to replicate. Then it enters the blood stream causing viremia (Kimura *et al*., 2010). Peripheral blood mononuclear cells (PBMCs) are thought to play a central role in JEV pathogenesis. Different mechanisms for JEV interaction with DCs and macrophages have been described including both Myd88 dependent and independent pathways (Aleyas *et al*., 2009). These interfering pathways lead to immunosuppression, viral survival and its dissemination to other parts of the body (Kaisho and Akira, 2001).

Since JEV interaction with DCs play a crucial role in viral dissemination and modulation of immune responses, it is imperative to recognize different JEV specific receptors/attachment factors on DCs, their viral ligands and contributions of these receptor-ligand interactions to JEV pathogenesis. Viral attachment factors are the nonessential cellular proteins which facilitates the virus binding to cell surface (Baribaud *et al*., 2002a). Two of the most comprehensively studied attachment factors include C-type lectins: DC-SIGN (CD209) (Geijtenbeek *et al*., 2000) and DC-SIGNR (L-SIGN or CD209L) (Bashirova *et al*., 2001) [henceforth referred collectively as DC-SIGN (R)]. High level of DC-SIGN expression has been reported *in vitro* in monocyte-derived DCs (Geijtenbeek *et al*., 2000) as compare to *in vivo* in DCs ; Soilleux *et al*., 2002) and macrophages (Krutzik *et al*., 2005). DC-SIGNR is expressed on micro-vascular endothelial cells (especially in lymph nodes and liver sinusoids) (Bashirova *et al*., 2001). According to various reports, DC-specific C type lectin DC-SIGN is an important attachment factor for dissemination of several pathogens. As viral attachment factors, DC-SIGN (R) can enhance the susceptibility of permissive cells to viral infection or allow non-permissive cells to bind and transmit captured pathogens to target cells *in trans* (Halary *et al*., 2002; Navarro-Sanchez *et al*., 2003; Mesman *et al*., 2012; Zhang *et al*., 2012; Borggren *et al*., 2013). These attachment factors have high affinity for the mannose containing carbohydrates on viral glycoproteins (Klimstra *et al*., 2003; Lin *et al*., 2003; Johnson *et al* 2011; Alen *et al*., 2012). Several studies have demonstrated the interaction of other flaviviruses with DCs via DC-SIGN (R) attachment factors.

In this study, the role of DC-SIGN (R) in JEV pathogenesis was investigated. It was observed that DC-SIGN can efficiently enhance the susceptibility of

permissive cells to JEV infection, whereas DC-SIGNR was not found to be as an important attachment factor in case of JEV infection.

MATERIALS AND METHODS

Cells, viruses and antibodies: Human embryonic kidney 293T cells and baby hamster kidney cells (BHK-21) were grown at 37°C and maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma–Aldrich, MO, USA) supplemented with 10% fetal bovine serum (FBS, Gibco). C6/36 cells were grown at 28°C and maintained in Modified Eagle's Medium (MEM, Sigma-Aldrich, MO, USA) supplemented with 10% FBS.

For generation of cell lines expressing DC-SIGN (R), full-length coding sequences of DC-SIGN (R) were cloned into pcDNA3.1. The constructs and vector were transfected into 293T cells. The expression of DC-SIGN and DC-SIGNR on cell surface was detected by the Indirect immunofluorescence assay (IFA) and western bolt. The 293T cells expressing DC-SIGN and DC-SIGNR henceforth will be referred as 293T-SIGN and 293T-SIGNR respectively and 293T-SIGN (R) collectively.

JEV wild type strain P3 was propagated in BHK-21 and C6/36 cells. Viral titers were determined by viral plaque assay on BHK cells as described by Wu *et al*. (2011). The anti-DC-SIGN and anti-DC-SIGNR antibodies from R & D system were used in this study. The anti-JEV polyclonal antibodies were generated in our laboratory. The Alexa Fluor® 555-labelled secondary anti-rabbit IgG and Alexa Fluor ® 488-labelled secondary anti-mouse IgG were purchased from Invitrogen.

IFA, blocking Assay and real-time PCR: Cultured cells were fixed in 4% paraformaldehyde and nonspecific binding sites were blocked with PBS containing BSA. Cells were then incubated with appropriate primary antibodies followed by florescence conjugated secondary antibodies. Nuclei were stained with DAPI (4', 6-diamidino-2-phenylindole) and analyzed using confocal microscope.

