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Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Utilization of human DC-SIGN and L-SIGN for entry and infection of host cells by the New World arenavirus, Junín virus



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ARTICLE INFO

Article history:

Received 3 October 2013

Available online 29 October 2013

Keywords:

Virus receptor

Junín arenavirus

Lectin

Virus entry

ABSTRACT

The target cell tropism of enveloped viruses is regulated by interactions between viral proteins and cellular receptors determining susceptibility at a host cell, tissue or species level. However, a number of additional cell-surface moieties can also bind viral envelope glycoproteins and could act as capture receptors, serving as attachment factors to concentrate virus particles on the cell surface, or to disseminate the virus infection to target organs or susceptible cells within the host. Here, we used Junín virus (JUNV) or JUNV glycoprotein complex (GPC)-pseudotyped particles to study their ability to be internalized by the human C-type lectins hDC- or hL-SIGN. Our results provide evidence that hDC- and hL-SIGN can mediate the entry of Junín virus into cells, and may play an important role in virus infection and dissemination in the host.

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1. Introduction

The family *Arenaviridae* is divided geographically and phylogenetically into two major complexes, the New World and the Old World complex. Seven of the known arenaviruses can cause viral hemorrhagic fever (VHF) in humans, with five of them, namely Junín, Guanarito, Machupo, Chapare, and Sabiá, belonging to the phylogenetic clade B of New World arenaviruses [1]. Rodents are the known reservoir of most arenaviruses and infection generally occurs after exposure to infected rodent excreta. Junín virus is the etiologic agent of Argentine Hemorrhagic Fever (AHF), an endemo-epidemic disease affecting populations living on the farming land of Argentina [2]. Despite the fact that a live attenuated vaccine, Candid#1, is available against Junín, the endemic region is expanding. In addition, the supply of convalescent serum for treatment of infected people is decreasing. Therefore, the arenavirus family remains a serious threat to public health and still a

growing concern that these viruses could be used as agents of bioterrorism.

Arenaviruses are enveloped viruses that contain two segments of ambisense single-stranded RNA. The smaller segment (S), encodes for the nucleocapsid-associated protein, (NP) and the glycoprotein precursor (GPC). The GPC precursor is proteolytically processed to produce GP1, involved in receptor binding; GP2, a membrane-anchored domain necessary for fusion [3] and the stable signal peptide, required for efficient trafficking to the plasma membrane and subsequent fusion of the viral envelope with the cellular membrane of infected cells [1].

Entry of human pathogenic arenaviruses into host cells has been studied extensively [4], in particular with regard to receptor utilization. Several members of the Old World complex and the New World clade C complex use α -dystroglycan as a cellular receptor [5,6]. New World arenaviruses utilize the transferrin receptor (TfR1) from their natural host [7] and it has been shown that all pathogenic members of the New World group use human TfR1 (hTfR1) as a receptor to infect human cells [8,9]. However, experiments using siRNA knockdown of TfR1 in mouse cells resulted in no reduction in titer relative to the controls suggesting that murine TfR1 plays no role in entry into these cells [10,11] and suggesting multiple receptors exist.

In addition to having a primary receptor, it is possible that arenaviruses may use non-specific receptors or attachment factors during entry. The C-type lectins were originally demonstrated to mediate entry for HIV-1 [12,13]. It is known that viruses in various

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families use the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN, or CD209) and/or the DC-SIGN homologue, DC-SIGNR or L-SIGN (CD209L), for entry and dissemination [14,15]. Recently, C-type lectins were shown to play a role in the infection of Lassa virus and LCMV, both members of the Old World arenavirus group [16,17]. However, the role of these molecules during New World arenavirus infection remains unexplored.

DC- and L-SIGN interact via their C-terminal carbohydrate recognition domain with mannose residues on viral glycoproteins [18,19]. Viruses including HCV, SARS-CoV, HCMV, Dengue and Ebola bind to these lectins via their envelope glycoproteins [12,20–24]. Previous studies showed that Junín virus has terminal mannose residues on its glycoproteins [25] and that S-layer proteins of *Lactobacillus* inhibit Junín virus infection [26], both studies indicating a putative role for C-type lectins in Junín virus binding and entry.

