Chapter 8

Entry Studies of New World Arenaviruses

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Abstract

Identification of cell moieties involved in viral binding and internalization is essential since their expression would render a cell susceptible. Further steps that allow the uncoating of the viral particle at the right subcellular localization have been intensively studied. These "entry" steps could determine cell permissiveness and often define tissue and host tropism. Therefore applying the right and, when possible, straightforward experimental approaches would shorten avenues to the complete knowledge of this first and key step of any viral life cycle. Mammarenaviruses are enveloped viruses that enter the host cell via receptor-mediated endocytosis. In this chapter we present a set of customized experimental approaches and tools that were used to describe the entry of Junín virus (JUNV), and other New World mammarenavirus members, into mammalian cells.

Key words Virus, Receptor recognition, Endocytosis pathways

1 Introduction

Viral entry into the host cell is a key step in the infection cycle. The entry process involves an initial interaction between a virus and attachment factor(s) and/or receptors, leading to virus internalization, release of the genome, and subsequent infection. Thus, addressing the mechanisms used for viruses during early steps of infection is fundamental to determining virus tropism and developing novel antiviral therapies.

The study and characterization of viral entry requires a combination of techniques that allow the specific measurement of viral binding to its receptor together with the involvement of different, and perhaps novel, internalization pathways. Measuring direct virus binding to cells requires the optimization of methods to label and purify viral particles (i.e., radioisotope or fluorescent conjugation). Indirect detection by fluorophore-conjugated antibodies is also possible with minor downstream steps. However, multiple approaches are necessary to cover uncertainties of a single virus-bound readout.

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When the specific receptor used by the virus is known, a wider variety of tools become available, such as transient expression of the specific receptor in receptor-negative cell lines.

To determine the internalization pathway(s) and cellular structures involved in virus entry, different and complementary approaches are available. Drugs or small molecules specifically targeting gene product functions are commonly used and rely on the previously proven specificity of each compound. Additionally, genetic tools such as dominant-negative protein expression have been widely used during the last decades.

Direct visualization of the viral entry process can be achieved by electron microscopy (EM). Some endocytic structures are easily identified by EM due to their distinct morphology (i.e., clathrin-coated pits). For other not-so-morphologically-distinct structures, immuno-EM is necessary, labeling both the virus particle and the cell structure of interest with specific antibodies. However, labeling with gold-conjugated antibodies depends on the existence of a strong and specific antibody-antigen interaction.

JUNV is the causative agent of Argentine hemorrhagic fever. For decades it has been locally studied, and in recent years it gained worldwide attention due to its relevance as a bioterrorism agent. Thus, according to the National Institute for Allergy and Infectious Diseases (NIAID, USA) and its Biodefense program, arenaviruses like Junín virus belong to Category A of priority pathogens.

Here we described our experience in establishing and optimizing methods to determine key features of JUNV entry into host cells. We have used JUNV or JUNV glycoprotein complex (GPC)-pseudotyped particles to study their ability to be recognized by the human C-type lectins hDC- or hL-SIGN. These findings provided evidence that hDC- and hL-SIGN can mediate the entry of JUNV into cells, in the absence of its specific cellular receptor hTfR1, suggesting an important role in virus infection. Using radioactive virus-binding assays, we showed that JUNV infects polarized lines preferentially through the apical surface [1]. Also performing the above-described studies, we have successfully defined cytoskeleton components essential for virus entry [2]. Our studies demonstrated that early JUNV infection of Vero cells relies on both an intact actin network and a dynamic microtubule network.

Our findings have shown that clathrin-mediated endocytosis is the main JUNV internalization pathway into Vero cells [3] (Fig. 1). Virus entry was dependent on dynamin 2 GTPase and EPS15. In addition, we have shown that after virus internalization, JUNV traffics through Rab5 (early) and Rab7 (late) endosomes in its pH-dependent entry [4]. Taken together, our experimental designs and protocols allowed us to explore a broad spectrum of JUNV entry features. From new putative attachment factors/receptor