Triplicate wells of 293T cell lines expressing DC-SIGN(R) were treated with 4mg/ml of mannan (Sigma) or 40µg/ml of anti-DC-SIGN(R) mAb, at 37°C for 1h prior to infection with viruses. qRT-PCR experiments were carried out by the Primer Script RT reagent Kit (TaKaRa) and SYBR Green Real time PCR Master Mix (TaKaRa) according to the manufacturer's instructions. The forward and reverse gene-specific primers of JEV C gene are FP: GGCTCATATCACGTTCTTCAAGTTTT and RP: TGCTTTCCATCGGCCYAAA.

Statistical analysis: Statistics were calculated using GraphPad Prism (version 5.01; GraphPad Software, Inc.). A value of $P<0.05$ was considered statistically significant.

RESULTS

Expression of DC-SIGN greatly enhances the susceptibility of 293T cells to infection by JEV: To determine whether JEV binding to cells can be influenced by cell surface expression of DC-SIGN (R), 293T-SIGN (R) cells were infected with JEV**.** The confocal microscopic analysis revealed that JEV protein can co-localize with 293T-SIGN and 293T-SIGNR cells throughout the cell surface. 48 hours post infection, confocal analysis revealed the increased internalization of JEV to 293T-SIGN cells as compare to 293T-SIGNR and control (Fig. 1).

In order to further investigate the interaction between DC-SIGN(R) and JEV, 293T-SIGN and 293T-SIGNR cells were infected with serial dilutions of JEV. In agreement with the previous findings, significantly enhanced level ($P<0.05$) of JEV was found in 293T-SIGN cells as compare to the control. This increase in the magnitude of virus production was found to be more than 10-fold at 0.0001 MOI in 293T-SIGN cells. Whereas in contrast to the previous findings with West Nile virus (WNV) and Dengue virus (Tassaneetrithep *et al*., 2003; Davis *et al*., 2006) the level of JEV in 293T-SIGNR infected cells was not found to be significant as compare to control (Fig. 2). Results of this experiment revealed a dose dependent increase in viral replication in 293T-SIGN cells. To further validate the results qRT-PCR was performed. Supporting the viral plaque assay results, the data of qRT-PCR also showed increased viral replication in 293T-SIGN cells, demonstrated by increased copies of virus C gene ($P<0.05$) at each time point. From these experiments it can be concluded that the expression of DC-SIGN can increase the viral infectivity in a dose dependent manner.

JEV derived from mosquito and mammalian cell lines do not confer a similar infection: It has already been demonstrated in case of WNV and Sindbis virus that C6/36 mosquito cell line derived virus can interact with DC-SIGN (R) in a more efficient manner and this increased affinity is the result of incorporation of high-mannose glycans (Klimstra *et al*., 2003; Davis *et al*., 2006). Since JEV is a mosquito-borne pathogen so it was examined whether JEV from C6/36 and BHK-21 derived cells could behave differently in their interactions with C-type lectins. After infecting the 293T-SIGN (R) cells with JEV (MOI=0.01), qRT-PCR was performed at different time points (24s, 48 h and 72 h). Interestingly C6/36 derived JEV produced more viral C gene at 24 and 48 hpi ($P<0.01$) but after 72 hpi C gene level was found to be same in mammalian and mosquito derived cell lines (Fig. 3A & B). It was supposed due to the internal equilibrium status (saturation) achieved. In addition to qRT-PCR, viral plaque assay was also performed at 72 hpi which revealed significantly increased viral production from C6/36 derived JEV (Fig. 3C). So these results are in complete harmony with the previous findings regarding other flaviviruses that mosquito derived virus can interact with DC-SIGN more efficiently. But interestingly this study demonstrate an increased affinity of insect derived virus for DC-SIGN not for DC-SIGNR, a reverse of this has already been reported in case of WNV (Davis *et al*., 2006).

Specific inhibitors block DC-SIGN mediated increased susceptibility of the 293T-SIGN cells: To substantiate that DC-SIGN is indeed involved in increased susceptibility of cells to JEV, inhibitors of DC-SIGN were used to block JEV infection on 293 T-SIGN cells. The