Here we performed a more comprehensive study of the role of the C-type lectins hDC-SIGN and hL-SIGN on Junín virus entry and infection. We used infectious Junín virus as well as pseudotyped retroviral particles carrying functional GPC (JUNV-pp). Our results show that both Junín virus and JUNV-pp infection is enhanced by the presence of hDC-SIGN and L-SIGN.

2. Materials and methods

2.1. Viruses

The naturally attenuated Junín IV4454 strain [27] was used in all experiments performed under BSL2 containment at Buenos Aires University. Junín virus was propagated in Vero cells. Virus yields were then determined by plaque formation (PFU) assays in Vero cells.

2.2. Cell culture

Monolayers of Vero cells (ATCC CCL 81) were grown in Minimum Essential Medium (MEM) (Invitrogen) containing 5% heat-inactivated fetal bovine serum (FBS) (Invitrogen) and supplemented with 50 µg/ml gentamycin. Maintenance medium (MM) consisted of MEM containing 1.5% fetal calf serum. HEK 293T (ATCC CRL-11268), NIH/3T3 cells (ATCC CCL 1658), BHK-21 (ATCC CCL 10) and 3T3-derived hDC-SIGN and hL-SIGN cells (provided by the NIAID AIDS Research and Reference Reagent Program) were grown in DMEM (Cellgro) or MEM supplemented with 10% FBS, penicillin/streptomycin and were supplemented with HEPES (20 µM).

2.3. Reagents and antibodies

FITC-goat anti mouse and mannan were purchased from Sigma. Anti-JUNV monoclonal antibody MAb SA05-BG12 was kindly donated by Dr. A. Sanchez (Centers for Disease Control, Atlanta GA, USA). Anti-hDC-SIGN and anti-hDC/L-SIGN MAbs, respectively (9E9A8, 14EG7) were obtained from the NIAID AIDS Research and Reference Reagent Program or from Santa Cruz Biotechnology Inc. Polyclonal anti-hDC-SIGN (ab97526) and hL-SIGN (ab58603) were obtained from Abcam. The anti-VSV antibody P5D4 was obtained from Sigma.

2.4. Plasmids

Constructs pcDNA3.1-hDC-SIGN and pcDNA3.1-hL-SIGN (NIAID AIDS Research and Reference Reagent Program) were used to transiently express C-type lectins using Lipofectamine2000 as

recommended by the manufacturer. Plasmid pEGFP-C1 (Clontech) or pcDNA3.1(-) (Invitrogen) were used as controls. A codon-optimized version of GPC was synthesized using the sequence of Junín virus strain IV4454, GenBank: DQ272266.3 (GeneArt) and subsequently subcloned into the pcDNA3.1 expression plasmid (Invitrogen). pcDNA3.1 plasmids expressing the human (*Homo sapiens*), feline (*Felis catus*) and canine (*Canis lupus familiaris*) transferrin receptors were provided by Dr. Colin Parrish (James A. Baker Institute for Animal Health, Cornell University). The cDNA coding for murine transferrin receptor (*Mus musculus*) was obtained from Origene and subcloned into the pcDNA3.1 expression plasmid.

2.5. Inhibition of Junín virus infectivity

Vero cells were pre-treated with different concentrations (50 or 100 µg/ml) of mannan or anti-DC/L-SIGN blocking antibodies respectively (9E9A8 or 14EG7) (20 µg/ml) for 1 h. Cells were then infected with JUNV (MOI = 1) in the presence of the blocking agent. After infection, the inoculum was removed and viral supernatants were collected after 24 h. Junín virus yields were determined by plaque assay.

2.6. Immunofluorescence assays

3T3 and Vero cells were treated with mannan (50 or 100 µg/ml) or anti-human DC-/L-SIGN MAb (20 µg/ml) for 1 h and then infected with Junín virus (MOI = 1). After a 24 h cells were fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100 and incubated with a MAb specific for JUNV NP. Anti-JUNV antibody was detected by incubation with FITC-goat anti-mouse antibody. Samples were examined using an Olympus BX51 microscope and the average number of positive cells on each coverslip was calculated as the number of virus positive cells over the number of total cells of 20 optical fields chosen randomly in 3 independent experiments.