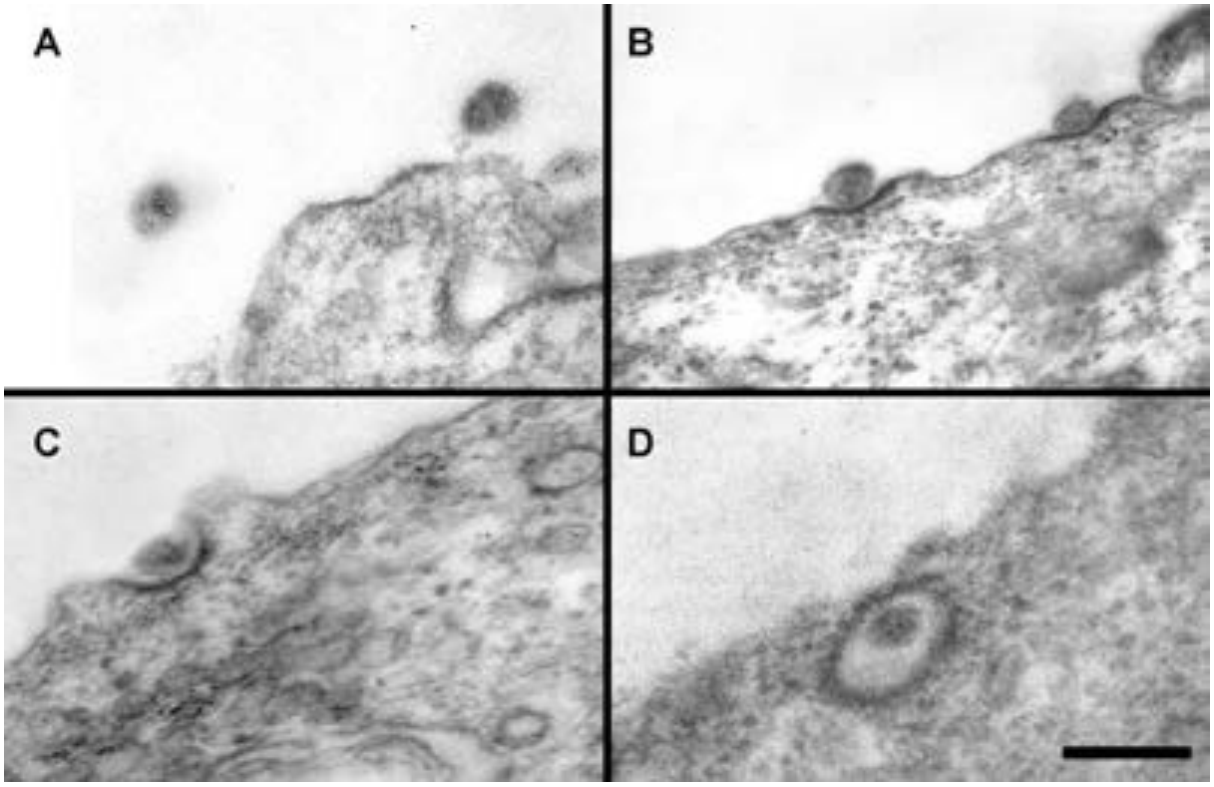

Fig. 1 Ultrastructural analysis of JUNV entry into Vero cells via clathrin-coated vesicles. Vero cells were incubated with concentrated JUNV for 60 min at 4 °C. Infection was initiated by shifting the temperature to 37 °C. After 15 min at 37 °C, cells were washed in PBS and fixed in 1.5% glutaraldehyde. Thin sections were made for ultrastructural analysis by transmission EM. (**a**) Population of JUNV outside Vero cells. (**b**) Binding of JUNV particles at the plasma membrane of Vero cells. (**c**) Uptake of JUNV by clathrin-coated pits. (**d**) JUNV is internalized within a clathrin-coated vesicle. *Bar*: 200 nm

molecules to cellular pathways used during internalization, all these features are essential to complete the gap in our knowledge about JUNV infection of specific cells.

2 Materials

2.1 Strains of Viruses, VLP, Cells, and Media Used in Entry Assays

1. Naturally attenuated Junín virus strain: IV_{4454} was propagated in Vero cells at BSL2 containment [5]. JUNV stock titers between 2×10^6 and 1×10^7 PFU/mL were aliquoted and stored at −80 °C.
2. Pseudotyped virion suspensions expressing the JUNV envelope glycoprotein were produced in 293T cells as described in Subheading 3.6. [6]. Pseudotyped stock titers in Vero cells were between 1×10^7 and 5×10^7 RLU/mL.
3. Vero cells (ATCC CCL81) were cultured at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% fetal bovine serum (FBS), 100 U penicillin/mL, and 100 μg streptomycin/mL.

4. 293T (ATCC CRL-11268), BHK-21 (ATCC CCL10), and 3T3 cells (ATCC CCL1658), and 3T3-derived hDC-SIGN and hL-SIGN cells (NIAID AIDS Research and Reference Reagent Program) were grown in DMEM containing 10% FBS.
5. CHO-TRVb cell cultures (devoid of detectable cell-surface TfR) provided by Dr. Colin Parrish (James A. Baker Institute for Animal Health, Cornell University) were grown in Ham's F12 containing 10% FBS. All cell cultures were grown under 5% CO_2 in humidified incubator.

2.2 Viral Attachment and Adsorption Studies

1. Purified, [^{35}S]methionine-labeled JUNV stock: 5×10^7 PFU/mL, 1×10^8 dpm/mL produced as described in [7].
2. 24-well plates.
3. Phosphate-buffered saline (PBS).
4. Blocking solution: PBS containing 1% FBS, 0.1% glucose, and 0.5% BSA.
5. Lysis buffer: 0.1 M NaOH containing 1% SDS.
6. Scintillation solution: dissolve 10 g of PPO (2,5-diphenyloxazole) and 150 mg of POPOP 1,4-bis[-2(5-phenyloxazole)-benzene] in 237 mL of Triton X-100. Complete with toluene to 1 L. Filter the scintillation solution to remove any particles and store in a brown bottle to prevent deterioration by light.
7. Liquid scintillation counter.

2.2.1 Quantifying Virus Binding Using Radiolabeled Virions

2.2.2 Quantifying Virus Binding Using Non-radiolabeled Virions

1. Purified non-radiolabeled stock or JUNV pseudotyped particle suspensions.
2. 24-well plates.
3. PBS.

2.2.3 Imaging Virus Binding by Confocal Microscopy

1. Purified non-radiolabeled JUNV stock.
2. 24-well plates.
3. Blocking solution: 4% BSA in PBS.
4. Anti-JUNV antibody GB03-BE08 (for antibody details, *see* Table 1).
5. Rabbit anti-hDC-SIGN antibody.
6. Anti-hTfR1 antibody.
7. Reagents for IF listed in Subheading 2.5.
8. Confocal microscope (Olympus FV 1000 or similar).

Table 1
Antibodies and their specificities

Antibody/compound	Characteristics	Working concentration (dilution)	Time of incubation	Refs./provider
IC06-BE10	Monoclonal anti-JUNV against the NP protein	4 μg/mL (1/250)	60 min at 37 °C/ ON at 4 °C	[8]
GB03-BE08	Monoclonal anti-JUNV against the GPC protein	4 μg/mL (1/250)	60 min at 37 °C/ ON at 4 °C	[8]
Anti-JUNV rabbit	Rabbit anti-JUNV polyclonal serum	1/50	60 min at 37 °C/ ON at 4 °C	[9]
P5D4	Monoclonal anti-VSV antibody	10 μg/mL (1/100)	60 min at 37 °C/ ON at 4 °C	Sigma-Aldrich
13-6800	Monoclonal anti-hTfR1 antibody	5 μg/mL (1/100)	60 min at 37 °C/ ON at 4 °C	Zymed
T-5158	Monoclonal anti-α-tubulin antibody clon B512	25 μg/mL (1/200)	60 min at 37 °C/ ON at 4°C	Sigma-Aldrich
DC-SIGN (D7F5C) XP	Rabbit anti-hDC-SIGN monoclonal antibody	1/200	60 min at 37 °C/ ON at 4 °C	Cell signaling
9E9A8	Monoclonal anti-hDC-SIGN antibody	5 μg/mL (1/300)	60 min at 37 °C/ ON at 4 °C	NIAID AIDS
97526	Polyclonal anti-hDC-SIGN antibody	1/300	60 min at 37 °C/ ON at 4 °C	Abcam
58603	Polyclonal anti-hL-SIGN antibody	2 μg/mL (1/300)	60 min at 37 °C/ ON at 4 °C	Abcam