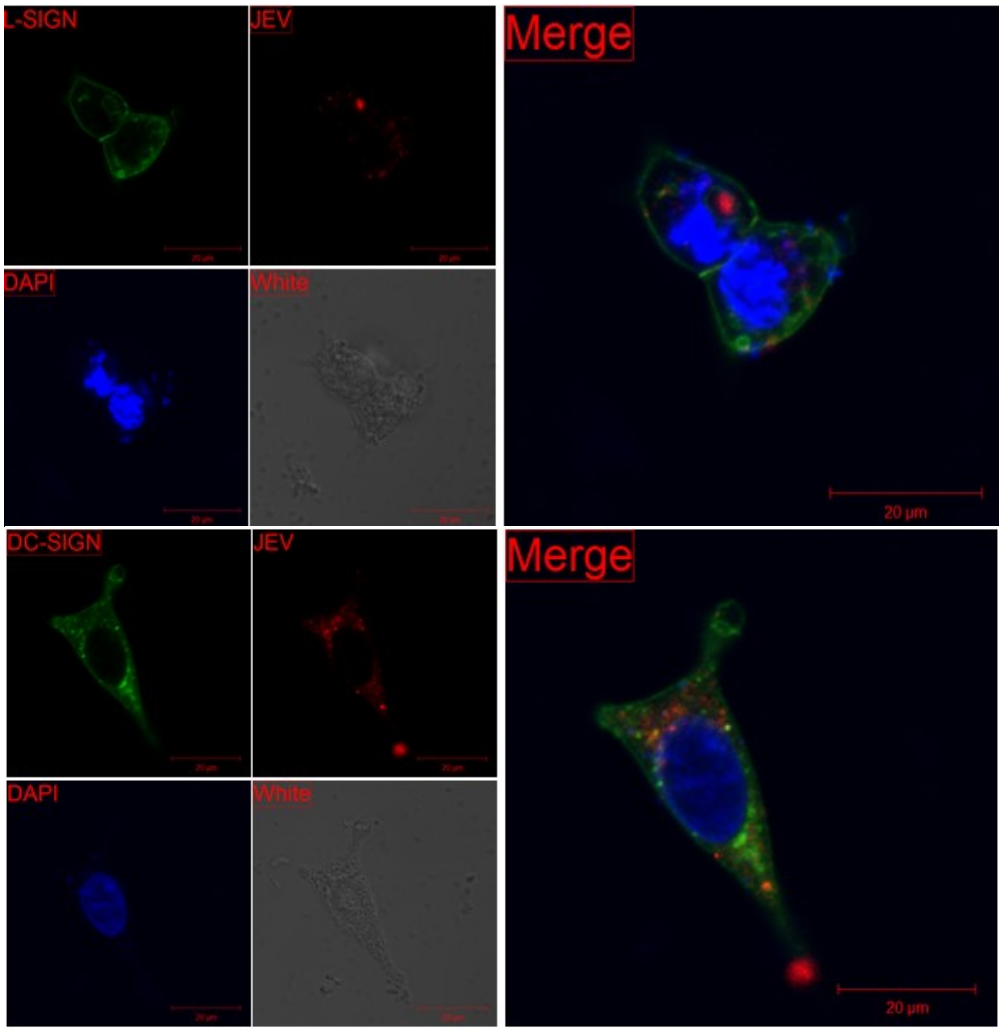

Fig. 1: Colocalization of DC-SIGN(R) and JEV proteins. 293T cells expressing DC-SIGN(R) were infected with JEV. Lectins expression were detected by MAb161 (anti-DC-SIGN) and MAb162 (anti-DC-SIGNR) (green). JEV proteins were detected by anti-JEV polyclonal antibody (red).Confocal images show that DC-SIGN(R) proteins distributed throughout the cell surface and cytoplasm. Co-localization of DC-SIGN and JEV was evidenced by merged images (yellow).

results showed that both mannan and anti-DC-SIGN can effectively block DC-SIGN mediated enhancement of JEV infection as demonstrated by viral plaque assay. Mannan and anti-DC-SIGN mAb reduce viral levels 91 folds (P=0.0006) and 21 folds (P=0.0007) lower than of untreated groups respectively. No significant difference was found between mannan and anti-DC-SIGN mAb in their abilities to block the infection. To further investigate the role of DC-SIGNR, a blocking assay with 293T-SIGNR cells was performed. The viral plaque assay suggested that 293T-SIGNR cells pre-incubated with blocking agents has no effect on JEV infection (Fig. 4A & B).

DISCUSSION

C-type lectins such as DC-SIGN and DC-SIGNR, which bind mannose rich carbohydrate modifications of pathogen proteins, have been shown to facilitate viral infection either *cis* or *trans* (Klimstra *et al*., 2003). Several early targets of arbovirus infection (dendritic cells and cells of reticuloendothelial system) express DC-SING (R) receptors (Klimstra *et al*., 2003), making them important to understand how these lectins interact with JEV. Interestingly in this study it was found that JEV interacts efficiently with DC-SIGN whereas no significant interaction difference was observed between 293T-SIGNR and controls. The preferential binding of DC-SIGN by any other flavivirus has not previously been observed although Davis *et al.* (2006), has described the preferential binding of WNV to DC-SIGNR. In agreement with previous findings for other viruses, it was also observed that JEV derived from mosquito cell line interacts more efficiently with DC-SIGN as compare to mammalian cell line derived virus. It is likely that the interaction of mammalian and insect cell derived JEV carbohydrates with DC-SIGN is of much higher affinity than that of DC-SIGNR.

