Receptor determinants of zoonotic transmission of New World hemorrhagic fever arenaviruses

Sheli R. Radoshitzky*, Jens H. Kuhn*†, Christina F. Spiropoulou‡, César G. Albariño‡, Dan P. Nguyen§, Jorge Salazar-Bravo¶, Tatyana Dorfman*, Amy S. Lee*, Enxiu Wang‖, Susan R. Ross‖, Hyeryun Choe§, and Michael Farzan*,**

*Department of Microbiology and Molecular Genetics and New England Primate Research Center, Harvard Medical School, Southborough, MA 01772; †Department of Biology, Chemistry, and Pharmacy, Freie Universität Berlin, 14195 Berlin, Germany; ‡Special Pathogens Branch, Centers for Disease Control and Prevention, Atlanta, GA 30333; §Department of Pediatrics, Children's Hospital, Harvard Medical School, Boston, MA 02115; ¶Department of Biological Sciences, Center for Epidemiology and Zoonoses, Texas Tech University, Lubbock, TX 79409; and ‖Department of Microbiology and Abramson Family Cancer Center, University of Pennsylvania, Philadelphia, PA 19104

Edited by Peter Palese, Mount Sinai School of Medicine, New York, NY, and approved December 27, 2007 (received for review September 28, 2007)

Transferrin receptor 1 (TfR1) is a cellular receptor for the New World hemorrhagic fever arenaviruses Machupo (MACV), Junín (JUNV), and Guanarito (GTOV). Each of these viruses is specifically adapted to a distinct rodent host species, but all cause human disease. Here we compare the ability of these viruses to use various mammalian transferrin receptor 1 (TfR1) orthologs, including those of the South American rodents that serve as reservoirs for MACV, JUNV, and GTOV (*Calomys callosus*, *Calomys musculinus*, and *Zygodontomys brevicauda*, respectively). Retroviruses pseudotyped with MACV and JUNV but not GTOV glycoproteins (GPs) efficiently used *C. callosus* TfR1, whereas only JUNV GP could use *C. musculinus* TfR1. All three viruses efficiently used *Z. brevicauda* TfR1. TfR1 orthologs from related rodents, including house mouse (*Mus musculus*) and rat (*Rattus norvegicus*), did not support entry of these viruses. In contrast, these viruses efficiently used human and domestic cat TfR1 orthologs. We further show that a local region of the human TfR1 apical domain, including tyrosine 211, determined the efficiency with which MACV, JUNV, and GTOV used various TfR1 orthologs. Our data show that these New World arenaviruses are specifically adapted to the TfR1 orthologs of their respective rodent hosts and identify key commonalities between these orthologs and human TfR1 necessary for efficient transmission of these viruses to humans.

Calomys | Junín virus | Machupo virus | transferrin receptor 1

Arenaviruses are enveloped, single-stranded, bisegmented RNA viruses with ambisense genomes (1). The family Arenaviridae consists of a single genus (*Arenavirus*) composed of at least 24 viruses (2, 3). Based on their antigenic properties, arenaviruses have been classified into two major groups: the Old World arenaviruses, which include lymphocytic choriomeningitis virus (LCMV) and Lassa virus (LASV), and the New World arenaviruses, which are further divided into clades A, B, and C. The South American viruses Machupo (MACV), Junín (JUNV), Guanarito (GTOV), and Sabiá (SABV) belong to clade B and cause Bolivian, Argentinian, Venezuelan, and Brazilian hemorrhagic fevers, respectively. MACV, JUNV, and GTOV are classified as National Institute of Allergy and Infectious Disease Category A Priority Pathogens, Select Agents, and Class 4 Biosafety Pathogens, in part due to their high lethality (2, 4).

Rodents of the Muridae family are the natural hosts of most arenaviruses, and the geographic distribution of each arenavirus is determined by the range of its corresponding host. New World arenaviruses are found in the murid subfamily Sigmodontinae in specialized ecologic niches in South and North America (5, 6). *Calomys callosus* (large vesper mouse), *Calomys musculinus* (drylands vesper mouse), and *Zygodontomys brevicauda* (cane mouse) are the principal hosts for MACV, JUNV, and GTOV, respectively. The host of SABV has not been identified (7–10). The phylogenetic diversity of arenaviruses is likely the result of long-term coevolution of the viruses and their corresponding hosts (11, 12).

Entry of arenaviruses into their target cells is facilitated by the two noncovalently linked surface glycoproteins GP1 and GP2. Both proteins are synthesized in infected cells as a single glycoprotein precursor (GPC), which is proteolytically processed to these two mature subunits (13–16). As with other class I fusion proteins, the GP1 subunit associates with a cellular receptor (17–20). The GP2 subunit is a transmembrane protein that mediates fusion of the viral and cellular membranes after internalization of the virus into acidified endosomes (21–24).

α-Dystroglycan is a cellular receptor for New World clade C and Old World arenaviruses (25–27). Four New World clade B arenaviruses (MACV, JUNV, GTOV, and SABV) use transferrin receptor 1 (TfR1) as a cellular receptor (28). Several properties of TfR1 support its critical role in arenaviral replication and disease. It is rapidly and constitutively internalized by clathrin-mediated endocytosis into an acidic compartment, consistent with the pathway and pH dependence of arenavirus cell entry (23, 29). It is expressed ubiquitously and is expressed at high levels on activated or rapidly dividing cells, including macrophages and activated lymphocytes, which are major targets of arenaviruses *in vivo* (30–33). TfR1 is also highly expressed on endothelial cells (34–36), thought to be central to the pathogenesis of hemorrhagic fevers (35, 37). TfR1 up-regulation on immune cells activated in response to infection may accelerate viral replication in these cells and may in part explain the higher lethality of New World hemorrhagic fevers compared with Lassa fever. Several studies also indicate that additional or alternative receptors for clade B viruses exist (38–41). For example, the nonpathogenic clade B viruses Amapari and Tacaribe use a receptor on human cells distinct from TfR1 and α-dystroglycan (38, 41).

Here we describe the ability of MACV, JUNV, and GTOV GP to mediate entry into cells expressing a range of TfR1 orthologs. We observed that JUNV and MACV but not GTOV efficiently used the TfR1 ortholog of the MACV host species, *C. callosus*, whereas only JUNV used the TfR1 ortholog of its host species, *C. musculinus*. Although GTOV could not use either *Calomys* species TfR1 ortholog, it efficiently used that of its principal reservoir, *Z. brevicauda*. House mouse (*Mus musculus*), rat (*Rattus norvegicus*), and dog TfR1 orthologs were inefficient receptors for all three viruses, whereas cat and human TfR1 supported their efficient

Author contributions: S. R. Radoshitzky and M.F. designed research; S. R. Radoshitzky, J.H.K., C.F.S., C.G.A., D.P.N., T.D., A.S.L., E.W., and H.C. performed research; J.S.-B., E.W., and S. R. Ross contributed new reagents/analytic tools; S. R. Radoshitzky and M.F. analyzed data; and S. R. Radoshitzky and M.F. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. EU164540, EU164541, and EU340259).