(continued)

Table 1
(continued)

Antibody/compound	Characteristics	Working concentration (dilution)	Time of incubation	Refs./provider
14EG7	Monoclonal anti-hDC-/L-SIGN antibody	2 μg/mL (1/300)	60 min at 37 °C/ ON at 4 °C	NIAID AIDS
Anti-mouse AF 488	Goat anti-mouse	0.2 μg/mL (1/1000)	60 min at 37 °C	Molecular Probes
Anti-rabbit AF 555	Goat anti-rabbit	0.2 μg/mL (1/1000)	60 min at 37 °C	Molecular Probes
Anti-mouse AF 568	Goat anti-mouse	0.2 μg/mL (1/1000)	60 min at 37 °C	Molecular Probes
Phalloidin-FITC	Actin staining	10 μg/mL	60 min at 37 °C	Sigma-Aldrich
Transferrin-TRITC	Transferrin-TRITC acoplated	20 μg/mL	60 min at 37 °C	Sigma-Aldrich
Cholera toxin-TRITC	β-Subunit cholera toxin-TRITC acoplated	0.5 μg/mL	60 min at 37 °C	Sigma-Aldrich
DAPI	Nuclear staining	1 μg/mL	5 min at room temperature	Sigma-Aldrich
Hoechst 33342	Nuclear staining	1 μg/mL	5 min at room temperature	Sigma-Aldrich

2.3 Viral Internalization Studies

1. Proteinase K solution: 1 mg/mL proteinase K in PBS.
2. PMSF solution: 1 mM PMSF in PBS containing 3% BSA.
3. Reagents listed in Subheading 2.2.1.

2.3.1 Quantifying Virus Internalization Using Radiolabeled Virions

2.3.2 Quantifying Virus Internalization Using Non-radiolabeled Virions

1. TRIzol (Invitrogen).
2. Isopropanol (analytic grade).
3. Chloroform (analytic grade).
4. 75% ethanol solution.
5. Bi-distilled nuclease-free water.
6. Real mix (Biodynamics, Argentina).
7. dNTPs 10 mM (Biodynamics, Argentina).
8. Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Promega).
9. 1 μg or 50 pmol random primers (Biodynamics, Argentina).
10. Primer ZF: 5′-ATGGGCAACTGCAACGGGGCATC-3′.
11. Primer ZR: 5′-GTTGCCATCAATGACCCCTTCA-3′.
12. Primer GAPDH-F151 (Invitrogen): 5′-GTTGCCATCAAT GACCCCTTCA-3′.
13. Primer GAPDH-R339 (Invitrogen): 5′-CAGCCTTCTCCAT GGTGGTG-3′
14. Thermocycler equipment (e.g., iCycler iQ Bio-Rad and its software iQ5 2.1.97.1001).
15. cDNA premix (per reaction): 3 μg RNA, 1 μg or 50 pmol random primers, enough nuclease-free water to complete 10 μL per reaction.
16. cDNA Mix (per reaction): 1 μL 10 mM dNTPs, 1 μL M-MLV RT, 4 μL nuclease-free water.
17. Reagents listed in Subheading 2.3.1.

2.3.3 Reagents and Materials Used for Electron Microscopy

1. 24-well plates.
2. Ice-cold 1.5% glutaraldehyde in 0.2 M phosphate buffer pH 7.2.
3. 0.2 M phosphate buffer.
4. 0.32 M sucrose solution.
5. 1.5% osmium tetroxide.
6. Graded ethanol solution.
7. Uranyl acetate.
8. Epon 812 resin (TAAB).
9. Diamond knife.

10. Reynold's solution.
11. C10 Zeiss electron microscope.
12. Kodak 4489 film.

2.4 Viral Entry Inhibition Studies

2.4.1 Cell Viability Assay

1. 5 mg/mL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] stock solution dissolved in DMSO or PBS (Sigma-Aldrich). Store at −20 °C in the dark.
2. 96-well plates.
3. Graded ethanol solution.
4. Spectrophotometer: reading at 570 nm and background at 630–690 nm.

2.4.2 Receptor-Specific Modulators

1. Mannan (Sigma-Aldrich) stock solution 50 mM in sterile water.
2. 24-well plates.
3. Anti-JUNV SA02-BG12 antibody (for antibody details, *see* Table 1).
4. Anti-hDC-SIGN 9E9A8 antibody.
5. Anti-hDC-/L-SIGN 14EG7 antibody.
6. Anti-hDC-SIGN 97526.
7. Anti-VSV P5D4 antibody.
8. Anti-mouse AF 488 antibody.
9. cDNA3.1-hDC-SIGN and pcDNA3.1-hL-SIGN constructs (NIAID AIDS).
10. pcDNA3.1 plasmids expressing different transferrin receptors: pcDNA3.1-hTfR1 (*Homo sapiens*), pcDNA3.1-fTfR1 (*Felis catus*), pcDNA3.1-cTfR1 (*Canis lupus familiaris*), and pcDNA3.1-mTfR1 (*Mus musculus*). All are provided by Dr. Colin Parrish, James A. Baker Institute for Animal Health, Cornell University.