The C-type lectin family of attachment factors/ receptors has biologically diverse functions. High expression level of DC-SIGN has been demonstrated on immature DCs and macrophage subpopulations abundant in the dermis of skin, peripheral tissues, at mucosal surfaces and in lymph nodes (Soilleux *et al*., 2002; van Kooyk *et al*., 2013). DCs and macrophages act as

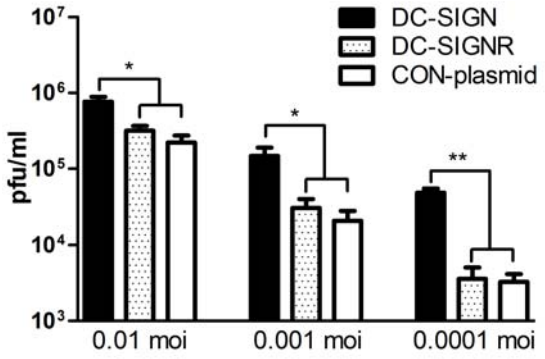

Fig. 2: DC-SIGN enhances the propagation of JEV in 293T cells. 293T cells were infected with JEV at MOI of 0.01, 0.001 and 0.0001, respectively. Titer of JEV in the media was determined by viral plaque assays on BHK cell monolayers. Data are expressed as mean and SD of triplicate wells. Representative data from two independent experiments are shown.

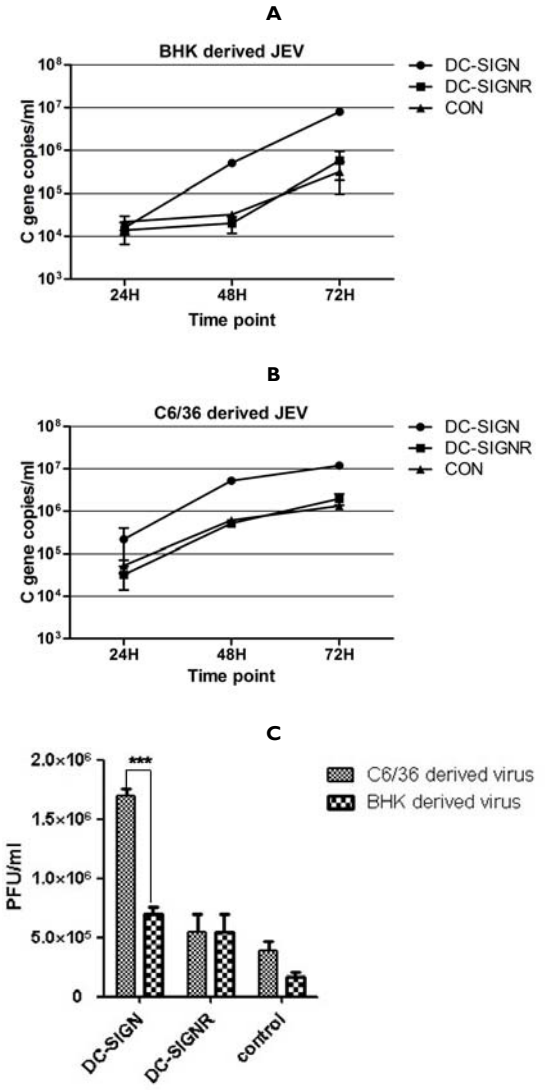

Fig. 3: C6/36 derived JEV demonstrates increased affinity for DC-SIGN as compare to DC-SIGNR. Cells were infected with C6/36 or BHK derived JEV at MOI of 0.01. JEV C gene replication kinetics was determined by qRT-PCR (A & B). Viral titers in the media were determined at 72hpi in BHK cells (C). Data are expressed as mean and SD of triplicate wells. Representative data from two independent experiments are shown.