**To whom correspondence should be addressed. E-mail: farzan@hms.harvard.edu.

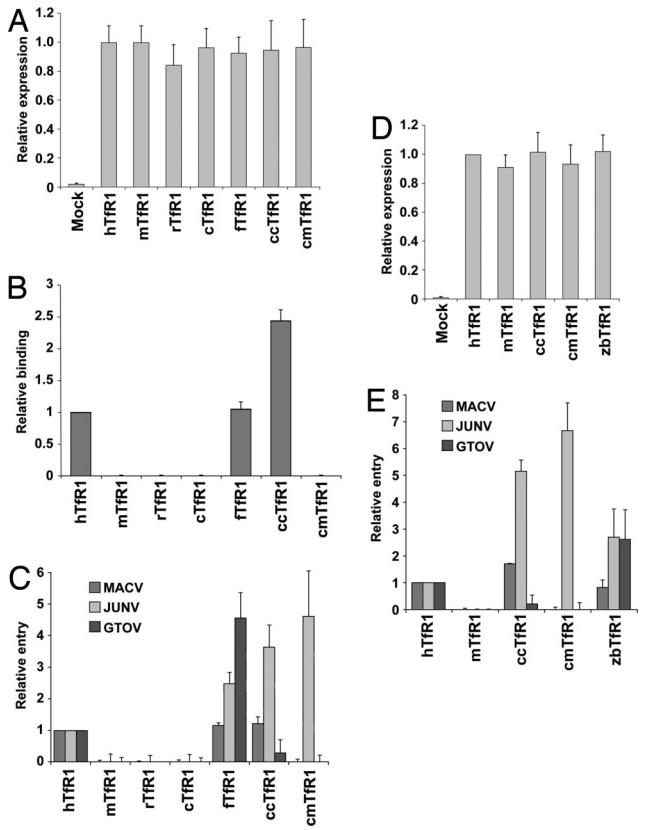

Fig. 1. New World arenavirus entry mediated by TfR1 orthologs. (*A*) CHO cells were transfected with plasmids encoding TfR1 orthologs from human (hTfR1), *M. musculus* (mTfR1), *R. norvegicus* (rTfR1), *C. familiaris* (cTfR1), *F. domesticus* (fTfR1), *C. callosus* (ccTfR1), or *C. musculinus* (cmTfR1) tissues. Cell surface expression was determined by flow cytometry using an anti-FLAG antibody recognizing a tag present at the C terminus of each of the TfR1 variants. (*B*) In parallel, transfected cells were incubated with 200 nM of the MACV GP1 truncation variant, GP1Δ, fused to the Fc domain of human IgG1 (GP1Δ-Fc), and TfR1 association was determined by flow cytometry. Mean fluorescence values were normalized to those of hTfR1. Error bars indicate the standard deviation of five experiments. (*C*) An aliquot of the cells used in *A* and *B* were transduced with MLVs expressing enhanced GFP and pseudotyped with the glycoproteins of MACV, JUNV, or GTOV. Forty-eight hours after transduction, cell entry was measured by flow cytometry. Mean fluorescence values were normalized to those of hTfR1-expressing cells. Error bars indicate the standard deviation of five experiments. (*D*) An experiment similar to that in *A*, except that CHO cells were transfected with plasmid encoding the *Z. brevicauda* TfR1 ortholog (zbTfR1), as well as those encoding hTfR1, mTfR1, ccTfR1, and cmTfR1. (*E*) An experiment similar to that in *C* in which cell entry efficiency was measured by using an aliquot of the cells used in *D*.

entry. Six residues from the apical domain of human TfR1 converted house mouse TfR1 to an efficient receptor for each of these arenaviruses. These studies localized the GP1-binding region to the C terminus of α-helix 2 and to a prominent loop between β strands 1 and 2 in the apical domain of TfR1. This latter loop includes tyrosine 211, which is common to human, cat, *C. callosus*, *C. musculinus*, and *Z. brevicauda* TfR1 orthologs but absent from rat and house mouse orthologs. We conclude that similarities in this region between human TfR1 and the TfR1 orthologs of the MACV, JUNV, and GTOV host species facilitate efficient transmission of these pathogenic New World arenaviruses to humans. Our data further establish a critical biologic role for TfR1 in New World arenavirus replication and South American hemorrhagic fevers.

Results

Utilization of TfR1 Orthologs by New World Hemorrhagic Fever Arenaviruses. To obtain insight into zoonotic transmission of New World arenaviruses and to help identify TfR1 determinants critical to its role as an arenaviral receptor, we compared human TfR1 (hTfR1) to TfR1 orthologs from *M. musculus* (house mouse; mTfR1), *R. norvegicus* (rat; rTfR1), *Canis familiaris* (dog; cTfR1), *Felis domesticus* (cat; fTfR1), *C. callosus* (large vesper mouse; ccTfR1), *C. musculinus* (drylands vesper mouse; cmTfR1), and *Z. brevicauda* (cane mouse; zbTfR1). *C. callosus*, *C. musculinus*, and *Z. brevicauda* serve as reservoirs for MACV, JUNV, and GTOV, respectively (5). Chinese hamster ovary (CHO) cells, which are refractory to transduction by Moloney murine leukemia virus (MLV) pseudotyped with the glycoproteins of MACV, JUNV, or GTOV (28, 41), were transfected with plasmids expressing these TfR1 orthologs tagged in their C-terminal ectodomains. Transfected CHO cells then were characterized for TfR1 expression by flow cytometry. In parallel, a chimeric protein in which residues 79–258 of MACV GP1 were fused to the Fc region of human IgG1 (MACV GP1Δ-Fc) was assayed for its ability to bind transfected cells. This truncation variant has been previously shown to bind human TfR1 with higher affinity than full-length GP1-Fc (28). Finally, transfected cells were transduced with MLV expressing GFP and pseudotyped with the GP of MACV, JUNV, or GTOV (MACV-MLV, JUNV-MLV, or GTOV-MLV, respectively), and cell entry was measured as GFP fluorescence. When roughly equivalent cell surface expression of each TfR1 ortholog was observed (Fig. 1*A*), MACV GP1Δ-Fc efficiently bound hTfR1, fTfR1, and ccTfR1 but not mTfR1, rTfR1, cTfR1, or cmTfR1 (Fig. 1*B*). The ability of MACV GP1Δ-Fc to bind cells transfected with TfR1 orthologs correlated with the ability of MACV-MLV to enter these cells (Fig. 1*C*). Similarly, neither JUNV-MLV nor GTOV-MLV could enter cells expressing mTfR1, rTfR1, or cTfR1. Strikingly, cells expressing fTfR1 were more efficiently transduced by JUNV-MLV and GTOV-MLV than hTfR1-expressing cells. Expectedly, MACV-MLV and JUNV-MLV each efficiently entered cells expressing the TfR1 orthologs of their respective host species, ccTfR1 and cmTfR1. However, MACV GP could not use cmTfR1. Also, the GTOV GP did not mediate entry into cells expressing either ccTfR1 or cmTfR1 (Fig. 1 *C* and *E*) (5). However, it did mediate efficient entry into cells expressing the TfR1 of the GTOV host species *Z. brevicauda*, as did MACV and JUNV GP molecules (Fig. 1*E*). These data demonstrate that MACV, JUNV, and GTOV are specifically adapted to the TfR1 orthologs of their respective hosts. The unexpected observation that feline TfR1 is a highly efficient receptor for each of these viruses raises the possibility that a felid, perhaps a predator of these rodent hosts, may serve as a transmission intermediate for one or more New World hemorrhagic fever arenaviruses.