2.7. Production of pseudotyped virions

Pseudotyped virions were produced as described previously: 293T cells were cotransfected at a ratio of 1:1:1 with an MLV-based transfer vector encoding luciferase, an MLV Gag-Pol packaging construct, and an envelope glycoprotein expressing vector (pcDNA3.1-JUNV GPC or VSV-G) by using Turbofect (Fermentas), as recommended by the manufacturer. Cells were incubated at 37 °C for 48 h, and after harvesting the supernatants were filtered through 0.45-µm pore sized membranes (Sarstedt).

2.8. Transduction of 3T3 cells

3T3 cells plated in 12 well plates were transfected (Turbofect) with 500 ng of plasmid coding for transferrin receptor (pcDNA3.1-hTfR1, pcDNA3.1-ftfR1, pcDNA3.1-mTfR1 or pcDNA3.1-ctfR1), pcDNA3.1-hDC-SIGN or pcDNA 3.1-hL-SIGN, using pEGFP-C1 (clontech) as a control. In a different set of experiments, 3T3 cells control or stably expressing DC- or L-SIGN were transfected with 500 ng of plasmid coding for transferrin receptors. In all the cases, 24 h after transfection, transfected cells were plated in 48 well plates. Transduction with the pseudotyped virions was performed the next day at 37 °C for a minimum of 4 h. The inoculum was removed and luciferase activity was measured 48 h post-transduction using a Luciferase Assay Kit (Promega) and light emission measured by using a Glomax 20/20 luminometer (Promega).

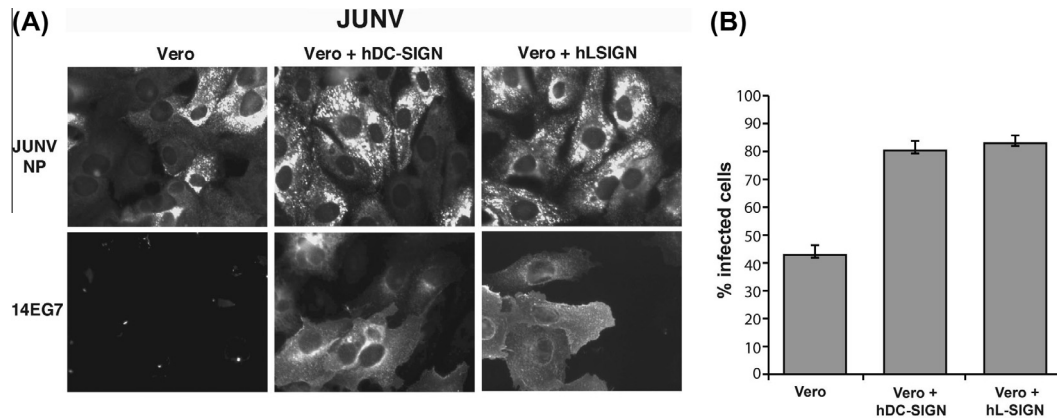


Fig. 1. Enhancement of virus entry in Vero cells by Junin virus in the presence of hDC-SIGN and hL-SIGN. Vero cells were transfected with hDC-SIGN or hL-SIGN. 24 h post-transfection, cells were infected with Junin virus (JUNV) at a MOI of 1. Cells were fixed at 24 h post infection and stained for immunofluorescence microscopy with MAbs against JUNV NP (SA05BG12) or MAbs against hDC-SIGN or hL-SIGN (14EG7). Images from three independent experiments were captured and quantified. Data are indicated in each figure as a percentage of virus positive cells over the number of total cells. Magnification 400 times. (B) Quantification of data was performed for panel (A) as indicated in Section 2. Errors represent the standard deviation of the mean.

