Guinea pig transferrin receptor 1 mediates cellular entry of Junín virus and other pathogenic New World arenaviruses

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**Running Head:** Role of TfR1 in JUNV infection in guinea pigs

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Abstract

Several clade B New World arenaviruses (NWAs) can cause severe and often fatal hemorrhagic fever, for which preventive and therapeutic measures are severely limited. These NWAs use human transferrin receptor 1 (hTfR1) as a host cell receptor for virus entry. The most prevalent of the pathogenic NWAs is Junín virus (JUNV), the etiological agent of Argentine hemorrhagic fever. Small animal models of JUNV infection are limited because most laboratory rodent species are refractory to disease. Only guinea pigs are known to develop disease following JUNV infection, but the underlying mechanisms are not well characterized. In the present study, we demonstrate marked susceptibility of Hartley guinea pigs to uniformly lethal disease when challenged with as few as 4 plaque-forming units of the Romero strain of JUNV. In vitro, we show that infection of primary guinea pig macrophages results in greater JUNV replication compared to infection of hamster or mouse macrophages. We provide evidence that the guinea pig TfR1 (gpTfR1) is the principal receptor for JUNV, while hamster and mouse orthologs fail to support viral entry/infection of pseudotyped murine leukemia viruses expressing pathogenic NWA glycoproteins or JUNV. Together, our results indicate that gpTfR1 serves as the primary receptor for pathogenic NWAs, enhancing viral infection in guinea pigs.

Importance

JUNV is one of five known NWAs that cause viral hemorrhagic fever in humans. Countermeasures against JUNV infection are limited to immunization with the Candid#1 vaccine and immune plasma, which are available only in Argentina. The gold-standard small animal model for JUNV infection is the guinea pig. Here, we demonstrate high sensitivity of this species to severe JUNV infection and identify gpTfR1 as the primary receptor. Use of hTfR1 for host
cell entry is a feature shared by pathogenic NWAs. Our results show that expression of gpTfR1 or hTfR1 comparably enhances JUNV virus entry/infectivity. Our findings shed light on JUNV infection in guinea pigs as a model for human disease and suggest that similar pathophysiological mechanisms related to iron sequestration during infection and regulation of TfR1 expression may be shared between humans and guinea pigs. A better understanding of the underlying disease process will guide development of new therapeutic interventions.

Keywords: Arenavirus, Junín virus, transferrin receptor 1, viral hemorrhagic fever, guinea pig

Introduction

*Mammarenaviruses* are bisegmented, negative-sense, single-stranded, rodent-borne RNA viruses divided into two lineages: Old World mammarenaviruses (OWAs) and New World mammarenaviruses (NWAs), with each lineage containing viruses that can cause severe disease in humans. The most prevalent of these viruses is Lassa virus (LASV), an OWA that infects an estimated 300,000 people per year resulting in approximately 5,000 deaths annually (1). The NWAs are divided into four clades A, B, C, and D. To date, all the known pathogenic NWAs belong to clade B and cause viral hemorrhagic fever. These viruses are: Junín (JUNV), Guanarito (GTOV), Machupo (MACV), Sabiá (SBAV), and Chapare (CHPV), causing Argentine, Venezuelan, Bolivian, Brazilian, and Chapare hemorrhagic fever, respectively. Treatment and preventive measures for pathogenic NWA infections are available only for Junín virus and are limited to the use of convalescent plasma and the Candid#1 vaccine, which are only approved for human use in Argentina (2).
Host cell entry of arenaviruses is mediated by the viral envelope glycoprotein (GP) complex consisting of three noncovalently linked subunits, GP1, GP2 and a stable signal peptide (SSP) (3). GP1 contains the receptor binding site, GP2 is a class I fusion protein, and SSP is involved in glycoprotein maturation and membrane fusion. Host cellular receptor usage for viral attachment and internalization differs between arenaviruses. OWAs and the clade C NWAs use alpha-dystroglycan (4, 5), while the receptor(s) used by the clade A and D NWAs are currently unknown. The clade B NWAs use their respective host species transferrin receptor 1 (TfR1) ortholog for viral attachment and internalization, with a high degree of species specificity (6, 7). An important shared feature of the pathogenic NWAs is the ability to use human TfR1 (hTfR1) as the primary receptor for virus entry (6-8). Characterization of the interaction between the GP complex of pathogenic NWAs and hTfR1 has identified the apical domain of TfR1, and more specifically the tyrosine residue at position 211, as essential for viral GP attachment (7).

While several species of non-human primates have been shown to be susceptible to disease caused by pathogenic NWA (9-12), few small animal models are currently available because most commonly used laboratory rodent species are refractory to severe disease. To date, guinea pigs, immunocompromised mice and suckling mice and rats have been shown to be susceptible to disease following pathogenic NWA challenge (reviewed in (13, 14)). For JUNV, the gold-standard small animal model is the Hartley guinea pig (15). This model has been used to evaluate a limited number of therapeutics and vaccines; however, the underlying mechanisms rendering the Hartley guinea pig susceptible to lethal JUNV challenge have not been fully characterized. In the present study, we demonstrate marked susceptibility of guinea pigs to JUNV and identify guinea pig TfR1 (gpTfR1) as the principal host cell receptor mediating viral entry of pathogenic NWAs. We also show that JUNV infection of primary guinea pig
macrophages results in greater viral replication compared to macrophages isolated from mice and hamsters, which are species that are refractory to disease. Taken together, our findings provide new insights into guinea pig susceptibility to JUNV infection and disease, and support the use of the guinea pig model to investigate TfR1 regulation linked to iron sequestration that occurs during infection, as a means to control JUNV replication.