Localization of the Arenaviral GP1-Binding Determinants on Human TfR1. Next, we characterized a series of chimeras of hTfR1 and mTfR1 (represented in Fig. 2*A*) for their ability to bind MACV GP1Δ-Fc and support MACV, JUNV, and GTOV GP-mediated entry. Chimeras 1–6 have been previously described (42). All receptor chimeras were expressed on the cell surface to similar levels as determined by flow cytometry (Fig. 2*B*). As shown in Fig. 2*C*, MACV GP1Δ-Fc efficiently bound human TfR1 as well as a TfR1 chimera consisting of the hTfR1 apical domain and the mTfR1 protease-like and helical domains (chimera 7), thus localizing the GP1-binding region to the hTfR1 apical domain. Comparison of chimera 5, which did not bind GP1Δ-Fc, with chimera 6, which did, identifies a binding determinant between residues 325 and 366 of the TfR1 apical domain. Comparison of chimeras 7 and 8, which differ only by the presence of mTfR1 amino acids between residues 208 and 212, identifies a second potential binding determinant. Unlike chimera 7, chimera 8 did not associate with GP1Δ-Fc, suggesting that these residues in hTfR1 bind MACV GP1. The ability of chimeras 9 and 10 to bind MACV GP1Δ-Fc efficiently is consistent with a role for these two GP1-binding determinants (Fig. 2*C*). Chimera 9 contains human sequences that include both regions (188–229 include 208–212, and 325–383 include 325–366) in a largely murine receptor. Chimera 10 also contains these

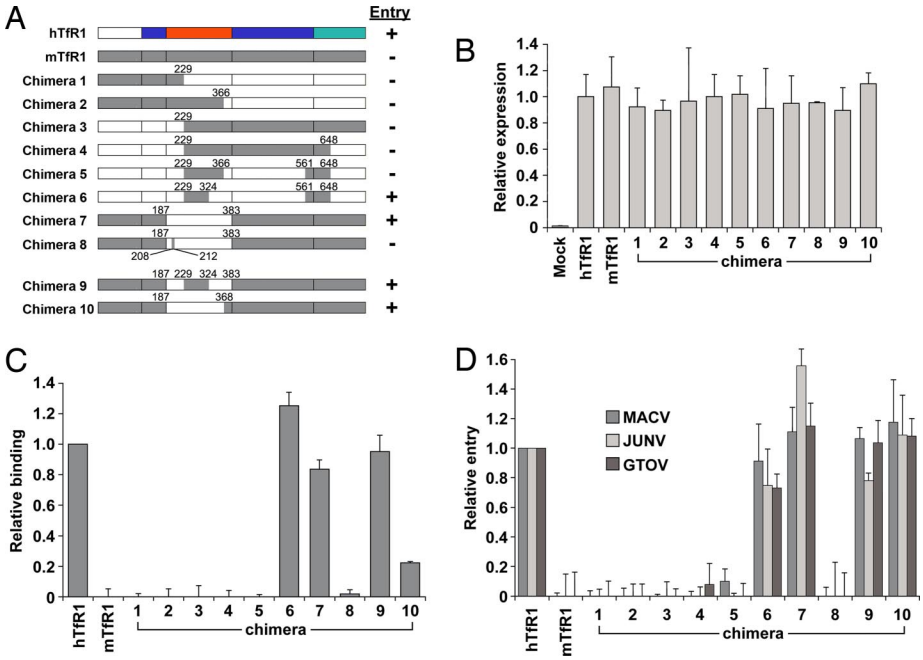

Fig. 2. MACV, JUNV, and GTOV entry mediated by chimeras of human and mouse TfR1. (*A*) A representation of the TfR1 structural domains and human/mouse TfR1 chimeras. In the top bar, the protease-like, apical, and helical domains of human TfR1 are indicated as blue, red, and cyan, respectively. The N-terminal cytoplasmic domain and transmembrane domain are shown in white. Individual mouse/human chimeras are represented in gray, indicating murine sequence, and in white, indicating human sequence. A plus sign to the right of each chimera indicates efficient MACV GP1Δ-Fc association and MACV, JUNV, and GTOV GP-mediated entry, shown in *C* and *D*. (*B*) CHO cells were transfected with plasmids encoding hTfR1, mTfR1, and chimeras of these receptors. Cell surface expression was analyzed as in Fig. 1*A*. Mean fluorescence values were normalized to hTfR1. Error bars indicate the standard deviation of three experiments. (*C*) In parallel, cell surface binding of MACV GP1Δ-Fc was determined by flow cytometry, as in Fig. 1*B*. Mean fluorescence values were normalized to those of hTfR1-expressing cells. Error bars indicate the standard deviation of three experiments. (*D*) An aliquot of the cells used in *B* and *C* was transduced with MACV, JUNV, or GTOV pseudoviruses and analyzed as in Fig. 1*C*. Mean fluorescence values were normalized to hTfR1. Error bars indicate the standard deviation of three experiments.

regions. TfR1 chimeras that bound GP1Δ-Fc efficiently (6, 7, 9, and 10) also supported efficient entry mediated by MACV, JUNV, or GTOV GP (Fig. 2*D*). These data indicate that arenaviral GP-mediated entry depends on at least two determinants in the hTfR1 apical domain that are separated in the TfR1 primary sequence but adjacent in the tertiary structure (43). Consistent with a role for the TfR1 apical domain in arenaviral entry, an anti-human TfR1 antibody previously shown to inhibit replication of infectious MACV, JUNV, GTOV, and SABV (28) recognized chimera 7 but not mTfR1 or chimera 8 (data not shown). Thus, this antibody, like MACV GP1Δ-Fc, recognizes the human TfR1 apical domain, probably including hTfR1 residues 208–212. Also consistent with a central role for the TfR1 apical domain in arenaviral entry, neither soluble transferrin (28) nor overexpressed human hemochromatosis protein HFE (not shown) altered the efficiency of MACV entry. These TfR1-binding proteins associate with TfR1 helical and protease-like domains and do not contact the apical domain (44, 45).