2.4.3 Endocytic Pathway-Affecting Compounds

1. Pharmacological compounds (*see* Table 2).
2. 24-well plates.
3. Maintenance medium (MM): complete DMEM containing 1.5% FBS.
4. Serum-free DMEM.
5. Anti-JUNV IC06-BE10 antibody.
6. Anti-mouse AF 568 antibody.
7. Transferrin-TRITC.
8. β-Subunit cholera toxin-TRITC (*see* **Note 1**).
9. Reagents for IF are listed in Subheading 2.5. Antibodies and fluorescent marker details are in Table 1.
10. Olympus BX51 microscope.

Table 2
Entry and cytoskeleton inhibitors and their specificities

	Inhibitor compound [a]	Working concentration ranges	Time of incubation	Refs.
Entry step affected				
Acid pH-dependent entry	Ammonium chloride	5–10 mM	30 min + virus adsorption	[10]
Dinamin-dependent endocytosis	Concanamycin A (ConcA)	5–10 mM	60 min + virus adsorption	[b]
	Dynasore (Dyn)	90–150 μM		
Clathrin-mediated endocytosis	Chlorpromazine (CPZ) (*see* **Note 21**)	20–50 μM	120 min + virus adsorption	[3, 4]
Cholesterol- dependent entry	Nystatin (NT)	70–120 μM	120 min	[3, 4]
	Methyl-β-cyclodextrin (MβCD)	5–10 μM	30 min	[b]
Cytoskeleton component affected				
Actin (polymerization)	Cytochalasin D (CitD)	1–2 μM		
	Latrunculin A (LatA)	1–5 μM		
Actin (depolymerization)	Jasplakinolide (Jas)	0.5 μM	30 min + virus adsorption	[2]
Microtubules (polymerization)	Nocodazole (Noc)	20–40 μM		
Microtubules (depolymerization)	Paclitaxel (Pac)	40–100 μM		

[a]All compounds used in Table 2 were acquired from Sigma-Aldrich
[b]Unpublished data

2.4.4 Cytoskeleton-Affecting Compounds

1. Inhibitory compounds (*see* Table 2).
2. 24-well plates.
3. Anti-tubulin T-5158 antibody.
4. Anti-JUNV IC06-BE10 antibody.
5. Anti-mouse AF 568 antibody.
6. Phalloidin-FITC.

2.4.5 Dominant-Negative Constructions Inhibiting Virus Entry

1. Plasmids encoding wt or dominant-negative constructions of EPS15 (GFP-EΔ95-295) [11], dynamin 2 (GFP-K44A) [12], and Rab5 (GFP-S34 N) [13] fused to GFP.
2. Lipofectamine 2000 (Invitrogen).
3. OptiMEM media (Invitrogen).
4. Anti-JUNV IC06-BE10 antibody.
5. Anti-mouse AF 568 antibody.

6. Transferrin-TRITC.
7. Reagents for IF listed in Subheading 2.5 and antibodies and fluorescent markers details in Table 1.
8. Olympus BX51 microscope.

2.5 Immunofluorescence (IF) and Light Microscopy

1. PBS.
2. 0.2% Triton X-100 solution in PBS.
3. 16% paraformaldehyde (PFA) solution (Electron Microscopy Sciences). Prepare a 4% working solution by dilution 1:4 in PBS.
4. Blocking solution: Prepare a 2% (g/v) stock solution of porcine skin gelatin in PBS (10× solution). Autoclave and keep sterile. Prepare a working blocking solution (1×) by making a 1/10 dilution of the 10× stock.
5. 2 mg/mL poly-L-lysine (PLL, Sigma-Aldrich) stock solution in water is used to coat coverslips. Filter to sterilize. Put 1 mL aliquots into sterile microcentrifuge tubes and store at −20 °C. To make a working stock, thaw and dilute 1:100 to 20 μg/mL in sterile water (*see* **Note 2**).
6. 12 mm round glass coverslips (thickness #1.5).
7. Mounting solution: Prolong diamond antifade medium (Molecular Probes).
8. DAPI or Hoechst (*see* **Note 3**).
9. Anti-JUNV IC06-BE10.
10. Anti-JUNV polyclonal serum.
11. Anti-tubulin T-5158 antibody.
12. Rabbit anti-hDC-SIGN antibody.
13. Phalloidin-FITC, transferrin-TRITC, cholera toxin-TRITC.
14. Anti-mouse AF 488, anti-mouse AF 568, and anti-rabbit AF 555.
15. Fluorescence microscope equipped with lamp and filters for excitation and detection of triple-stained samples (BFP/GFP/RFP). High-sensitivity camera and objectives with magnification of 20× or 10× and 60× or 100× (*see* **Note 4**).

2.6 Plaque-Forming Unit (PFU) Assay

1. Vero cell cultures.
2. 24-well plates.
3. 1.4% methylcellulose solution (Sigma-Aldrich) (*see* **Notes 5** and **6**).
4. MM of 2× concentration.
5. Plaque semisolid medium: composed of half parts of 1.4% methylcellulose and 2× MM.
6. 10% paraformaldehyde.

7. Crystal violet solution: dissolve 1 g crystal violet in 10 mL of 96% ethanol, and then complete up to 100 mL with distilled water.
8. Bench transilluminator.

2.7 Pseudotyped Virion Production

1. 293T cell cultures.
2. 24-well plate.
3. MLV-based transfer vector encoding luciferase.
4. MLV Gag-Pol packaging construct.
5. pEGFPC1 (Clontech) or pcDNA3.1(−) plasmid (Invitrogen).
6. Codon-optimized version of GPC of Junín virus strain IV4454 GenBank: DQ272266.3 (GeneArt) subcloned into the pcDNA3.1 expression plasmid (Invitrogen).
7. pcDNA3.1 expression plasmid encoding VSV-G as a control.
8. TurboFect Transfection Reagent (Thermo Fisher).
9. 0.45-μm pore-sized membrane filter (Sarstedt).
10. Luciferase Assay Kit (Promega).
11. GloMax 20/20 Luminometer (Promega).

3 Methods

3.1 Viral Attachment Studies

In the following experimental approaches, use the JUNV-susceptible cell line of your choice and change the culture media accordingly.