**Results**

**Susceptibility of guinea pigs to JUNV infection.** Hartley guinea pigs have been shown to be highly susceptible to infection with several pathogenic strains of JUNV (15-17), however, the 50% lethal dose (LD$_{50}$) has not been defined. To further evaluate the susceptibility of guinea pigs to the Romero strain of JUNV (JUNV-R), we challenged animals with ten-fold serial dilutions of virus stock ranging from 4 to 4,000 plaque-forming units (PFU) by intraperitoneal (IP) injection. Uniform lethality was observed in all infected groups with the animals exposed to the highest challenge dose succumbing earlier during the course of the study (Figure 1A). Weight gain in some of the infected animals started to plateau at approximately 8 days post-infection (p.i.), followed by precipitous weight loss as the condition of the animals rapidly declined during the terminal stages of disease (Figure 1B). The effects on body weight lagged behind the onset of the febrile response with the guinea pigs challenged with 40 to 4,000 PFU spiking fevers as early as day 6, and the 4 PFU challenge group progressing more gradually to elevated body temperatures, followed by a rapid decline as the animals reached a moribund stage requiring euthanasia (Figure 1C). The results demonstrate the marked susceptibility of Hartley guinea pigs to lethal JUNV-R infection with the LD$_{50}$ estimated to be 1.27 PFU.
**Susceptibility of guinea pig macrophages to JUNV infection.** Macrophages are believed to be an important cell population targeted by JUNV (18, 19). Infection of peritoneal macrophages is likely to be a key step in the process towards the marked susceptibility of guinea pigs to severe disease. To evaluate the susceptibility of macrophages to JUNV infection, resident macrophages of guinea pigs, hamsters and mice were collected by peritoneal cavity lavage and inoculated with JUNV-R and Tacaribe virus (TCRV, non-pathogenic clade B NWA) for comparison. As shown in Figure 2A, JUNV-R infection of guinea pig macrophages resulted in a persistent amplification of the virus measured longitudinally in culture supernatant. In contrast, JUNV-R replicated significantly less efficiently in hamster and mouse macrophages. Virus replication in TCRV-infected guinea pig, hamster and mouse macrophages was comparable across most of the time points (Figure 2B). The data suggest that replication in primary macrophages by the pathogenic JUNV-R may be a key to lethal infection in guinea pigs, as both immune competent mice (20) and hamsters (Figure 3) are refractory to disease.

**The apical domain of gpTfR1 contains a putative entry determinant for pathogenic NWA GPs.** Because of the susceptibility of guinea pigs to JUNV-R challenge and the greater replicative capacity of JUNV-R in guinea pig primary macrophages, we hypothesized that gpTfR1 serves as the main receptor that mediates efficient host cell entry of JUNV. Previous studies have shown that JUNV and other related pathogenic NWAs use hTfR1 for host cell entry and a tyrosine residue at position 211 of the apical domain is essential for GP1 binding and viral entry (7). Sequence alignment comparing gpTfR1 to published TfR1 amino acid sequences of *Calomys musculinus*, the natural host of JUNV, and to TfR1 ortholog amino acid sequences of several other species known to be susceptible to severe disease following JUNV challenge,
shows a high degree of apical domain homology including the presence of the critical tyrosine residue at position 211 of hTfR1 (Table 1). In contrast, common laboratory rodent species refractory to JUNV infection and disease were divergent in their apical domain amino acid sequence.

**Expression of gpTfR1 in hamster cells results in efficient entry by pseudotyped retroviruses expressing pathogenic NWA GPs.** To investigate whether gpTfR1 mediates viral entry of pathogenic NWAs, we used pseudotyped reporter retroviruses displaying various arenaviral GPs to measure viral entry facilitated by different TfR1 orthologs of interest. Human, guinea pig, hamster, and mouse TfR1 orthologs were expressed in Chinese hamster ovary cells (CHO-K1) and the cells were exposed to murine leukemia virus (MLV)-based pseudoviruses displaying the GPs of JUNV, TCRV, GTOV, MACV, lymphocytic choriomeningitis virus (LCMV, prototypical OWA) or LASV. Pseudovirus entry was assessed by measuring eGFP reporter expression 48 h after infection. As shown in Figure 4, only CHO-K1 cells expressing gpTfR1 or hTfR1 were highly susceptible to entry by pseudotyped MLVs displaying JUNV, GTOV or MACV GPs, but not TCRV, LCMV or LASV. Equivalent expression of the TfR1 orthologs was confirmed in parallel by assessment of immunofluorescence in transfected CHO-K1 cells through detection of the FLAG tag engineered into the C-terminus of the TfR1 orthologs (Figure 5A). The data indicate that expression of gpTfR1 enhances pathogenic NWA cellular entry comparable to hTfR1.