Tyrosine 211 in the Human TfR1 Apical Domain Is a Critical Determinant of MACV, JUNV, and GTOV Cell Entry. Comparison of residues 208–212 and 325–366 among the TfR1 orthologs characterized in Fig. 1 suggested that tyrosine 211 might be a critical determinant of MACV, JUNV, and GTOV entry and of variation in host susceptibility to these viruses. Most TfR1 orthologs with a tyrosine at this position supported entry of at least one of these arenaviruses, whereas orthologs lacking this tyrosine did not function as arenaviral receptors (critical differences among TfR1 orthologs are represented in summary in Fig. 3). Alteration of tyrosine 211 of hTfR1 to aspartic acid or alanine prevented surface expression of hTfR1. However, one hTfR1 variant, in which tyrosine 211 was altered to threonine (Y211T), expressed efficiently (Fig. 4*A*). We also generated human TfR1 variants in which asparagine 292 and asparagine 348 were altered to their mTfR1 counterparts, glutamic acid (N292E) and lysine (N348K), respectively. Asparagine 348 is adjacent to tyrosine 211 in the tertiary structure of hTfR1 (Fig. 3*C*). MACV GP1Δ-Fc efficiently bound hTfR1 and the N292E hTfR1 variant. In contrast, hTfR1 variants N348K and Y211T did not support MACV GP1Δ-Fc binding (Fig. 4*B*). Again, these observations are consistent with the ability of these receptor variants to mediate entry of MACV-MLV, JUNV-MLV, and GTOV-MLV (Fig. 4*C*). Together, these data suggest a critical role for residues 211 and 348 of human TfR1 in mediating entry of New World hemorrhagic fever arenaviruses. Fig. 3*C* shows the crystal structure of human TfR1 apical domain (43) in which residues 211 and 348, proximal in the hTfR1 tertiary structure, are indicated.

Introduction of Six hTfR1 Residues Convert mTfR1 to an Efficient New World Arenavirus Receptor. Tyrosine 211 is located within an exposed loop of hTfR1 between β-strands 1 and 2. We introduced this five-residue loop (RLVYL) of hTfR1 into mTfR1, replacing its four-residue loop (NLDP). We also constructed a second mTfR1 variant in which lysine 348 was altered to asparagine (K348N). A third mTfR1 variant (RLVYL+K348N) includes both of these alterations. Although all variants expressed as efficiently as hTfR1 and mTfR1 (Fig. 5*A*), only mTfR1 variants with the RLVYL loop bound MACV GP1Δ-Fc (Fig. 5*B*). GP1Δ-Fc associated most efficiently with RLVYL+K348N. Similarly, MACV-MLV and GTOV-MLV could enter cells expressing the RLVYL mTfR1 variant and, more efficiently, the RLVYL+K348N mTfR1 variant (Fig. 5*C*). The RLVYL variant mediated JUNV-MLV entry as efficiently as hTfR1, and the K348N change had no additional effect. These data show that hTfR1 residues 208–212, including tyrosine 211, are critical for MACV, JUNV, and GTOV entry and that MACV and GTOV entry is partially disrupted by lysine 348, which is present in mTfR1. JUNV may better tolerate this lysine because the TfR1 of its host species, *C. musculinus*, also has a lysine at position 348 (Fig. 3*D*).

Effect of Potential *N*-glycosylation of *C. callosus* and *C. musculinus* TfR1 Asparagine 205 on MACV-MLV, JUNV-MLV, and GTOV Entry. *C. callosus*, *C. musculinus*, and *Z. brevicauda* TfR1 possess a potential *N*-glycosylation site proximal to tyrosine 211, whereas human TfR1 lacks such a site (Fig. 3*D*). We constructed ccTfR1 and cmTfR1 variants lacking this glycosylation motif at asparagine 205 (ccTfR1 N205A and cmTfR1 N205A) and assayed their ability to mediate entry of MACV-, JUNV-, and GTOV-MLV (Fig. 6 *A* and *B*). Removal of the glycosylation motif from

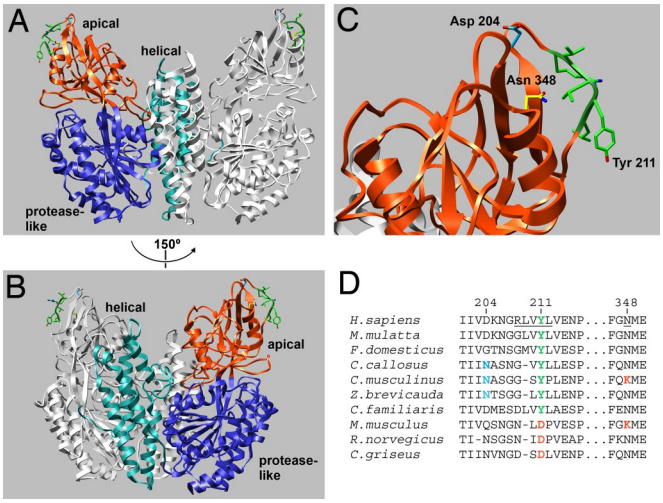

Fig. 3. TfR1 determinants of zoonotic transmission of New World hemorrhagic fever arenaviruses. (*A*) The structure of the human TfR1 dimer is shown oriented with the cellular membrane at the bottom. Protease-like, apical, and helical domains are indicated as blue, red, and cyan, respectively, on one monomer. The other monomer is shown in white. A loop composed of residues 208–212, critical for New World arenaviral entry, is indicated in green. (*B*) As in *A*, except that human TfR1 dimer is rotated 150° about the twofold axis of the dimer. (*C*) As in *B*, except that the apical domain is enlarged. Aspartic acid 204, corresponding to a potential glycosylation site in the *Calomys* species, is indicated in blue. Asparagine 348, necessary together with residues 208–212 to convert mTfR1 to an efficient MACV and GTOV receptor, is shown in yellow. Tyrosine 211, within the 208–212 loop, is also indicated. (*D*) An alignment of amino acid sequences from two proximal regions of the indicated TfR1 orthologs. Human TfR1 residues that convert mouse TfR1 to an efficient MACV, JUNV, and GTOV receptor are underlined. Tyrosine 211 and lysine 348 are shown in green and red, respectively. Potential glycosylation sites present in *Calomys* species and *Z. brevicauda* TfR1 orthologs are indicated in blue. *Macaca mulatta* (rhesus macaque) and *Cricetulus griseus* (Chinese hamster) TfR1 regions are shown with those of the TfR1 orthologs characterized here. Rhesus macaques can be used as a model for MACV and JUNV infection (46, 47, 52). Hamster CHO and BHK cell lines are refractory to entry mediated by MACV, JUNV, and GTOV GP (28, 41).