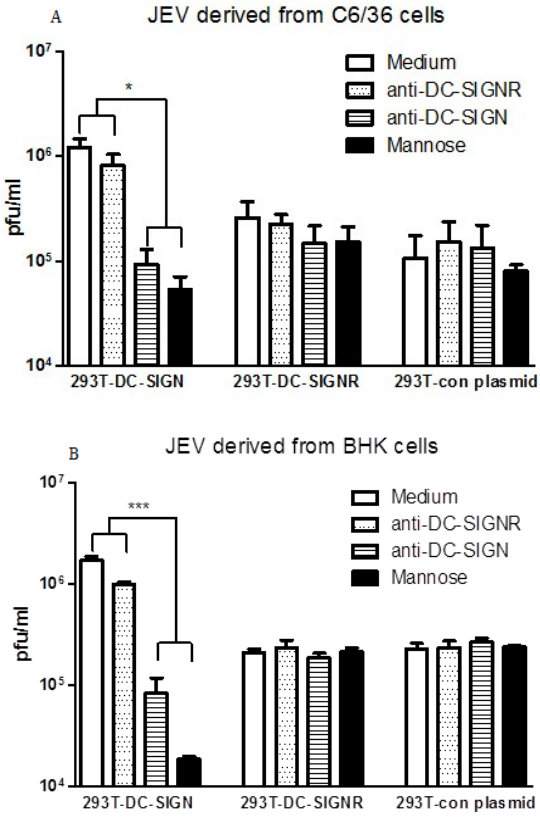

Fig. 4: Specific inhibitors block JEV infection in 293T-SIGN cells. 293T cells expressing DC-SIGN(R) were either treated with mannan or mAb (anti-DC-SIGN or anti-DC-SIGNR) at 37 for 1h prior to infection with virus [C6/36 cell derived virus (A) or mammalian derived virus (B)] at a MOI of 0.01. Virus titer was determined by viral plaque assays on BHK cell monolayers. Data are expressed as mean and SD of triplicate wells. Representative data from two independent experiments are shown.

professional antigen presenting cells, any invading pathogen is sensed, engulfed, processed and antigens are presented. Several studies have confirmed DC-SIGN (R) as important receptor for many pathogens. HIV (Boily-Larouche *et al*., 2012), Ebola virus, Cytomegalovirus (Baribaud *et al*., 2002b), Dengue virus (Tassaneetrithep *et al*., 2003), and WNV (Davis *et al*., 2006) have been shown to gain entry into DCs by utilizing these receptors. In this study it was demonstrated that DC-SIGN not only confers binding to 293T cells but also enhance 293Tcells susceptibility to JEV infection. This enhanced susceptibility to infection was found to be in a dose dependent manner which has previously been proved in case of Dengue virus (Tassaneetrithep *et al*., 2003) so very low MOIs are sufficient for enhanced infection, replication and production of JEV in DC-SIGN expressing cells. But it is still remains to be determined whether DC-SIGN can promote spreading of infection as it has been documented in case of Dengue virus (Tassaneetrithep *et al*., 2003). Interestingly JEV virus can discriminate between DC-SIGN and DC-SIGNR receptors but it is worth mentioning that JEV only utilizes DC-SIGN and no interaction with DC-SIGNR was found. Inhibitors blocking assay further proved that the increased susceptibility observed in this study is indeed due to the DC-SIGN expression. Although this is a preliminary study conducted on 293T cells but as it has already been

reported by Davis *et al*. (2006), that expression of DC-SIGN (R) on 293T cells was indeed yielded same results for SupT1, BHK, CHO-K1, and Primary cell types. So in authors opinion the results will not differ much in case of primary cell types but the need of study to be conducted on the primary cell types cannot be negated.

In this study, the role of DC-SIGN (R) in case of JEV pathogenesis was investigated which revealed that DC-SIGN but not the DC-SIGNR is an important VAR. It was also observed that insect derived JEV has higher affinity for DC-SIGN as compare to mammalian derived virus. This preferential utilization of DC-SIGN by JEV needs to be explored in depth to further reveal the differences between the other closely related flavivirus like WNV which preferentially bind to DC-SIGNR. These results should be considered the first report of DC-SIGN acting as a pathogen recognition receptor of JEV and also that JEV can discriminate between DC-SIGN and DC-SIGNR attachment factors. It will be of great interest to investigate the viral glycoproteins that contribute in this discrimination. Furthermore, the findings regarding utilization of DC-SIGN by JEV can further serve to probe the pathogenesis of JEV infection. These findings are significant addition in the existing pool of literature regarding JEV utilization of receptors and entry pathways.

Acknowledgment: This research work was supported by 973 Project of China (No. 2010CB530100), Natural Sciences Foundation of China (31172325) and National Training Programs of Innovation and Entrepreneurship for Undergraduates (201210504002).

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