**JUNV infection of hamster cells expressing gpTfR1 results in increased viral attachment and replication.** To evaluate the role of gpTfR1 on viral attachment and replication of native
NWAs, transfected CHO-K1 cells expressing hTfR1, gpTfR1, hamster (ham)TfR1, or mouse (m)TfR1 were exposed to the Candid#1 strain of JUNV (JUNV-C1) or TCRV. Considering safety and constraints associated with Select Agent research, we performed native virus attachment and replication experiments using JUNV-C1 (BSL-2, non-Select Agent). Although highly attenuated (avirulent in guinea pigs), JUNV-C1 retains the ability to efficiently use hTfR1 for entry into human cells similar to that observed for pathogenic strains of JUNV (21). Viral attachment was measured by infecting cells at 4°C to prevent endocytosis, washing away unbound virus, releasing bound virions by freezing-thawing, and titrating the cell lysates on Vero cells. Expression of gpTfR1 or hTfR1 resulted in substantial JUNV attachment, with low levels of virus present in samples collected from hamTfR1, mTfR1 or mock-transfected cells (Figure 6A). As expected, there was no difference in attachment by TCRV (Figure 6C), consistent with our pseudovirus assay findings (Figure 4). To measure the impact of gpTfR1 expression on JUNV-C1 infection, supernatants were collected from parallel cultures of TfR1- or mock-transfected CHO-K1 cells exposed to JUNV-C1 or TCRV. After washing away unbound virions, cells were cultured for 48 h at 37°C and 5% CO₂ and supernatants titrated for infectious virus. As shown in Figure 6B, expression of gpTfR1 or hTfR1 significantly enhanced JUNV replication compared to mock-transfected controls. As observed with viral attachment, there were no differences in the levels of TCRV replication in the various TfR1-expressing and mock-transfected cultures (Figure 6D). These data indicate that expression of gpTfR1 enhances native JUNV attachment and infection.

**Expression of gpTfR1 or hTfR1 in refractory MDBK cells results in a productive JUNV infection.** Unlike CHO-K1 cells where non-TfR1-mediated JUNV-C1 entry can be measured,
Madin-Darby bovine kidney (MDBK) cells are completely refractory to infection by JUNV-C1 either due to lack of a functional receptor(s) or because the cells are not permissive to replication following virus entry. To further evaluate the role of gpTfR1 and hTfR1 in JUNV entry, MDBK cells stably expressing different TfR1 orthologs were exposed to JUNV-C1 or TCRV and viral attachment and replication were assessed as described above. JUNV only attached to MDBK cells expressing gpTfR1 or hTfR1 (Figure 7A) and viral replication was supported (Figure 7B) suggesting that the block in JUNV-C1 infection of MDBK cells is due to the lack of a suitable receptor rather than the replicative capacity of the cell. Comparable expression of the various TfR1 orthologs was verified in parallel transfections in MDBK cells by detection of the FLAG tag present at the C-terminus of the receptors (Figure 5B). No differences in TCRV attachment or replication were observed in the various MDBK cell lines expressing different TfR1 proteins (Figure 7C, D). The results demonstrate that expression of gpTfR1 or hTfR1 is necessary and sufficient to render MDBK cells susceptible to productive JUNV-C1 infection.

**Knockout (KO) of TfR1 results in reduced JUNV entry, attachment and replication.** We next investigated the impact of knocking out TfR1 to determine whether infection of guinea pig or human cells devoid of TfR1 would be less capable of supporting viral infection. Using the CRISPR/Cas9 system, we induced 8 and 34 nucleotide deletions in TfR1 exon 3 in JH4 (guinea pig lung) and HEK 293T (human kidney) cell lines, respectively, resulting in frameshift mutations and introduction of premature stop codons (Figure 8). Entry of pseudoviruses expressing pathogenic NWA GPs was significantly impaired in JH4 and HEK 293T TfR1 KO (TfR1−/) cells (Figure 9A and B). To further assess the contribution of TfR1-mediated entry on JUNV infection in the TFR1 KO guinea pig and human cell lines, the JH4 and HEK 293T TfR1−/−
cells were transfected to express TfR1 orthologs or mock-transfected. TfR1 KO cells complemented with hamTfR1 and mTfR1 failed to support entry by JUNV, GTOV, MACV, TCRV, LCMV or LASV pseudoviruses (Figure 9A and B). In contrast, entry of MLVs expressing pathogenic NWA GPs was fully restored to levels comparable to wild-type (WT) controls in JH4 and HEK 293T TfR1−/− cells complemented with gpTfR1 or hTfR1.

To measure how the absence of TfR1 affects native NWA attachment, TfR1−/− JH4 and HEK 293T cells were transfected to express TfR1 orthologs or mock transfected as described above. Then, along with their WT counterparts, the various transfectants were exposed to JUNV-C1 or TCRV and viral attachment and replication were measured. JUNV-C1 attachment to the TfR1−/− JH4 and HEK 293T cells was significantly reduced compared to the respective WT cell lines (Figure 9C. However, the decreased JUNV-C1 attachment was restored by complementation with gpTfR1 or hTfR1. Viral replication in the WT TfR1−/− JH4 and HEK 293T cells was consistent with the attachment results showing markedly reduced viral loads in cells not complemented with gpTfR1 or hTfR1 (Figure 9D. Similar expression levels of the selected TfR1 orthologs were confirmed in parallel transfections of HEK 293T and JH4 TfR1−/− cells by detection of the C-terminal FLAG present in the receptors (Figure 5C and 5D).