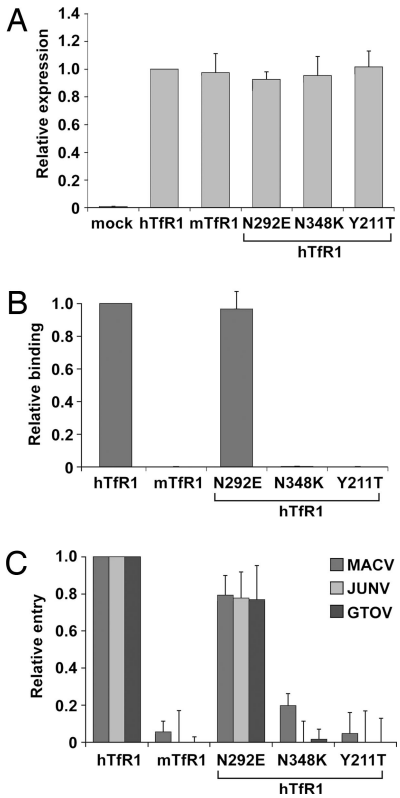

Fig. 4. The arenavirus GP1-binding site of human TfR1. (*A*) CHO cells were transfected with plasmids encoding hTfR1, mTfR1, or human TfR1 variants N292E, N348K, or Y211T. Cell surface expression was analyzed as in Fig. 1. Mean fluorescence values were normalized to those of hTfR1-expressing cells. Error bars indicate the standard deviation of three experiments. (*B*) In parallel, cell surface binding of MACV GP1Δ-Fc was determined by flow cytometry. Mean fluorescence values were normalized to those of hTfR1-expressing cells. Error bars indicate the standard deviation of three to five experiments. (*C*) An aliquot of the cells used in *A* and *B* was transduced with MACV, JUNV, or GTOV pseudoviruses and analyzed as in Fig. 1*C*. Mean fluorescence values were normalized to hTfR1. Error bars indicate the standard deviation of three experiments.

ccTfR1 had a modest effect on MACV entry (≈30% increase). However, this variant substantially increased JUNV GP-mediated entry (>2-fold over wild-type ccTfR1) and, unlike wild-type ccTfR1, was an efficient receptor for GTOV-MLV. Removal of the glycosylation motif from cmTfR1 increased the entry efficiency of each virus but to a lesser extent than observed with ccTfR1. These data suggest that the efficiency of hTfR1 as a receptor for MACV, JUNV, and GTOV may in part be due to the absence of this glycosylation site. They also underscore the greater efficiency with which these viruses use the TfR1 orthologs of their natural hosts, despite apparent interference from glycosylation at asparagine 205. The analogous residue of hTfR1, aspartic acid 204, is shown in Fig. 3*C*.

Discussion

TfR1 is a cellular receptor for New World hemorrhagic fever arenaviruses (28) and is likely to play a critical role in their zoonotic transmission and the pathogenesis of arenaviral hemorrhagic fevers. Here we identify the GP1-binding site on human TfR1. Specifically, residues 208–212, which are localized to a TfR1 apical-domain loop between β-strands 1 and 2, and residue 348, which is adjacent to this loop (Fig. 3*C*), participate in MACV GP1 binding and in MACV, JUNV, and GTOV GP-mediated entry into target cells. By introducing these residues into house mouse (*M. musculus*) TfR1, we converted it to an efficient receptor for New World hemorrhagic fever arenaviruses. These results are consistent with the presence of tyrosine 211 in TfR1 orthologs from the MACV, JUNV, and GTOV rodent hosts, *C. callosus*, *C. musculinus*, and *Z. brevicauda*, respectively (Fig. 3*D*). Tyrosine 211 is present in cat TfR1, a very efficient receptor for all three New World hemorrhagic fever arenaviruses. TfR1 from the rhesus macaque, a non-human primate model for MACV and JUNV infections (46, 47), also has a tyrosine at position 211. In contrast, position 211 is an aspartic acid in house mouse and rat TfR1 orthologs, preventing efficient arenavirus entry. Furthermore, hamster TfR1 also possesses an aspartic acid at this position, consistent with the resistance of hamster cell lines, including CHO and BHK cells, to New World arenavirus GP-mediated transduction (28, 41). Thus, the presence of tyrosine 211 in the human receptor is likely to be critical for zoonotic transmission of hemorrhagic fever arenaviruses.

Although tyrosine 211 is necessary for New World arenaviral infection, it is not sufficient. The dog TfR1 ortholog, which expresses this tyrosine, does not function as an efficient receptor for MACV, JUNV, or GTOV. Neither MACV nor GTOV uses the TfR1 ortholog of the JUNV host species, *C. musculinus*, which also has this tyrosine. Residues in the local vicinity of tyrosine 211 likely account for these differences. For example, dog TfR1 has two acidic residues at positions 206 and 208 (Fig. 3*D*) that are not present in receptors that mediate efficient arenavirus entry. Similarly, *C. musculinus* TfR1 includes a lysine at residue 348, which we have shown to interfere with MACV and GTOV entry but which does not affect entry of JUNV (Fig. 5*C*).

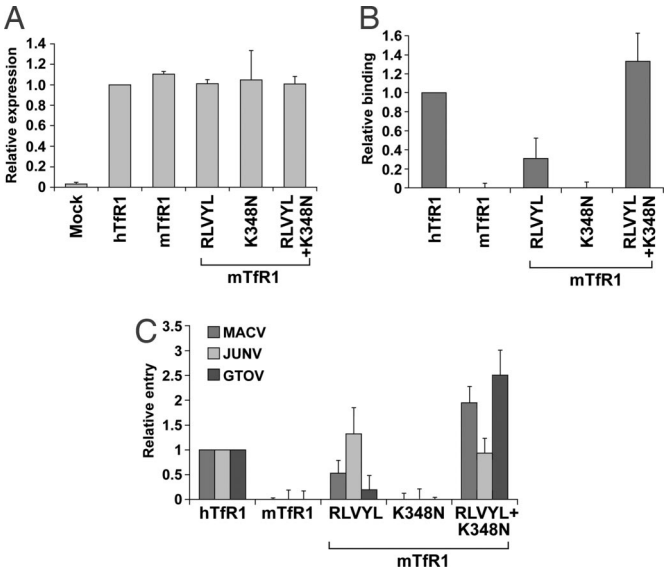

Fig. 5. Conversion of house mouse TfR1 to an efficient arenavirus receptor. (*A*) CHO cells were transfected with plasmids encoding hTfR1, mTfR1, and mTfR1 mutants (RLVYL, K348N, and RLVYL+K348N). Cell surface expression was analyzed as in Fig. 1. Mean fluorescence values were normalized to hTfR1. Error bars indicate the standard deviation of three experiments. (*B*) In parallel, cell surface binding of MACV GP1Δ-Fc was determined by flow cytometry. Mean fluorescence values were normalized to hTfR1. Error bars indicate the standard deviation of three to five experiments. (*C*) An aliquot of the cells used in *A* and *B* was transduced with MACV, JUNV, or GTOV pseudoviruses and analyzed as in Fig. 1*C*. Mean fluorescence values were normalized to hTfR1. Error bars indicate the standard deviation of three experiments.