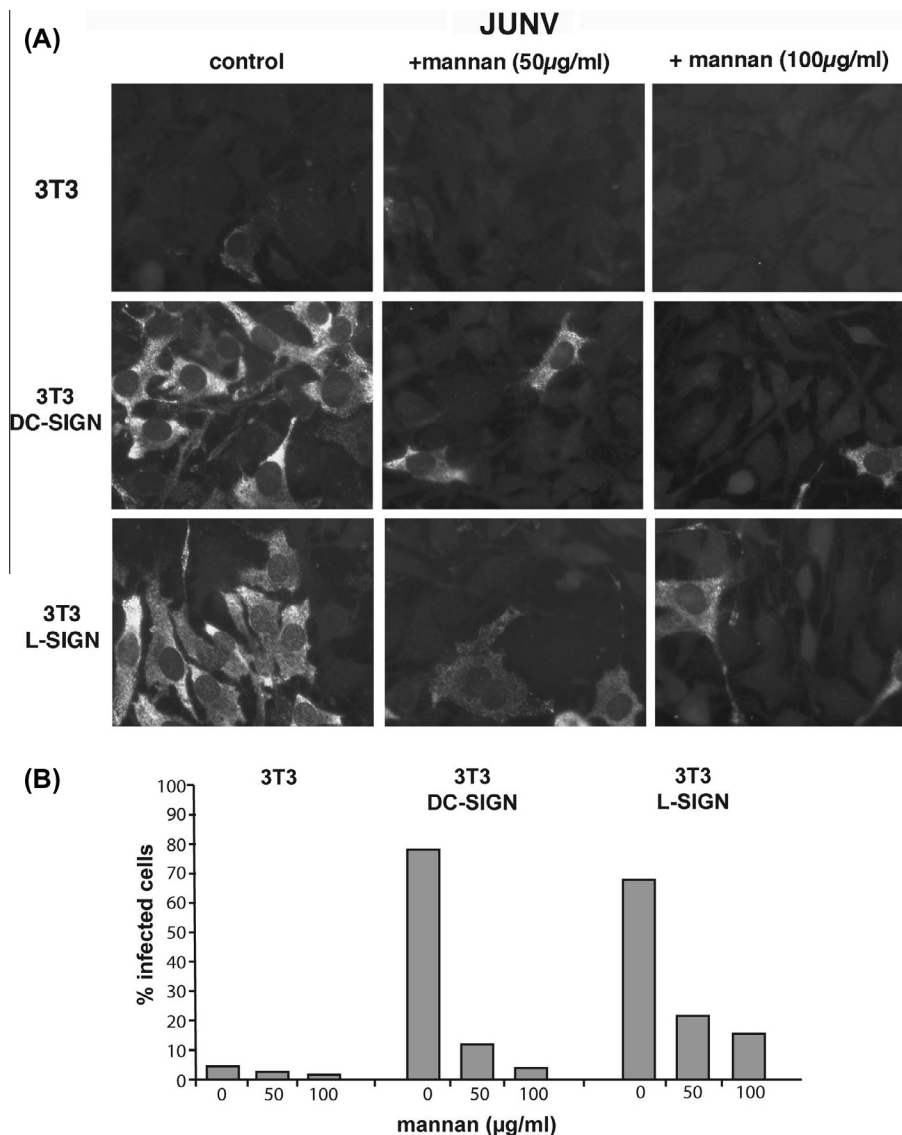


Fig. 2. Rescue of virus entry in 3T3 cells by Junin virus in the presence of hDC-SIGN and hL-SIGN and blockage of rescue by mannan. (A) 3T3, 3T3-hDC-SIGN or 3T3-hL-SIGN cells, pretreated with mannan (50 µg/ml or 100 µg/ml), were infected with Junin virus (JUNV) at a MOI of 1. Cells were fixed at 24 h post infection and stained for immunofluorescence microscopy with MAbs against JUNV NP (SA05BG12). Images from three independent experiments were captured and quantified. Magnification 400 times. (B) Quantification of data was performed for panel (A) as indicated in Section 2.

3. Results

3.1. hDC-SIGN and hL-SIGN augment Junín virus infection

We assessed the role of hDC-SIGN and hL-SIGN in the context of the infection of Vero cells with Junín virus. Vero cells were transfected with hDC-SIGN or hL-SIGN and infected with Junín virus. Infection was allowed to proceed for 24 h before cells were fixed and stained with anti-JUNV NP, or with MAb 14EG7 that recognizes both DC/L-SIGN. Expression of either lectin markedly increased Junín virus infectivity in Vero cells confirming that hDC-SIGN and L-SIGN expression augment infection (Fig. 1). Mannan binds to lectins and pretreatment of cells with mannan has been shown to reduce viral infectivity [21,29,30]. To confirm that the enhancement of Junín virus infection was due to hDC-SIGN or hL-SIGN, we treated relatively non-susceptible 3T3 cells or stably expressing hDC- or hL-SIGN cells for 1 h with mannan (50 or 100 µg/ml) and then infected with Junín virus, in the presence of mannan as a competitor of binding (Fig. 2). Mannan pre-treatment reduced Junín virus infection to levels found in the absence of lectin expression. Control experiments with vesicular stomatitis virus (VSV) showed no enhancement of infection based on lectin expression (data not shown), confirming that hDC- and hL-SIGN show specific effects on JUNV.