**Discussion**

TfR1 has been identified as the principal receptor that mediates human host cell entry by pathogenic clade B NWAs (8). Furthermore, binding of the viral GP to a specific TfR1 ortholog appears to define whether a species is susceptible to severe disease or may serve as a reservoir for the clade B NWAs (6, 22). The specific region of hTfR1 that is essential for the binding of MACV GP has been mapped (7). Based on sequence homology with hTfR1 (Table 1) and the
marked susceptibility of guinea pigs to JUNV Romero (Figure 1), we hypothesized that gpTfR1 mediates efficient entry by pathogenic NWAs. Our investigation employing pseudotyped and native virus assays, as well as gene deletion and complementation strategies, establishes gpTfR1 as the principal receptor facilitating entry by pathogenic NWAs in guinea pigs. Moreover, we observed that expression of gpTfR1 enhances entry mediated by pathogenic NWA GPs comparable to the levels conferred by hTfR1 expression. Our findings also show that JUNV is able to infect guinea pig and hamster cells through non-TfR1 pathways as previously described for mice (22, 23). While entry by non-TfR1 mechanisms is likely a less efficient process and these secondary receptor(s) may not be present on target cell populations that play a central role in infection and disease in an adult immunocompetent rodent, the alternative entry pathways appear to be sufficient for JUNV to productively infect and cause lethal disease in suckling and immunocompromised mice (14, 20, 24, 25). TCRV, which infects guinea pig, mouse and hamster cells independent of TfR1, been shown to induce higher levels of antiviral type I interferon (IFN) responses through more potent activation of protein kinase R (PKR) (26), which may contribute to its inability to cause disease in rodents other than newborn or type I and type II IFN receptor-deficient mice (27, 28).

Infection of guinea pigs with certain strains of JUNV, MACV or GTOV results in 100% lethality, whereas limited mortality is observed with other strains of JUNV and MACV, as well as SBAV (10, 15, 16, 29-37). Thus, while the use of TfR1 results in enhanced infection by hemorrhagic fever NWAs, variability in the outcome of guinea pig infection following challenge with different strains of JUNV and MACV indicate that other factors beyond entry are also important. This is underscored by inapparent disease in guinea pigs challenged with the Candid#1 strain of JUNV, which enters efficiently through gpTfR1, but contains attenuating
mutations affecting stages of the virus replication cycle beyond the viral GP-host TfR1 interaction. It is also unclear what factors dictate whether infection via TfR1 result in a transient or persistent infection. While infection of humans, non-human primates and guinea pigs result in an acute disease course marked by viral clearance in survivors, infection of adult rodent host species often results in a persistent carrier state (38, 39). The different infection outcomes are likely due to the type of host immune response mounted against the infection coupled with the ability of the virus to subvert the response. The importance of the type I and type II IFN responses has been established (20, 40, 41) and viral interference with these responses likely contributes to persistence in reservoir hosts. Additional work is needed to characterize the nature of immune responses during transient infection and disease in susceptible species, and in viral persistence underlying the carrier state in reservoir hosts.

The biology and regulation of TfR1 may favor the virus during the natural host response to infection. As the main receptor that mediates the endocytosis of iron, TfR1 expression is tightly regulated by intracellular iron content (42). During periods of adequate iron supply, TfR1 is expressed at low levels on most cell types (43). Upregulation of TfR1 generally occurs in two ways. First, cells undergoing rapid division, differentiation, or activation, such as immune cells responding to infection, upregulate TfR1 expression to meet the increased demand for iron (44-47). Second, low serum iron levels, as seen in individuals initiating inflammatory responses, result in increased TfR1 expression from iron-starved cells. This condition, often termed “hypoferremia of inflammation”, is thought to be an evolutionary innate immune response to deprive iron-dependent pathogens and is orchestrated by the expression of the proinflammatory interleukin-6 (IL-6) (48, 49). Produced during the initial stages of inflammation, a primary effector function of IL-6 is to stimulate hepatic cells to produce hepcidin, a key iron regulatory
peptide (48). Binding of hepcidin to the iron export protein ferroportin inhibits dietary absorption of iron and the release of iron stores from hepatocytes and macrophages (50). In turn, extracellular pools of iron are depleted by continued demand from non-hepatic cells, resulting in increased TfR1 expression. Indeed, it has been shown that periods of iron deficiency correlate with increased tissue TfR1 expression (51, 52). Notably, increased circulating levels of IL-6 have been documented in severe cases of Argentine hemorrhagic fever (53). These scenarios could enhance infection by viruses that utilize TfR1 as an entry receptor, such as the pathogenic NWAs, by inducing expression of TfR1 by iron-deprived cells and immune system cells undergoing activation, resulting in greater availability of viral receptors.