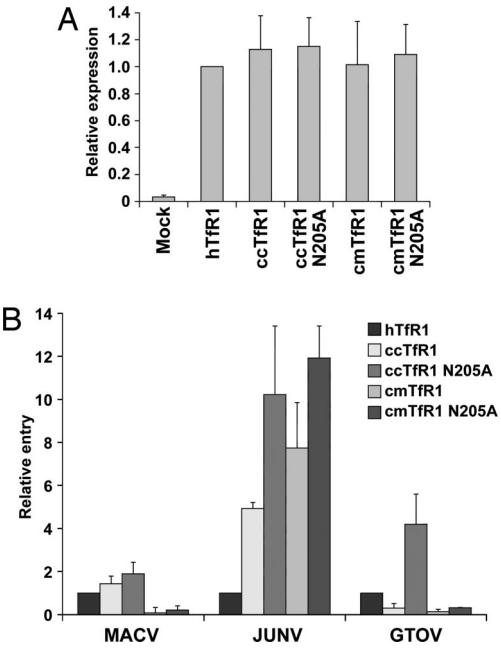

Fig. 6. Effect of *C. callosus* and *C. musculinus* asparagine 205 glycosylation on MACV, JUNV, and GTOV entry. (*A*) CHO cells were transfected with plasmids encoding hTfR1, *C. callosus* TfR1 (ccTfR1), *C. musculinus* TfR1 (cmTfR1), or N205A glycosylation mutants (ccTfR1 N205A or cmTfR1 N205A). Cell surface expression was analyzed as in Fig. 1. Mean fluorescence values were normalized to hTfR1. Error bars indicate the standard deviation of three experiments. (*B*) An aliquot of the cells used in *A* was transduced with MACV, JUNV, or GTOV pseudoviruses and analyzed as in Fig. 1*C*. Mean fluorescence values were normalized to hTfR1. Error bars indicate the standard deviation of three experiments.

These arenaviruses are specific for the TfR1 orthologs of their respective host species but, paradoxically, also use the less related cat and human orthologs. For example, MACV uses the *C. callosus* TfR1 efficiently, but it cannot use the closely related *C. musculinus* TfR1. GTOV cannot use TfR1 from MACV or JUNV host species. Nonetheless, all three viruses use human TfR1 efficiently, despite the lack of obvious opportunity to adapt to the human receptor. Our data show that a potential glycosylation site adjacent to the GP-binding site of the *C. callosus* and *C. musculinus* TfR1 interferes with entry of MACV, JUNV, and GTOV (Fig. 6*B*). Human and cat TfR1 lack this glycosylation motif which, combined with sequence similarities to *Calomys* and *Zygodontomys* TfR1 orthologs around tyrosine 211, may account for the relatively efficient use of these receptors.

Our data also have implications for the future study of arenaviral hemorrhagic fevers and for efforts to prevent or treat these fevers. First, use of a common binding region by MACV, JUNV, and GTOV suggests that a single antibody or small molecule, including mimetics of the GP-binding site, may be useful in controlling replication of all three viruses. Second, our observations may be useful in identifying species that may serve as additional reservoirs or intermediates in the transmission of New World arenaviruses. Third, our observations suggest an approach for generating an adult murine model of arenaviral hemorrhagic fever, namely by modifying the murine *Tfr1* gene to include the six residues identified here.

Finally, our data underscore the biologic role of TfR1 in New World arenavirus replication. We have shown that MACV, JUNV, and GTOV GPs use the TfR1 orthologs of their respective hosts specifically and with high efficiency. Similarities between the GP-binding sites of these TfR1 orthologs with hTfR1 provide an explanation for the efficient zoonotic transmission of these pathogenic arenaviruses. The absence of a house mouse or rat model of South American hemorrhagic fevers (48–50) is consistent with the inefficiency of mouse and rat TfR1 orthologs as receptors for MACV, JUNV, and GTOV. Collectively, these data indicate that TfR1 is central to the replication of these viruses in humans and their natural hosts.

Materials and Methods

Cells and Plasmids. Human embryonic kidney 293T cells [American Type Culture Collection (ATCC) no. CRL-11268] were maintained in Dulbecco modified Eagle's medium, and CHO (ATCC no.CCL-61) epithelial cells in Ham F12 medium. Both cell lines were supplemented with 10% fetal bovine serum (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin (Cellgro). Plasmids encoding *F. domesticus* (cat) and *C. familiaris* (dog) TfR1 (fTfR1 and cTfR1, respectively) were generously provided by Colin Parrish (Cornell University, Ithaca, NY). *R. norvegicus* TfR1 (rTfR1) was cloned from a rat liver cDNA library (BioChain) by using the following primers: 5′-AATAACTACTTCGAAGCCACCATGGATCAAGCCAGATCAGCATTCTC-3′ and 5′-AATAACTACCTCGAGTTAAAACTCATTGTCAATATTCCAAATGTCACCAG-3′.

C. callosus (large vesper mouse), *C. musculinus* (drylands vesper mouse), and *Z. brevicauda Tfr1* genes (ccTfR1, cmTfR1, and zbTfR1, respectively) were cloned from frozen liver tissues (GenBank accession nos. EU164540, EU164541, and EU340259, respectively). RNA was isolated from tissues by using an RNAqueous kit (Ambion), and cDNA was generated by using the SuperScript III First-Strand Synthesis system for RT-PCR (Invitrogen). The following primers were used to amplify the cmTfR1, ccTfR1, and zbTfR1 genes: 5′-ACAACTATGATGGATCAAGCCAGATCAGCA-3′ and 5′-ACAACTACATTTAAAACTCATTGTCAATATTCCAAATGTC-3′. Coding regions of human, *M. musculus* (house mouse) (28), *F. domesticus*, *C. familiaris*, *R. norvegicus*, *C. callosus*, *C. musculinus*, and *Z. brevicauda* TfR1 were cloned into the pcDNA 3.1(+) expression vector (Invitrogen) with a C-terminal FLAG tag. Chimeras of *M. musculus* and human TfR1 (mTfR1 and hTfR1, respectively) were generated using gene splicing by overlap extension (51). All chimeras were cloned into the pcDNA 3.1(+) expression vector (Invitrogen) with a C-terminal FLAG tag. Site-directed mutagenesis of mTfR1 and hTfR1 was performed by using Expand Long-Range dNTPack (Roche). Plasmids encoding the MACV Carvallo strain GP1Δ deletion variant (residues 79–248) fused to the Fc region of human IgG1 (GP1Δ-Fc), as well as

MACV, JUNV (MC2), and GTOV (INH-95551) GPC, have been described previously (28).