3.2. Viral production is enhanced in cells expressing hDC-SIGN or hL-SIGN and blocked in the presence of mannan or hDC-SIGN/hL-SIGN antibodies

3T3 cells stably expressing hDC-SIGN or hL-SIGN were infected with Junín virus. 24 h post-infection, supernatants were collected and analyzed by plaque assay in Vero cells. Our results showed that viral production is enhanced by hDC-SIGN or hL-SIGN expression (Fig. 3A) and virus production was inhibited by mannan (Fig. 3B). To confirm that the enhancement of Junín virus infection by C-type lectin was specific, we treated wild type 3T3 cells, hDC-SIGN or hL-SIGN stably-expressing 3T3 cells with the MAbs 14EG7 or 9E9A8 for 1 h. Viral production was greatly reduced in cells pretreated with MAb 14EG7, which cross-reacts with both lectins. Viral production was also reduced in hDC-SIGN expressing cells pretreated with the hDC-SIGN specific MAb 9E9A8 (Fig. 3C), which does not

cross react with h-LSIGN [31], only slightly inhibiting infection in hL-SIGN expressing cells that were pre-treated with the same antibody.

3.3. JUNV pseudovirus particles transduction of non-susceptible cells transiently expressing hDC or hL-SIGN and specific TfR1s

Our previous assays suggested that JUNV interacts with hDC/L-SIGN through the glycoprotein complex. To study this possibility, avoiding down-stream viral events we studied specifically the interaction between the lectins and JUNV glycoproteins using murine leukemia virus particles pseudotyped with JUNV GPC (JUNVpp), according to a previously described protocol [28]. Cleaved GPC was incorporated into pseudoparticles as determined by Western blotting (data not shown). Relatively non-susceptible mouse 3T3 cell lines were transfected with plasmid vectors containing hDC-SIGN or hL-SIGN. Cells were then transduced with equal amounts of JUNVpp and infectivity was measured 48 h after transduction (Fig. 4). 3T3 cells transiently expressing hDC-SIGN or hL-SIGN were susceptible to JUNVpp infection, with hL-SIGN expression more conducive to transduction. Control 3T3 cells expressing GFP did not mediate JUNVpp entry (Fig. 4A). These data suggest that hDC- and hL-SIGN promote transduction of 3T3 cells with JUNVpp.

Human transferrin receptor 1 (hTfR1) has been identified as a receptor for Junín virus, along with feline TfR1 which can also be used by Junín virus; however the canine (cTfR1) and murine TfR1s (mTfR1) do not serve as functional receptors for Junín virus infection [8]. Based on these data, we performed control experiments to examine the relative efficiency of entry mediated by transferrin receptors of different species, in comparison to entry solely mediated by hDC-SIGN and hL-SIGN. 3T3 cells were transfected with hTfR1, mTfR1, fTfR1 or cTfR1s followed by transduction with JUNVpp (Fig. 4A). As shown in previous studies, hTfR1 and fTfR1 allowed virus entry, whereas mTfR1 and cTfR1 did not allow entry. The levels of pseudovirus transduction were similar for both hL-SIGN and hTfR1, while hDC-SIGN had reduced transduction in this experiment.

We next examined whether co-expression of a C-type lectin and a TfR1 would have a combinatorial effect on transduction levels. 3T3 cells or 3T3 stably expressing DC- or L-SIGN were transfected