The regulation of TfR1 expression linked to iron concentration provides a potential vulnerability that could be exploited to counter hemorrhagic fever of NWA etiology. However, therapeutic manipulation of tightly regulated serum and tissue iron levels, or interference of IL-6 or hepcidin production to reduce the expression of TfR1, presents significant challenges as it has yet to be reported whether such a strategy would result in the downregulation of TfR1 \textit{in vivo}. Proving that TfR1 can be downregulated in guinea pigs would pave the way towards therapeutic efficacy studies investigating this novel host-directed approach to lessen NWA infection and hemorrhagic fever disease. Taken together, our findings establish gpTfR1 as the primary receptor facilitating the host cell entry of pathogenic NWAs, supporting the translatability of guinea pig infection models to human infection and disease (8). Thus, the use of the guinea pig model for Argentine hemorrhagic fever may be a viable option for the assessment of promising therapies based on the management of iron, IL-6, or hepcidin as a mechanism to reduce the density of viral receptors and mitigate disease.
Materials and Methods

Ethics statement. All animal procedures complied with USDA guidelines and were conducted at AAALAC-accredited laboratory animal research facilities at Utah State University under protocol #10088, approved by the Utah State University Institutional Animal Care and Use Committee.

Animals. Outbred male Hartley guinea pigs (300-350 g) were obtained from Charles River (Wilmington, MA) and acclimated for one week prior to challenge or collection of peritoneal macrophages. IPTT-300 electronic transponders were implanted subcutaneously for identification and temperature measurement in conjunction with the DAS 6002 scanner (BMDS, Seaford, DE).

Cells, plasmids, and viruses. Human embryonic kidney 293T/17 (HEK 293T), Chinese hamster ovary (CHO-K1), guinea pig lung (JH4) and African green monkey kidney (Vero) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; HEK 293T), F-12 medium (CHO-K1 and JH4) or minimal essential medium (MEM; Vero) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). Plasmids encoding human and mouse TfR1 fused with a C-terminus FLAG tag were kindly provided by Sheli Radoshitzky (USAMRIID, Ft. Detrick, MD). TfR1 genes for guinea pig and hamster were cloned from their corresponding liver tissues. Briefly, RNA was isolated from liver tissues using the RNeasy kit (Qiagen, Hilden, Germany) and cDNA was generated using the SuperScript IV VILO Master Mix system (Thermo Fisher Scientific, Waltham, MA). PCR primers used to amplify the genes for gpTfR1: sense 5’-
AACTGGATCCGCCACCATGATGAATCAAGCCAGAGCCACG-3’ and anti-sense 5’-ACAACTCTCGAGAAACTCATTGTCAATATCCCAAATGTC-3’, and for hamTfR1: sense 5’-AACTGGATCCGCCACCATGATGATCAAGCCAGTCAGCA-3’ and anti-sense 5’-ACAACTCTCGAGAAACTCATTGTCAATATCCCAAATGTC-3’. The genes were then cloned into the pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA) which contained a FLAG tag on the C-terminus of the gene insert. The final constructs were verified by Sanger sequencing. Plasmids encoding for viruses pseudotyped with full-length GPC of JUNV (MC2; X15827.1), TCRV (TRVL 11573; M20304.1), MACV (Carvallo; KM198592.1), GTOV (INH-9551; AY129247.1), LCMV (Armstrong; KY514256.1) or LASV (Josiah; HQ688672.1) were kindly provided by Jonathan Abraham (Harvard Medical School, Boston, MA).

The Candid#1 vaccine strain of Junín virus (JUNV-C1; 1 passage in BSC-1, 2 passages Vero) was kindly provided by Robert Tesh (World Reference Center for Emerging Viruses and Arboviruses, University of Texas Medical Branch, Galveston, TX). The molecular cDNA clone of the Romero strain of JUNV (JUNV-R) was provided by Slobodan Paessler (University of Texas Medical Branch, Galveston, TX). JUNV-R was rescued in BHK-21 cells as previously described (54) and the stock used was prepared from a single passage in Vero cells. All work with JUNV-R was performed by Candid#1-vaccinated personnel in Select Agent-approved, enhanced BSL-3+ laboratories at Utah State University. Tacaribe virus (TCRV, strain TRVL 11573) was obtained from ATCC. The TCRV stock was generated from 2 passages in Vero cells.

**Guinea pig challenge dose titration study.** Guinea pigs were weighed on the day of infection and grouped to minimize weight differences across experimental groups (n=4 per virus challenge.
dose, n=2 for sham-infected controls). The animals were inoculated by IP injection of 0.1 ml containing 4, 40, 400 or 4,000 PFU of JUNV-R or sham infected with MEM. The guinea pigs were observed for 19 days for morbidity and mortality following JUNV-R challenge. Animal weights and body temperatures were recorded daily. Animals that lost 20% or more of body weight relative to their starting weight or which had a body temperature decline of 4°C or more relative to their baseline temperature were humanely euthanized.

**Collection and infection of peritoneal macrophages.** Peritoneal macrophages were harvested from guinea pig, hamster and mouse as previously described (55). Briefly, animals were euthanized by CO₂ asphyxiation and the peritoneal cavity was lavaged with phosphate-buffered saline (PBS). The lavage cells were washed and resuspended in RPMI (HyClone), counted and 10⁶ cells were seeded into 24-well plates and allowed to attached for 2 h. Unbound cells were washed away and the adherent cell populations were overlayed with RPMI supplemented with 10% FBS and 10 µg/ml gentamicin and incubated at 37°C, 5 % CO₂ for 48 h.

For the virus infections, the media was removed from the 48 h cultures and RPMI containing JUNV-R or TCRV was added. Following a 1.5 h incubation, the cells were washed and 1 ml of RMPI containing 2% FBS was added for extended incubation at 37°C, 5 % CO₂. Viral replication was assessed by removing 40 µl samples of culture supernatant every 24 h p.i. for 4 days and titration on Vero cells.