Binding Assays. To generate the MACV GP1Δ-Fc fusion protein, 293T cells were transfected with the appropriate plasmid according to the calcium phosphate method. The protein was purified from SFM II (Invitrogen) with protein A-Sepharose beads, eluted with 3 M $MgCl_2$, dialyzed in PBS, and concentrated. Association of MACV GP1Δ-Fc with TfR1 orthologs and variants was determined by flow cytometry. CHO cells were transfected with the different TfR1-encoding plasmids or with vector alone with FuGENE HD transfection reagent (Roche). Twenty-four hours after transfection, cells were detached with trypsin and seeded in 6- or 24-well plates for subsequent flow cytometric experiments assaying cell surface expression, MACV GP1Δ-Fc association, and pseudovirus transduction. After 24 h, CHO cells expressing TfR1 orthologs and variants were detached with cell dissociation buffer (GIBCO) and washed with 2% goat serum in PBS. Anti-FLAG M2 murine antibody (Sigma) or 200 nM MACV GP1Δ-Fc protein was added to 5×10^5 cells, and the mixture was incubated at 4°C for 1 h. Cells were washed once with PBS/2% goat serum and incubated for 45 min at 4°C with anti-mouse IgG-specific phycoerythrin conjugate (Sigma) or with anti-human IgG (Fc-specific) FITC conjugate (Sigma). Cells were again washed with PBS/2% goat serum, and cell surface binding was detected by flow cytometry, with 10,000 events counted per sample. Background fluorescence was determined by measuring cells treated only with anti-mouse IgG-specific PE conjugate or with anti-human IgG (Fc-specific) FITC conjugate. Each assay was performed in duplicate and repeated three to five times.

Pseudovirus Transduction. To generate retroviruses pseudotyped with arenaviral GP, 293T cells were transfected according to the calcium phosphate method with plasmids encoding MACV GPC, JUNV GPC, or GTOV GPC, together with the pQCXIX vector (BD Biosciences) expressing GFP, and plasmid encoding the MLV *gag* and *pol* genes by using equal concentrations of each plasmid. Cell supernatants were harvested 48 h after transfection, cleared of cellular debris by centrifugation, filtered through a 0.45-μm pore size filter (Corning Glass), and stored at −80°C. Supernatants containing pseudoviruses were added to CHO cells transfected to express TfR1 orthologs and variants in 24-well plates. After 1.5 h of incubation at 37°C, cells were washed once with PBS and replenished with fresh media. At 48 h after infection, cells were imaged by fluorescence microscopy and detached with trypsin for GFP fluorescence analysis by flow cytometry. Fluorescence was normalized to mock-transfected cells transduced with each of the pseudoviruses. Each assay was performed in duplicates and repeated three to five times.

ACKNOWLEDGMENTS. We thank Thomas Postler for comments and careful manuscript editing and Colin Parish for helpful advice and for plasmids expressing cat and dog TfR1.