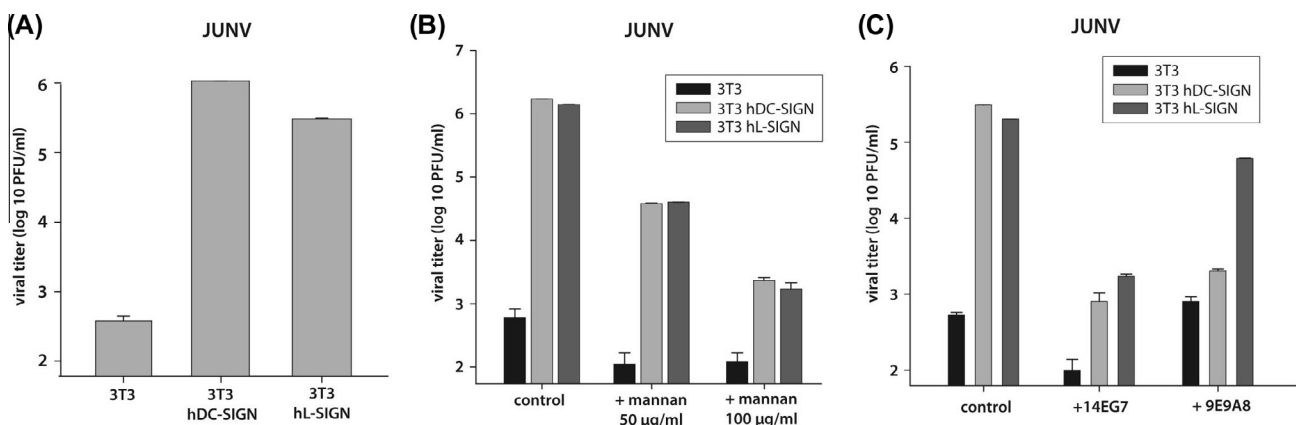


Fig. 3. Viral production is enhanced in cells expressing hDC-SIGN or hL-SIGN and blocked in the presence of mannan or hDC-SIGN/hL-SIGN antibodies. (A) 3T3, 3T3-hDC-SIGN or 3T3-hL-SIGN cells were infected with Junín virus (JUNV) at a MOI of 1. Supernatants were collected at 24 h post infection and viral production was determined by PFU assay in Vero cells. Data from three independent experiments were quantified. Error bars represent the standard deviation of the mean. (B) 3T3, 3T3-hDC-SIGN or 3T3-hL-SIGN cells pretreated with mannan (50 µg/ml or 100 µg/ml), were infected with Junín virus (JUNV) at a MOI of 1. Supernatants were collected at 24 h post infection and viral production was determined by PFU assay in Vero cells. Data from three independent experiments were quantified. Error bars represent the standard deviation of the mean. (C) 3T3, 3T3-hDC-SIGN or 3T3-hL-SIGN cells pretreated with MAbs against hDC-SIGN 9E9A8 (20 µg/ml) or 14EG7 (20 µg/ml), and then infected with Junín virus (JUNV) at an MOI of 1. Supernatants were collected at 24 h post infection and viral production was determined by PFU assay in Vero cells. Data from three independent experiments were quantified. Error bars represent the standard deviation of the mean.