**Sequence comparison.** The Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) was used to align the TfR1 apical domain sequences from several species. The sequences used in the analysis were: *Calomys musculinus*
(Drylands vesper mouse, GenBank accession no. EU255275.1); Homo sapiens (Human, NM_001128148.3); Macaca mulatta (Rhesus macaque, NM_001257303.1); Callithrix jacchus (Common marmoset, NM_001301847.1); Cavia porcellus (Guinea pig, NM_001251822.1); Mesocricetus auratus (Syrian golden hamster, XM_005071554.3); Cricetulus griseus (Chinese hamster, NM_001246819.1); Rattus norvegicus (Norway rat, NM_022712.1); Mus musculus (House mouse, AJ426432.1).

**Pseudovirus production and entry assay.** The production of the recombinant retroviruses pseudotyped with arenaviral GPC has been described previously (6, 7). Briefly, HEK 293T cells were transfected using Lipofectamine (Thermo Fisher Scientific, Waltham, MA) at a 1:1:1 ratio of three plasmids: 1) a plasmid encoding the murine leukemia virus gag and pol genes, 2) a pCAGGS expression vector encoding arenaviral GPs (JUNV, GTOV, MACV, TCRV, LCMV or LASV), and 3) the pQCXIX retroviral vector (BD Biosciences, San Jose, CA) coding for enhanced green fluorescent protein (eGFP). Pseudovirus-containing cell culture supernatant was harvested 48 h post-transfection and filtered through a 0.45-μm filter. To quantify inoculation doses, 0.1 ml of pseudovirus-containing supernatant was added to HEK 239T cells for 1 h before washing and overlay with fresh culture media containing 2% FBS. Cells were incubated for 48 h, at which time eGFP expression was quantified by measuring fluorescence intensity from the wells. Inoculation doses for subsequent pseudovirus entry experiments were normalized to eGFP expression.

To study the effect of TfR1 expression on arenaviral entry, CHO-K1 cells were transfected with the pcDNA3.1 plasmid encoding the human, guinea pig, mouse or hamster TfR1
or mock-transfected using Lipofectamine 2000 (Thermo Fisher). Twenty-four h after transfection, the cells were split and either seeded in 24-well plates for TfR1 expression analysis or 96-well plates for pseudovirus infection. TfR1 expression levels were assessed at 48 h post-transfection by incubation of the transfected cells with an anti-FLAG M2-fluorescein isothiocyanate (FITC) antibody and measuring FITC expression by fluorometric analysis (Synergy HT; BioTek, Winooski, VT). TfR1 expression levels were normalized to that of hTfR1-transfected cells. In parallel, transfected cells in 96-well plates were infected with the pseudoviruses for 3 h, then washed and overlayed with fresh culture medium and incubated at 37°C, 5 % CO2. Expression of eGFP was quantified by fluorometric analysis after a 48 h incubation period by measuring fluorescent intensity and expressed as percent relative entry compared to mock transfected cells. In addition, to allow comparison of absolute and relative entry measurements, eGFP expression expressed in relative fluorescent units (RFUs) is shown in Figure 4B.

**Measurement of arenavirus attachment and replication in cells expressing rodent and human TfR1 orthologs.** For measurement the of viral attachment, endocytosis (virus internalization) was inhibited by cooling WT and TfR1 transfected cells to 4°C prior to the addition of media containing either JUNV-C1 or TCRV at a multiplicity of infection (MOI) of 0.01. Cells were incubated at 4°C for 1.5 h, then washed 3 times with ice-cold PBS and the attached virions released by lysing the cells by cooling to -80°C and warming to room temperature. The thawed cell lysates were serially diluted and the fifty percent cell culture infectious dose (CCID50) was determined by titration on Vero cells as previously described (56). To measure the effect of TfR1 expression on viral replication and production, parallel cultures of
transfected cells were infected with the same MOI of 0.01 of JUNV-C1 or TCRV and incubated at 37°C, 5 % CO₂ for 1.5 h. The cells were then washed to remove free virions, provided with fresh culture media containing 2% FBS and incubated at 37°C, 5 % CO₂. Culture supernatant was removed at 48 h p.i. and titrated on Vero cells using the same infectious virus endpoint dilution assay.

**Transfection of MDBK cells with TfR1 orthologs and measurement of viral replication.** We have found MDBK cells to be refractory to JUNV-C1 infection. To assess whether expression of guinea pig or human TfR1 can confer susceptibility to JUNV infection, MDBK cells were transfected with the various TfR1 ortholog-encoding plasmids as described above. Due to the low transfection efficiency of 10-20%, G418 was applied to select for a population of cells that expressed the transfected TfR1. After selection, equivalent TfR1 expression levels were verified as described above for the CHO-K1 cell transfections and the transfected MDBK cells were infected with either JUNV-C1 or TCRV at a MOI of 0.01, extensively washed to remove unbound virions, and provided with fresh MEM culture media containing 2% FBS and 10 μg/ml gentamicin. Culture supernatants collected at 48 h p.i. were titrated by endpoint dilution to determine viral loads.