1. Oldstone MB (2002) Arenaviruses I: The epidemiology molecular and cell biology of arenaviruses. *Curr Top Microbiol Immunol* 262:V–XII.
2. Charrel RN, de Lamballerie X (2003) Arenaviruses other than Lassa virus. *Antiviral Res* 57:89–100.
3. Lecompte E, Ter Meulen J, Emonet S, Daffis S, Charrel RN (2007) Genetic identification of Kodoko virus, a novel arenavirus of the African pigmy mouse (*Mus Nannomys minutoides*) in West Africa. *Virology* 364:178–183.
4. Borio L, *et al.* (2002) Hemorrhagic fever viruses as biological weapons: medical and public health management. *J Am Med Assoc* 287:2391–2405.
5. Salazar-Bravo J, Ruedas LA, Yates TL (2002) Mammalian reservoirs of arenaviruses. *Curr Top Microbiol Immunol* 262:25–63.
6. Clegg JC (2002) Molecular phylogeny of the arenaviruses. *Curr Top Microbiol Immunol* 262:1–24.
7. Johnson KM, Mackenzie RB, Webb PA, Kuns ML (1965) Chronic infection of rodents by Machupo virus. *Science* 150:1618–1619.
8. Johnson KM, Kuns ML, Mackenzie RB, Webb PA, Yunker CE (1966) Isolation of Machupo virus from wild rodent *Calomys callosus*. *Am J Trop Med Hyg* 15:103–106.
9. Fulhorst CF, *et al.* (1999) Natural rodent host associations of Guanarito and pirital viruses (Family Arenaviridae) in central Venezuela. *Am J Trop Med Hyg* 61:325–330.
10. Mills JN, *et al.* (1991) Junin virus activity in rodents from endemic and nonendemic loci in central Argentina. *Am J Trop Med Hyg* 44:589–597.
11. Hugot JP, Gonzalez JP, Denys C (2001) Evolution of the Old World Arenaviridae and their rodent hosts: Generalized host-transfer or association by descent? *Infect Genet Evol* 1:13–20.
12. Bowen MD, Peters CJ, Nichol ST (1997) Phylogenetic analysis of the Arenaviridae: Patterns of virus evolution and evidence for cospeciation between arenaviruses and their rodent hosts. *Mol Phylogenet Evol* 8:301–316.
13. Buchmeier MJ, Southern PJ, Parekh BS, Wooddell MK, Oldstone MB (1987) Site-specific antibodies define a cleavage site conserved among arenavirus GP-C glycoproteins. *J Virol* 61:982–985.
14. Kunz S, Edelmann KH, de la Torre JC, Gorney R, Oldstone MB (2003) Mechanisms for lymphocytic choriomeningitis virus glycoprotein cleavage, transport, and incorporation into virions. *Virology* 314:168–178.
15. Lenz O, ter Meulen J, Klenk HD, Seidah NG, Garten W (2001) The Lassa virus glycoprotein precursor GP-C is proteolytically processed by subtilase SKI-1/S1P. *Proc Natl Acad Sci USA* 98:12701–12705.
16. Beyer WR, Popplau D, Garten W, von Laer D, Lenz O (2003) Endoproteolytic processing of the lymphocytic choriomeningitis virus glycoprotein by the subtilase SKI-1/S1P. *J Virol* 77:2866–2872.
17. Hughson FM (1995) Structural characterization of viral fusion proteins. *Curr Biol* 5:265–274.
18. Kielian M, Rey FA (2006) Virus membrane-fusion proteins: More than one way to make a hairpin. *Nat Rev Microbiol* 4:67–76.
19. Kunz S, Borrow P, Oldstone MB (2002) Receptor structure, binding, and cell entry of arenaviruses. *Curr Top Microbiol Immunol* 262:111–137.
20. Schibli DJ, Weissenhorn W (2004) Class I, class II viral fusion protein structures reveal similar principles in membrane fusion. *Mol Membr Biol* 21:361–371.
21. Gallaher WR, DiSimone C, Buchmeier MJ (2001) The viral transmembrane superfamily: Possible divergence of Arenavirus and Filovirus glycoproteins from a common RNA virus ancestor. *BMC Microbiol* 1:1.
22. York J, Agnihothram SS, Romanowski V, Nunberg JH (2005) Genetic analysis of heptad-repeat regions in the G2 fusion subunit of the Junin arenavirus envelope glycoprotein. *Virology* 343:267–274.
23. Castilla V, Mersich SE, Candurra NA, Damonte EB (1994) The entry of Junin virus into Vero cells. *Arch Virol* 136:363–374.
24. Eschli B, *et al.* (2006) Identification of an N-terminal trimeric coiled-coil core within arenavirus glycoprotein 2 permits assignment to class I viral fusion proteins. *J Virol* 80:5897–5907.
25. Cao W, *et al.* (1998) Identification of alpha-dystroglycan as a receptor for lymphocytic choriomeningitis virus and Lassa fever virus. *Science* 282:2079–2081.
26. Kunz S, Rojek JM, Perez M, Spiropoulou CF, Oldstone MB (2005) Characterization of the interaction of lassa fever virus with its cellular receptor alpha-dystroglycan. *J Virol* 79:5979–5987.
27. Spiropoulou CF, Kunz S, Rollin PE, Campbell KP, Oldstone MB (2002) New World arenavirus clade C, but not clade A, B viruses, utilizes alpha-dystroglycan as its major receptor. *J Virol* 76:5140–5146.
28. Radoshitzky SR, *et al.* (2007) Transferrin receptor 1 is a cellular receptor for New World haemorrhagic fever arenaviruses. *Nature* 446:92–96.
29. Martinez MG, Cordo SM, Candurra NA (2007) Characterization of Junin arenavirus cell entry. *J Gen Virol* 88:1776–1784.
30. Oldstone MB (2002) Arenaviruses II: The molecular pathogenesis of arenavirus infections. *Curr Top Microbiol Immunol* 263:V–XII.
31. Daniels TR, Delgado T, Rodriguez JA, Helguera G, Penichet ML (2006) The transferrin receptor part I: Biology and targeting with cytotoxic antibodies for the treatment of cancer. *Clin Immunol* 121:144–158.
32. Gatter KC, Brown G, Trowbridge IS, Woolston RE, Mason DY (1983) Transferrin receptors in human tissues: their distribution and possible clinical relevance. *J Clin Pathol* 36:539–545.
33. Terrell TG, Stookey JL, Eddy GA, Kastello MD (1973) Pathology of Bolivian hemorrhagic fever in the rhesus monkey. *Am J Pathol* 73:477–494.
34. Jefferies WA, *et al.* (1984) Transferrin receptor on endothelium of brain capillaries. *Nature* 312:162–163.
35. Peters CJ, Zaki SR (2002) Role of the endothelium in viral hemorrhagic fevers. *Crit Care Med* 30:S268–S273.
36. Soda R, Tavassoli M (1984) Transendothelial transport (transcytosis) of iron-transferrin complex in the bone marrow. *J Ultrastruct Res* 88:18–29.
37. Andrews BS, *et al.* (1978) Replication of dengue and junin viruses in cultured rabbit and human endothelial cells. *Infect Immun* 20:776–781.
38. Flanagan ML, *et al.* (2008) New World clade B arenaviruses can use transferrin receptor 1 (TfR1)-dependent and independent entry pathways, and glycoproteins from human pathogenic strains are associated with the use of TfR1. *J Virol* 82:938–948.
39. Oldenburg J, Reignier T, Flanagan ML, Hamilton GA, Cannon PM (2007) Differences in tropism and pH dependence for glycoproteins from the Clade B1 arenaviruses: Implications for receptor usage and pathogenicity. *Virology* 364:132–139.
40. Reignier T, *et al.* (2006) Receptor use by pathogenic arenaviruses. *Virology* 353:111–120.
41. Rojek JM, Spiropoulou CF, Kunz S (2006) Characterization of the cellular receptors for the South American hemorrhagic fever viruses Junin, Guanarito, and Machupo. *Virology* 349:476–491.
42. Wang E, Albritton L, Ross SR (2006) Identification of the segments of the mouse transferrin receptor 1 required for mouse mammary tumor virus infection. *J Biol Chem* 281:10243–10249.
43. Lawrence CM, *et al.* (1999) Crystal structure of the ectodomain of human transferrin receptor. *Science* 286:779–782.
44. Bennett MJ, Lebron JA, Bjorkman PJ (2000) Crystal structure of the hereditary haemochromatosis protein HFE complexed with transferrin receptor. *Nature* 403:46–53.
45. Cheng Y, Zak O, Aisen P, Harrison SC, Walz T (2004) Structure of the human transferrin receptor-transferrin complex. *Cell* 116:565–576.
46. Kastello MD, Eddy GA, Kuehne RW (1976) A rhesus monkey model for the study of Bolivian hemorrhagic fever. *J Infect Dis* 133:57–62.
47. McKee KT, Jr., Mahlandt BG, Maiztegui JI, Eddy GA, Peters CJ (1985) Experimental Argentine hemorrhagic fever in rhesus macaques: Viral strain-dependent clinical response. *J Infect Dis* 152:218–221.
48. Webb PA, Johnson KM, Mackenzie RB, Kuns ML (1967) Some characteristics of Machupo virus, causative agent of Bolivian hemorrhagic fever. *Am J Trop Med Hyg* 16:531–538.
49. Webb PA, Justines G, Johnson KM (1975) Infection of wild and laboratory animals with Machupo and Latino viruses. *Bull WHO* 52:493–499.
50. Tesh RB, Jahrling PB, Salas R, Shope RE (1994) Description of Guanarito virus (Arenaviridae: Arenavirus), the etiologic agent of Venezuelan hemorrhagic fever. *Am J Trop Med Hyg* 50:452–459.
51. Warrens AN, Jones MD, Lechler RI (1997) Splicing by overlap extension by PCR using asymmetric amplification: An improved technique for the generation of hybrid proteins of immunological interest. *Gene* 186:29–35.
52. Kenyon RH, *et al.* (1992) Aerosol infection of rhesus macaques with Junin virus. *Intervirology* 33:23–31.