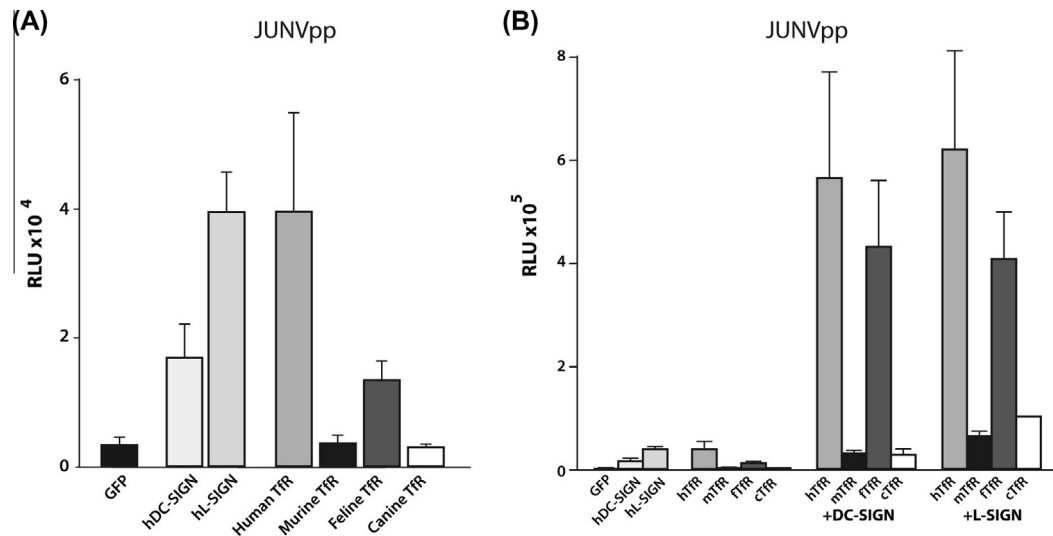


Fig. 4. JUNV pseudovirus particles transduction of non-susceptible cells transiently expressing hDC or hL-SIGN and specific TfR1s. (A) 3T3 cells were transfected with pcDNA3-hDC-SIGN, pcDNA3hL-SIGN or plasmids encoding human, feline, canine or murine TfR1 (hTfR, fTfR, cTfR or mTfR), or with C1 plasmid, carrying only GFP, as a control. 24 h post-transfection cell cultures were transduced with the retroviral pseudotypes carrying JUNV GPC. After 48 h at 37 °C cells were lysed and luciferase activity was measured as RLU. Error bars represent standard deviation of the mean. (B) 3T3, 3T3-hDC-SIGN or 3T3-hL-SIGN cells were transfected with plasmids encoding human, feline, canine or murine TfR1 (hTfR, fTfR, cTfR or mTfR), or with C1 plasmid, carrying only GFP, as a control. 24 h post-transfection cell cultures were transduced with the retroviral pseudotypes carrying JUNV GPC. After 48 h at 37 °C cells were lysed and luciferase activity was measured as RLU. Error bars represent standard deviation of the mean.

with TfR1s from different species, and transduced with JUNVpp (Fig. 4B) or with VSVGpp as a control (data not shown). As shown in Fig. 4B, the combination of hTfR1 or fTfR1 with either hDC-SIGN or hL-SIGN produced substantially higher levels of transduction than either receptor expressed alone. Control experiments using murine leukemia virus particles pseudotyped with VSV G protein (VSVGpp) showed no overall differences in virus entry indicating that the effects observed were specific to JUNVpp (data not shown).

4. Discussion

In this paper the interaction between C-type lectins and Junin virus, or a retroviral pseudotype carrying JUNV GPC, was investigated. We demonstrate that expression of hDC-SIGN, or its homologue hL-SIGN, significantly enhances Junin virus entry and infection of susceptible cells, with expression of hDC- or hL-SIGN being enough to markedly increase susceptibility of a relatively non-susceptible murine cell culture. Our data show that Junin virus can use either hDC- or hL-SIGN expressed on the cell surface, resulting in productive infection of mouse 3T3 cells that express these lectins. There was an increase in virus yield of over 3 log units in lectin-expressing cells (Fig. 3) compared to an approximately 16-fold increase in primary infection (Fig. 2), suggesting that expression of hDC-SIGN and hL-SIGN also increased production of infectious virus particles. This is significant as over-expression of binding factors can lead to blockage of virus release in some virus systems, and leads to the conclusion that hDC-SIGN and hL-SIGN are functionally important *in vivo*.

Despite the wide use of C-type lectins as attachment factors by many viruses, the mechanistic understanding of the interplay between the virus and the host is still not clear. For HIV gp120 and Ebola virus glycoprotein, it has been demonstrated that its carbohydrate processing can show different patterns depending on the cell type they were produced in, which alters the interactions of these glycoproteins with C-type lectins [32,33]. For this reason, binding to hDC- or hL-SIGN can differ according to the phases of the virus replication cycle, within the same or different hosts.

Junin virus is one of the many pathogens that can interact with hDC- or hL-SIGN, nevertheless not all glycosylated viruses can interact with these lectins, and it has been demonstrated that these interactions show some specificity [34]. Results from the present study indicate that JUNV GPC is glycosylated in such a way as to allow its interaction with hDC- and hL-SIGN. Our study showing an enhancement of the infection in hDC- or hL-SIGN positive cells demonstrates the importance of researching JUNV GPC to determine if changes in this binding have an impact on virus infectivity, tropism and pathogenicity.

Acknowledgments

We thank Volker Vogt and Ruth Collins for advice during the course of this work, Nadia Chapman for technical assistance and all members of the Whittaker lab for helpful discussions. We also thank Dr. Colin Parrish for the kind provision of reagents. These studies were funded in part by travel grants from the American Society for Microbiology and the Journal of Cell Science (to MGM). MAB was supported by grant T32AI007618 (Training in Molecular Virology and Pathogenesis) from the National Institutes of Health. MGM and SMC were supported by CONICET (Consejo Nacional de Investigaciones Científicas y Tecnológicas) and by the following grants: ANPCyT 2007-761 and UBA 2008-035 to NAC.

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