3.1.1 Quantifying Radiolabeled JUNV Particle Binding

1. Plate around 1×10^5 cells (i.e., Vero) per well in 24-well plates to a 90-100% confluency.
2. Pretreat cells with blocking solution (*see* Subheading 2.2.1) at 37 °C for 1 h to avoid nonspecific binding.
3. Infect cells at MOI of 1 with 100 μL of radiolabeled virus (200,000 dpm) per well routinely; each condition is done in duplicate; take this into account when preparing radiolabeled stock (*see* **Note 7**).
4. Incubate for 60 min at 4 °C.
5. Wash cells extensively with cold PBS and lyse in 500 μL lysis buffer per well and dissolve in scintillator solution (*see* Subheading 2.3.1).
6. Quantify cell-associated [^{35}S]-radioactivity using a liquid scintillation counter.
7. Determine total amount of cell-associated dpm in control infected cells vs. treated-infected cells. Values may also be expressed as percentages.

3.1.2 Quantifying Non-radiolabeled JUNV Particle Binding

1. Proceed as in Subheading 3.1.1 from 1 to 4 but using non-radiolabeled virus stock.
2. Wash cells extensively with cold PBS and lyse in 500 μL of PBS per well by freezing and thawing twice.
3. Quantify the amount of infectious bound virus by standard PFU assay (*see* Subheading 3.4).

3.1.3 Determining Virus Binding by Confocal Microscopy

1. Plate around 1×10^5 cells (i.e., 3T3-DC-SIGN, Vero) per well in 24-well plates to a 90–100% confluency.
2. If target cells express both TfR1 and DC-SIGN, preincubate cells with anti-hTfR1 antibody for 1 h at 4 °C (blocking one of the two molecules would allow detecting and binding to one another).
3. Infect the cultures with 100 μL of JUNV (MOI of 5) diluted in MM and in the presence of blocking agent (*see* **Note 8**), during 1 h at 4 °C and continuously shaking.
4. Wash gently the cultures three times with cold PBS and incubate with primary antibodies dilutions (*see* Table 1 and **Note 9**) for 1 h at 4 °C, continuously shaking.
5. Wash gently three times with cold PBS and then incubate for 20 min with PFA 4% at room temperature (RT) (*see* **Note 10**).
6. Wash three times with PBS at RT.
7. Process the cultures for IF assay to detect JUNV-GP1 and DC-SIGN (*see* Subheading 3.5). Use confocal microscopy to detect adsorbed particles and DC-SIGN in membrane (*see* **Notes 11** and **12**).

3.2 Viral Internalization Studies

For the internalization assay, follow instructions as in Subheading 3.1. After virus adsorption at 4 °C for 1 h, incubate cells at 37 °C for 1 h to allow virus penetration.

3.2.1 Quantifying Radiolabeled JUNV Particle Internalization

1. Wash cultures with PBS and treat with proteinase K solution to remove external adsorbed virus.
2. Stop protease treatment by adding PMSF solution for 3 min at RT.
3. Follow **steps 5** and **6** to quantify internalized radiolabeled virus.

3.2.2 Quantifying Non-radiolabeled JUNV Particle Internalization

Proceed as in Subheading 3.2.1 and alternatively wash and lyse infected cultures in TRIzol buffer (according to the manufacturer's instructions) and continue as follows:

1. Extract total RNA by adding 0.2 mL of chloroform per mL of TRIzol. Mix vigorously by hand for 15 s and centrifuge at $12{,}000 \times g$ in a bench centrifuge, 15 min at 4 °C.

2. Take upper phase containing total RNA and transfer to a sterile tube to precipitate RNA by adding 0.5 mL of isopropanol per mL of TRIzol. Incubate samples at RT for 10 min and centrifuge at 12,000 × *g*, 15 min at 4 °C.
3. Remove and discard gently all supernatant and wash pellet once with 1 mL of 75% ethanol per mL of TRIzol. Mix by vortexing and centrifuge at not more than 7,500 × *g* for 5 min at 4 °C.
4. Air-dry the RNA for 10–20 min and resuspend in 50 μL of RNase-free water (*see* **Note 13**). Incubate for 10 min at 55–60 °C and store at −80 °C until use.
5. Generate the cDNA using the following procedure: Prepare the cDNA premix and cDNA reaction mix from Subheading 2.3.2. Mix together the cDNA premix with the cDNA reaction mix, vortex, and centrifuge for a spin and incubate for 2 h at 42 °C. Store at −80 °C until use.
6. Quantification of the amount of cell-bound viral RNA was performed by qRT-PCR employing TaqMan technology as follows:
   (a) Amplify the cDNA PCR using specific viral gene primers (*see* Subheading 2.3.2).
   (b) Amplify housekeeping mRNA using gene-specific primers (*see* Subheading 2.3.2).
   (c) Normalize average viral RNA Ct values to the average Ct values of GAPDH, and set ΔΔCt-based fold-change calculations relative to untreated virus infected, determining the Ct values using software indicated (*see* **Note 14**).

3.2.3 Electron Microscopy Readout

1. Plate around 5×10^5 cells (i.e., 3T3-DC-SIGN, Vero) per well in a 6-well plate to a 90–100% confluency.
2. Infect the cultures with 100 μL of JUNV (MOI of 50) for 1 h at 4 °C.
3. Expose the cultures to 37 °C for 15 min.
4. Wash the cells three times with cold PBS.
5. Fix the cultures with 1.5% glutaraldehyde in 0.2 M phosphate buffer pH 7.2 during 4 h at RT.
6. Wash the cultures overnight in a 0.32 M sucrose in 0.1 M phosphate buffer solution at 4 °C.
7. Resuspend the cells and centrifuge at 500 × *g* for 10 min. Add 1.5% osmium tetroxide overnight at 4 °C for the post-fixation.
8. Dehydrate the cultures in ethanol solutions followed with propylene oxide.