**CRISPR/Cas9 functional knockout of TfR1 and restoration of JUNV infection by TfR1 complementation.** HEK 293T and JH4 cells were gene edited using the CRISPR/Cas9 system. A single synthetic guide RNA: GAGAACCAUUGUCAUAUACC, targeting exon 3 of the human and guinea pig TfR1 was designed using the CRISPR design tool from Synthego (Redwood City, CA). Cas9 guide RNA complexes were assembled as per the manufacture
instructions and transfected using the P3 Primary Cell 4D Nucleofector X Kit L and an Amaxa 4D-Nucleofector (Lonza, Allendale, NJ). To assess gene editing, restriction fragment length polymorphism (RFLP) was performed on genomic DNA isolated from colonies derived from single cell isolates. Briefly, PCR amplicons of the target TfR1 region were generated using the following primers: Human: forward: 5’-ACAGACTTCCAGAGTTGGT-3’ and reverse: 5’-GCCCAAGGAAGAATGTTGGT-3’, Guinea pig: forward: 5’-GCTGCTTGGAAAGGGGATCT-3’ and reverse: 5’-TTCCTCCTTGCACAATGGCA-3’.

Cycling conditions consisted of 94°C for 4 min, followed by 34 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, and a final extension for 5 min at 72°C. To assess if insertion or deletion (INDEL) of nucleotide base pairs occurred at the target site, the PCR amplicons were then digested with MspI enzyme according the manufacturer instructions (New England Biolabs, Ipswich, MA). PCR amplicons from colonies present for INDELS were then sequenced (ACGT Inc., Wheeling, IL) to determine if a frameshift had occurred. Colonies in which a frameshift was present were used for the subsequent experiments.

To assess the impact of the gpTfR1 and hTfR1 functional KO on JUNV-C1 infection, gene edited JH4 and HEK 293T cells were evaluated for virus attachment and replication as described in the previous sections. The TfR1<sup>−/−</sup> cells were also transfected with plasmids encoding human, guinea pig, hamster or mouse TfR1, or mock-transfected and comparable TfR1 expression levels were confirmed as described above for the CHO-K1 and MDBK cell transfections. WT and TfR1<sup>−/−</sup> HEK 293T and JH4 cell lines were used to evaluate GP-mediated virus entry using pseudotyped viruses, and native virus attachment and replication, as described.
in the previous sections. In experiments using pseudotyped viruses, measurements in RFU are included in Figure 9B.

**Statistical analysis.** A one-way analysis of variance (ANOVA) with Dunnett’s posttest to correct for multiple comparisons was used to compare differences in pseudovirus entry and viral titers. All statistical evaluations were performed using Prism 8 (GraphPad Software, La Jolla, CA).

**Acknowledgements**

We are grateful to Johnathan Abraham (Harvard University, Cambridge, MA) for providing the arenavirus glycoprotein pseudotyped murine leukemia virus plasmids and Sheli Radoshitzky (USAMRIID, Ft. Detrick, MD) for the human and mouse Tfr1 expression plasmids. We thank Eric Sefing, Luci Wandersee, Kevin Bailey, Brayden Stembridge and Joseph Hawkes for their technical support and are indebted to Korry Hintze for helpful discussions regarding Tfr1.
Table 1. Amino acid sequence comparison of selected species TfR1 apical domain.

<table>
<thead>
<tr>
<th>Species (Common Name)</th>
<th>TfR1 Apical Loop Amino Acid Sequence</th>
<th>Ascension No. (GenBank)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>JUNV host</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. musculinus</em> (Drylands vesper mouse)</td>
<td>206-tgd-sYlvenp-217</td>
<td>ABX54883.1</td>
</tr>
<tr>
<td><strong>Susceptible to severe JUNV disease</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. Sapiens</em> (Human)</td>
<td>205-ngrlvYlvenp-217</td>
<td>NP_001121620.1</td>
</tr>
<tr>
<td><em>M. mulatta</em> (Rhesus macaque)</td>
<td>205-ngglvYlvenp-217</td>
<td>NP_001244232.1</td>
</tr>
<tr>
<td><em>C. jacchus</em> (Common marmoset)</td>
<td>205-nsefvYlvenp-217</td>
<td>NP_001288776.1</td>
</tr>
<tr>
<td><em>C. porcellus</em> (Guinea pig)</td>
<td>210-nndlvYlvenp-222</td>
<td>NP_001238751.1</td>
</tr>
<tr>
<td><strong>Not susceptible to severe JUNV disease</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. auratus</em> (Syrian golden hamster)</td>
<td>203-ngd-snLvenp-214</td>
<td>XP_005071611.1</td>
</tr>
<tr>
<td><em>C. griseus</em> (Chinese hamster)</td>
<td>203-ngd-sdlvenp-214</td>
<td>NP_001233748.1</td>
</tr>
<tr>
<td><em>R. norvegicus</em> (Norway rat)</td>
<td>207-gsn-idpveap-218</td>
<td>NP_073203.1</td>
</tr>
<tr>
<td><em>M. musculus</em> (House mouse)</td>
<td>208-ngn-ldpvesp-219</td>
<td>NP_001344227.1</td>
</tr>
</tbody>
</table>

Alignment of known susceptible and non-susceptible species TfR1 sequences based on hTfR1 residues 206-216. The critical tyrosine residue for pathogenic NWA glycoprotein (GP) binding is capitalized, bolded, and shaded in grey.
References