9. Embed the cells in Epon resin and let it polymerize for 2 days at 70 °C.
10. Take ultrathin sections with a diamond knife.
11. Stain with 2% uranyl acetate and then with Reynold's solution.
12. Take micrographs with Zeiss electron microscope using Kodak 4489 film.

3.3 Using Inhibitors to Determine Cellular Components Involved in Virus Entry

The inhibitory effect of any experimental condition or compound on virus internalization can be measured by different experimental approaches. Initially it is important to establish the noncytotoxic range of a chosen compound for a particular cell line.

3.3.1 Measuring Cell Viability

1. Plate around 2×10^4 cells (i.e., Vero, 3T3) in a 96-well plate to a 90–100% confluency (*see* **Note 15**).
2. Prepare a gradient of compound concentrations to a total of 600 μL each (in the corresponding cell culture medium).
3. Remove growth medium and add 100 μL per well of the compound concentration to be tested. Add 100 μL per well of culture medium to control wells (*see* **Note 16**).
4. Incubate the plate for the time chosen to be tested.
5. Add 10 μL of MTT stock solution to each well and incubate 2 h at 37 °C.
6. Remove the supernatants and add 200 μL of ethanol to each well to solubilize the formazan crystals by vigorous shaking.
7. Measure absorbance in a microplate reader at 595 nm.
8. Calculate CC_{50} as the compound concentration necessary to reduce cell viability by 50%.

3.3.2 Modulating Virus Receptor-Specific Recognition

Inhibition of Virus Infection by C-Type Lectin-Binding Compounds

1. Plate around 1×10^5 cells (i.e., 3T3 or 3T3-derived hDC-SIGN and hL-SIGN cells) per well in 24-well plates to a 90–100% confluency (containing a coverslip for determination of viral infection by IF, *see* Subheading 3.5).
2. Prepare dilutions of the blocking agent of interest in DMEM 5% FBS in sterile plastic tubes. Use concentrations ranging from 50 to 100 μg/mL for mannan or 20 μg/mL of anti-DC-/L-SIGN blocking antibodies.
3. Gently remove the media from the cells in the 24-well plate and replace 300 μL of the media with the blocking agent or DMSO as a control.
4. Incubate 60 min at 37 °C.
5. Prepare virus dilutions in media for viral infection (MM warmed up at 37 °C) to a final MOI of 1 and add the appropriate concentration of the blocking agent. Gently remove the

media from the cells and replace it with the media containing the compound and the virus (100 μL).

6. Incubate for 60 min at 37 °C.
7. Gently remove the infectious media from the cells; do two very gentle washes with warm PBS.
8. Add 500 μL of warm DMEM 5% FBS media/well. Incubate for 24 h at 37 °C.
9. At 24-h post-infection, collect the supernatants in sterile plastic tubes.
10. Clarify supernatants by centrifugation at 10,000 × *g*, 10 min at 4 °C. Transfer the supernatant to a new sterile tube (*see* **Note 17**).
11. Virus yields will be determined by plaque assays in Vero cells (*see* Subheading 3.4).
12. For virus infection determination by IF, after the two washes, add 500 μL of warm DMEM 5% FBS media/well.
13. At 24-h post-infection, wash cells on the coverslips two times with warm PBS and fix and permeabilize cells for IF.
14. Follow protocol for indirect IF in Subheading 3.5 for the detection of infected cells.
15. Calculate mean of positive cells in three independent experiments as indicated in Subheading 3.5.

Pseudotype Transduction of Cells Expressing Different TfR1

1. Transfect 3T3 cells using Lipofectamine following manufacturers' recommendation. Use 500 ng of plasmid coding for transferrin receptor (*see* Subheading 2.4.2) and pEGFP-C1 as a control.
2. In parallel, transfect 3T3 cells control or stably 3T3DC- or L-SIGN with 500 ng of plasmid coding for transferrin receptors.
3. At 24 h after transfection, plate transfected cells in 24-well plates.
4. Transduce with 1×10^6 RLU of pseudotyped virion suspension (*see* Subheading 3.6) the next day at 37 °C for a minimum of 4 h.
5. Remove the inoculum and measure luciferase activity at 48 h post-transduction using a Luciferase Assay Kit (according to manufacturer's recommendation) and a GloMax 20/20 Luminometer.

3.3.3 Endocytic Pathway-Affecting Compounds

1. Plate around 1×10^5 cells (i.e., Vero, 3T3, TRVb) in a 24-well plate to 90–100% confluency per well (containing a coverslip for determination of viral infection by IF, *see* Subheading 3.5).
2. Treat with the corresponding concentration and time according to the drug (*see* Table 2) at 37 °C.

3. Remove the medium and infect with 100 μL of JUNV (MOI of 0.1) for 1 h at 37 °C in the presence of the same concentration of drug diluted in MM (*see* **Notes 18** and **19**).
4. After removing the inoculum, change the MM for fresh MM at 24 h at 37 °C.
5. Collect the supernatant to determine virus yields by PFU assay as in Subheading 3.4.
6. Wash the cells three times with PBS for 5 min, fix them with PFA 4% for 10 min at room temperature, and then permeabilize with Triton X-100 0.2% for the same period of time. Process the cells for IF assay to detect NP (*see* Subheading 3.5 and Table 1) and quantify the effect of compounds on virus entry (*see* **Notes 20** and **21**).

3.3.4 Inhibition of Virus Infection by Cytoskeleton-Disrupting Compounds

1. Plate 1×10^5 Vero cells in a 24-well plate (containing PLL-coated coverslips for determination of viral infection by IF; *see* Subheading 3.5).
2. Prepare compound solutions in sterile plastic tubes in increasing concentrations, diluted in DMEM 5% FBS (*see* Table 2).
3. Gently remove the media from the cells in the 24-well plate and replace 300 μL of media with the compounds or DMSO as a control.
4. Incubate 30 min at 37 °C.
5. Prepare virus dilutions in media for viral infection (MM warmed up at 37 °C) to a final MOI of 1 and add the appropriate concentration of the compound. Gently remove the media from the cells and replace with the media containing the compound and the virus (100 μL).
6. Incubate for 60 min at 37 °C.
7. Gently remove the infectious media from the cells; do two very gentle washes with warm PBS.
8. Add 500 μL of warm DMEM 5% FBS media/well. Incubate for 24 h at 37 °C.
9. At 24-h post-infection, collect the supernatants in sterile plastic tubes. Clarify supernatants by centrifugation at 500 × *g*, 10 min at 4 °C. Transfer the supernatant to a new sterile tube (*see* **Note 17**).
10. Virus yields will be determined by plaque assays in Vero cells (*see* Subheading 3.4).
11. Wash the cells three times with PBS for 5 min, fix them with 4% PFA for 10 min at room temperature, and permeabilize with Triton X-100 0.2% for the same period of time. Process the cells for IF assay to detect NP (*see* Subheading 3.5 and Table 1) and quantify the effect of compounds on virus entry (*see* **Notes 22** and **23**).

3.3.5 Dominant-Negative Mutations to Inhibit Virus Entry into Endosomes

1. Plate around 5×10^4 cells (i.e., Vero, 3T3) in a 24-well plate to 50–70% confluency.
2. Add 300 μL of OptiMEM solution and keep the cultures at 37 °C (*see* **Note 24**).
3. Mix 1 μg of plasmid DNA with 40 μL of OptiMEM for 10 min at room temperature (*see* **Notes 25** and **26**).
4. Mix 1 μL of Lipofectamine with 50 μL of OptiMEM per tube and incubate for 10 min at room temperature.
5. Add 50 μL of Lipofectamine mix prepared in **step 4** to each tube containing the plasmid mix in **step 3**, and incubate for 20 min at room temperature.
6. Add 100 μL of the mix containing the DNA-Lipofectamine complexes to the cells and incubate to 37 °C during 6 h. Then, replace with maintenance medium and incubate for 18 h.
7. Infect the cultures with 100 μL of JUNV (MOI of 1) for 1 h at 37 °C.
8. Remove the inoculum and add fresh MM for 24 h more.
9. Wash the cultures three times with PBS.
10. Fix the cultures with 4% PFA in PBS at room temperature for 10 min and then permeabilize them using 0.2% Triton X-100 in PBS for 10 min.
11. Process the cultures for IF assays to detect NP (*see* Subheading 3.5).

3.4 Plaque-Forming Unit (PFU) Assay

1. Collect the supernatant (1 mL) of each sample that needs PFU determination.
2. Incubate Vero cells until 80% confluency with 100 μL of serial dilutions (1/10–1/10,000) of the supernatants in MM at 37 °C for 1 h. Make duplicates for each dilution (*see* **Note 27**).
3. Remove the inoculum, wash, and add semisolid medium (*see* Subheading 2.6). Incubate for 7 days at 37 °C.
4. Fix the monolayers by adding 10% PFA for 30 min, wash exhaustively with tap water, and stain for 30 min with crystal violet solution. Wash again as before and let the plate air-dry.
5. Count the number of plaques in each well by using a bench transilluminator.
6. Calculate the PFU/mL using the following formula:

   $\text{PFU/mL} = X/V \times \text{Dil}$

   References: X, plaque number per well; V, volume of inoculum; Dil, dilution used.

3.5 Immunofluorescence Microscopy Readout

1. To prepare PLL-coated coverslips: in the tissue culture hood, put sterile round coverslips into 24-well plates. Add 0.5 mL PLL working stock/well and swirl to cover. Leave at room temperature for 20 min. Aspirate and let dry in hood with lid off.
2. Grow the cells on the PLL-coated coverslips, treat or infect them, and after the indicated time, wash three times with PBS during 5 min.
3. Fix the cultures using 4% PFA for 20 min at room temperature.
4. Add 0.2% Triton X-100 in PBS when permeabilization is required (*see* **Notes 28** and **29**).
5. Incubate the coverslips with the blocking solution (*see* Subheading 2.6) for 1 h at 37 °C (*see* **Note 30**).
6. Wash the cultures three times (5 min each wash) with PBS.
7. Incubate the cultures with the primary antibody (*see* Table 1), repeat the washing steps, and incubate with the second antibody 1 h at 37 °C (*see* Table 1).
8. Wash the cultures as in **step 7** and incubate with DAPI in PBS during 5 min.
9. Wash the cultures three times with PBS and then once using ultrapure water (5 min each wash).
10. Mount the glass coverslips with the mounting solution.
11. Calculate the mean of positive cells in three independent experiments. Quantify the number of JUNV positive cells over total cells in 20 randomly chosen optical fields for each experiment. Express values as a percentage of the corresponding control.

3.6 Production of Pseudotyped Virions

1. Plate 5×10^5 293T cells/well in 0.5 mL of DMEM 10% FBS in a 6-well plate and culture overnight at 37 °C.
2. Co-transfect 293T cells, at a ratio of 1:1:1, with an MLV-based transfer vector encoding luciferase [14], an MLV Gag-Pol packaging construct, and an envelope glycoprotein-expressing vector (pcDNA3.1-JUNVGPC) or VSV-G as a control by using TurboFect, as recommended by the manufacturer.
3. Incubate cells at 37 °C for 48 h. Collect the supernatants, filter them through 0.45 μm pore-sized membranes, and store at −80 °C. Determine titers by luciferase assay in Vero cells.

4 Notes

1. TRITC and FITC conjugates can be replaced by AF 568 or AF 488.
2. Keep it on ice while you work with PLL working solution.