Groups of 4 guinea pigs were challenged IP with the indicated dose of virus. (A) Survival, (B) percent body weight change, and (C) change in body temperature readings are shown. The percent change in body weight and temperature are represented as the group mean and standard error of mean (SEM) of the surviving animals relative of their starting weights or temperatures on the day of challenge. Sham-infected normal controls animals (n=2) are included for comparison.
Figure 2. Replication of JUNV-R and TCRV in primary guinea pig, hamster and mouse macrophages. Resident peritoneal macrophages were infected with the indicated viruses at a MOI of 0.1. The infection medium was removed after 1.5 h and the cells washed and culture medium added. A longitudinal growth curve analysis was performed by titrating aliquots of culture supernatant collected every 24 h for 4 days. The y-axis intercept indicates the assay limit of detection and the x-axis intercept indicates input (inoculum) virus. Data reflect means and standard deviations and are representative of 2 independent experiments. *, P < 0.05, **, P < 0.01 compared to mouse macrophages. δ, P < 0.05, ε, P < 0.01 compared to hamster macrophages.
Figure 3. Titration of JUNV-R infection in Syrian golden hamsters. Groups of female 7-8-week-old hamsters (n=4) were challenged IP with the indicated dose of virus. (A) Survival, and (B) percent body weight change are shown. The percent change in body weight is represented as the group mean and standard error of mean of the surviving animals relative of their starting weights on the day of challenge. Sham-infected normal controls animals (n=4) are included for comparison.
Figure 4. Expression of gpTfR1 results in enhanced JUNV, MACV and GTOV pseudovirus entry into CHO-K1 cells. CHO-K1 cells were transfected with expression vectors encoding human (h), guinea pig (gp), hamster (ham) or mouse (m) TfR1 orthologs and infected with the indicated pseudotyped viruses. Viral entry was assessed by measuring eGFP expression 48 h after infection and expressed as (A) relative entry compared to mock-transfected cells and (B) relative fluorescent units (RFU). Data reflect means and standard deviations and are representative of 4 independent experiments. *, $P < 0.001$ compared to respective mock-transfected controls.
Figure 5. TfR1 ortholog expression in cells 48 h post-transfection. TfR1 expression was assessed in transfected cells by fluorometric detection of anti-FLAG, FITC-conjugated antibodies binding to the FLAG tag present at the C-terminus of each TfR1 ortholog. Expression levels were normalized to hTfR1 expression. Shown are expression levels from A) CHO, B) MDBK, C) HEK 293T and D) JH4 cell lines. The data are representative of the mean and standard deviation for TfR1 expression for experiments using the specified cell lines.
Figure 6. Expression of gpTfR1 leads to increased attachment and replication of JUNV. CHO-K1 cell transfectants expressing TfR1 orthologs were inoculated with JUNV-C1 or TCRV at 4°C to inhibit internalization. After washing, bound virions were released by a freeze-thaw cycle and attachment was determined by titration of cell lysates on Vero cells. In parallel, infections were conducted at 37°C and following the wash steps, cultures were incubated for 48 h and supernatants collected for titration on Vero cells. (A) JUNV-C1 and (C) TCRV attachment to cells expressing TfR1 orthologs. (B) JUNV-C1 and (D) TCRV viral loads at 48 h p.i. The y-axis intercept indicates the assay limit of detection. Data reflect means and standard deviations and are representative of 3-5 independent experiments. *, P < 0.001 compared to mock (empty vector)-transfected controls.
Figure 7. Complementation of refractory MDBK cells with gpTfR1 and hTfR1 results in increased JUNV attachment and replication. Stable MDBK transfectants expressing rodent or human TfR1 proteins were exposed to JUNV-C1 or TCRV to assess attachment and replication as described in Figure 6. JUNV-C1 (A) attachment and (B) replication measured at 48 h.p.i. TCRV (C) attachment and (D) replication measured at 48 h.p.i. The y-axis intercept indicates the assay limit of detection. Data reflect means and standard deviations and are representative of 4 independent experiments. *, $P < 0.001$ compared to mock (empty vector)-transfected controls.
Figure 8. Schematic representation of CRISPR guided knockout of human and guinea pig TfR1. Schematic representation of the CRISPR-Cas9 TfR1 target site and resulting INDEL for human and guinea pig TfR1. Image was generated using BioRender.
Figure 9. Deletion of TfR1 in human and guinea pig cells limits JUNV attachment and replication. (A) Pseudovirus entry in HEK 293T and JH4 WT cells, TfR1<sup>−/−</sup> (KO) cells, and TfR1<sup>−/−</sup> cells complemented with human and rodent TfR1 orthologs. Entry expressed as (A) relative entry compared to mock-transfected cells and (B) relative fluorescent units (RFU). Transfection of TfR1 expression vectors, pseudovirus infection and eGFP measurement was performed as described in Figure 4. * P < 0.001 compared to respective TfR1<sup>−/−</sup> controls. JUNV-C1 and TCRV (C) attachment to, and (D) replication in, WT, TfR1<sup>−/−</sup> and TfR1-complemented HEK 293T and JH4 cells was determined as described in Figure 6. *, P < 0.05, **, P < 0.01, ***, P < 0.001 compared to respective TfR1<sup>−/−</sup> cells.