3. Both DAPI and Hoescht are amenable for nuclear staining.
4. Epifluorescence or confocal microscopes are both amenable for the readout in Subheading 3.5.
5. All the solutions should be prepared in cell culture quality water.
6. Prepare 1.4% methylcellulose solution in cell culture quality water. Agitate vigorously and autoclave.
7. To test the effect of a compound on virus adsorption infect in the presence or absence of the compound.
8. During infection and antibody incubations, always keep the anti-transferrin receptor antibody in the media to study DC-SIGN-associated binding.
9. Prepare primary and secondary antibody dilutions in the blocking solution (*see* Subheading 2.2.3).
10. For IF assays, the 4% PFA must be prepared fresh.
11. For staining two components, incubate at the same time with both antibodies prepared in 4% BSA in PBS.
12. As a control for nonspecific secondary staining, perform the same protocol without adding the primary antibody. Instead, incubate cells with the blocking solution for the same period of time.
13. Alternatively use 0.5% SDS solution by pipetting the solution up and down.
14. ΔCt: calculate the difference between viral media values and cellular media values. ΔΔCt: difference between the ΔCt in the infected condition and the ΔCt of the uninfected condition.
15. Each condition needs six replicates or more, so a standard test including ten different compound concentrations and proper controls will require a complete microplate.
16. Strong pipetting should be avoided to prevent cell detachment.
17. The samples can be stored at −80 °C for later determination of virus yield.
18. For NT and MβCD which present virucidal activity, wash the cells three times with PBS and infect without the compound.
19. Stock solution of compounds used in Subheading 3.3.3 should be prepared in DMSO solvent with the exception of MβCD which can be dissolved in water. Working solutions should be prepared in MM with the exception of MβCD and NT which may be prepared in a serum-free media.
20. As a control for compound activity, use the endocytic markers instead of the virus suspension. Incubate with transferrin-TRITC (clathrin-dependent internalization) or cholera toxin-TRITC (cholesterol-dependent internalization) for 20 or 30 min each after treatments with the compounds.

Internalization of endocytic markers should be inhibited by the specific treatments and conditions.

21. Pitstops 1 and 2 are novel compounds designed to inhibit clathrin-mediated endocytosis. Pitstop 2 is used more frequently owing to its cell permeability whereas Pitstop 1 retains value as an in vitro chemical probe, and it can be used for in-cell experiments if it is introduced by microinjection [15].
22. To verify the effect of the compounds in the cell cytoskeleton, incubate the cells with phalloidin-FITC for actin filament detection or with anti-tubulin antibody for microtubule detection.
23. 90 min after the treatments with the compounds, evaluate the recovery of the actin and microtubule distribution, by IF assay.
24. Transfection protocol in Subheading 3.3.5 was done following manufacturer's recommendations.
25. For control assays, use a plasmid with the same backbone expressing GFP.
26. To control the effect of dominant-negative constructions, incubate the transfected cells with transferrin-TRITC for 20 min. The transferrin internalization should be inhibited in cells expressing the dominant-negative proteins.
27. Do not allow the cells to dry out and agitate inoculum by swirling the plate every 15 min.
28. For membrane IF studies, do not use **step 4** in Subheading 3.5.
29. By **step 4** it is possible to save the plate containing the fixed cultures at −20 °C during 1 week. Make sure they were completely dried before freezing them.
30. During culture incubations with antibodies or blocking solutions, use humid incubator chambers to prevent evaporation of the antibody solution.

References

1. Cordo SM, Cesio y AM, Candurra NA (2005) Polarized entry and release of Junín virus, a new world arenavirus. J Gen Virol 86: 1475–1479
2. Martinez MG, Cordo SM, Candurra NA (2008) Involvement of cytoskeleton in Junín virus entry. Virus Res 138:17–25
3. Martinez MG, Cordo SM, Candurra NA (2007) Characterization of Junín arenavirus cell entry. J Gen Virol 88:1776–1784
4. Martinez MG, Forlenza MB, Candurra NA (2009) Involvement of cellular proteins in Junín arenavirus entry. Biotechnol J 4:866–870
5. Contigiani MS, Sabattini MS (1977) Virulencia diferencial de cepas de virus Junín por marcadores biológicos en ratones y cobayos. Medicina (B Aires) 37:244–251
6. Martinez MG, Bialecki MA, Belouzard S, Cordo SM, Candurra NA, Whittaker GR (2013) Utilization of human DC-SIGN and L-SIGN for entry and infection of host cells by the new world arenavirus, Junín virus. Biochem Biophys Res Commun 441:612–617
7. Damonte EB, Mersich SE, Candurra NA (1994) Intracellular processing and transport of Junín virus glycoproteins influences virion infectivity. Virus Res 34:317–326
8. Sanchez A, Pifat D, Kenyon RH, Peters CJ, McCormick JB, Kiley MP (1989) Junín virus monoclonal antibodies: characterization and

cross-reactivity with other arenaviruses. J Gen Virol 70:1125–1132

9. Damonte EB, Mersich SE, Candurra NA, Coto CE (1986) Cross reactivity between Junín and Tacaribe viruses as determined by neutralization test and immunoprecipitation. Med Microbiol Immunol 175:85–88
10. Castilla V, Mersich SE, Damonte EB (1991) Lysosomotropic compounds inhibiting the multiplication of Junín virus. Rev Argent Microbiol 23:86–89
11. Benmerah A, Bayrou M, Cerf-Bensussan N, Dautry-Varsat A (1992) Inhibition of clathrin-coated pit assembly by an EPS15 mutant. J Cell Sci 112:1303–1311
12. Damke H, Baba T, Warnock DE, Schmid SL (1994) Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. J Cell Biol 127:915–934
13. Li G, Stahl PD (1993) Structure-function relationship of the small GTPase rab5. J Biol Chem 15:24475–24480
14. Negre D, Mangeot PE, Duisit G, Blanchard S, Vidalain PO, Leissner P, Winter AJ, Rabourdin-Combe C, Mehtali M, Moullier P, Darlix JL, Cosset FL (2000) Characterization of novel safe lentiviral vectors derived from simian immunodeficiency virus (SIVmac251) that efficiently transduce mature human dendritic cells. Gene Ther 7:1613–1623
15. Robertson MJ, Deane FM, Stahlschmidt W, von Kleist L, Haucke V, Robinson PJ, McCluskey A (2014) Synthesis of the Pitstop family of clathrin inhibitors. Nat Protoc 9:1592–1606