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A Substitution in the Transmembrane Region of the Glycoprotein Leads to an Unstable Attenuation of Machupo Virus

Michael Patterson, Takaaki Koma, Alexey Seregin, Cheng Huang, Milagros Miller, Jennifer Smith, Nadezhda Yun, Jeanon Smith, Slobodan Paessler

Department of Pathology, University of Texas Medical Branch, Galveston, Texas, USA

Machupo virus (MACV) is the etiologic agent of Bolivian hemorrhagic fever (BHF). Utilizing a reverse-genetics system recently developed, we report the rescue of a rationally modified recombinant MACV containing a single mutation in the transmembrane region of the glycoprotein. Following challenge of susceptible mice, we identified a significant reduction in virulence in the novel virus. We also identified an instability leading to reversion of the single mutation to a wild-type genotype.

The etiologic agent of Bolivian hemorrhagic fever (BHF), Machupo virus (MACV), is classified as a Tier 1 Select Agent by the Centers for Disease Control and Prevention (CDC), requiring a biosafety level 4 (BSL4) laboratory environment (1). First identified during an outbreak in the Beni district of Bolivia, MACV has a reported case mortality rate of 25 to 35% (2–6). Since 2007, reports of MACV have increased dramatically, with a peak of over 200 human cases in 2008 (7). Exposure to the aerosolized excreta and secreta of the natural rodent reservoir *Calomys callosus* is predicted to be the primary route of exposure (3, 8, 9). However, nosocomial spread within family groups and at the hospital have been reported but are not believed to be the primary method of spread during an outbreak (10–12).

A member of the family *Arenaviridae*, MACV is a single-stranded, negative-sense RNA virus that utilizes an ambisense encoding strategy (2). The two segments, small (S [~3.3 kb]) and large (L [~7.2 kb]), encode four viral proteins (Fig. 1A). The S segment encodes the nucleoprotein (NP) and glycoprotein precursor (GPC), which is posttranslationally cleaved into GP1, GP2, and stable signal peptide (SSP). The L segment encodes the RNA-dependent RNA polymerase (L protein) and a RING finger matrix protein (Z) (13–18). The genes are separated by an intergenic region (IGR) serving as the transcription terminator and flanked by untranslated regions (UTR) at the 5′ and 3′ ends of each segment (2, 19, 20). The 19-nucleotide (nt) termini of each UTR are highly conserved across members of the *Arenaviridae* (21–23).

Clinically, BHF development is similar to that of Argentine hemorrhagic fever (AHF), caused by Junin virus (JUNV) (24). The disease incubation period after exposure to MACV is 3 to 16 days (25). The prodromal phase lasts from 1 to 5 days, from which one-third of patients progress into the hemorrhagic/neurological phase (25–28). Recovering patients have an extended 1- to 3-month convalescence period, during which hair loss, dizziness, and fatigue are reported.

There are no licensed or approved treatments for MACV. Immune plasma and ribavirin have been utilized in a limited number of cases, but no trials have been completed (10, 11). Candid#1, the attenuated vaccine strain of JUNV, is licensed for use in the region of endemicity of Argentina (29–31). Recent reports have identified a single mutation in the glycoprotein transmembrane region, F427I, of Candid#1 as playing a key role in attenuating JUNV in a murine model (32, 33). This transmembrane region is highly conserved between MACV, JUNV, and Candid#1 (Fig. 1B) (32, 34, 35). Utilizing a reverse-genetics system our laboratory published previously, we generated a rationally designed recombinant MACV (rMACV) strain containing a single F438I mutation at the GPC transmembrane region (referred to as rMACV-F438I here) by introducing a U-to-A mutation at nucleotide (nt) 1400 in the S segment (36). The rational generation of rMACV-F438I builds upon the first-generation methodology of reassortment used to generate the Lassa virus (LASV) vaccine candidate ML29 (37–39). The following report describes the *in vitro* and *in vivo* characterization of rMACV-F438I. Additionally, we identified instability of the single mutation following *in vivo* challenge, leading to a reversion to the wild-type genotype.

Following the rescue and single passage in Vero cells, the rMACV-F438I titer was 4×10^7 PFU/ml, comparable to those of MACV and rMACV (23, 36, 40). Sequencing of the stock virus confirmed the presence of the single mutation at nt 1400 along with two silent genetic markers at nt 808 and nt 1447 on the S segment: the first allows us to distinguish all recombinant viruses from MACV, and the second distinguishes rMACV-F438I from rMACV (Fig. 2A and B). To determine if the F438I mutation had an impact on viral growth *in vitro*, we infected interferon (IFN)-incompetent Vero cells and IFN-competent A549 cells at a multiplicity of infection (MOI) of 0.01. Tissue culture supernatant (TCS) was collected in triplicate at 0, 24, 48, 72, and 96 h postinfection. The growth curve of rMACV-F438I was similar to those of MACV and rMACV in both cell types (Fig. 2C and D).

To examine the impact of the single mutation on the virulence of rMACV-F438I, we challenged IFN-αβ/γ receptor knockout (IFN-αβ/γ $R^{-/-}$) mice with 10,000 PFU of rMACV ($n = 9$) or rMACV-F438I ($n = 7$) through intraperitoneal injection (36, 41). Changes in body temperature, weight, and disease development were monitored throughout the study. Animals challenged with rMACV followed a similar disease progression as described previously (36). A period of significant ($P < 0.01$, two-way analysis of variance

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Address correspondence to Slobodan Paessler, slpaessl@utmb.edu.

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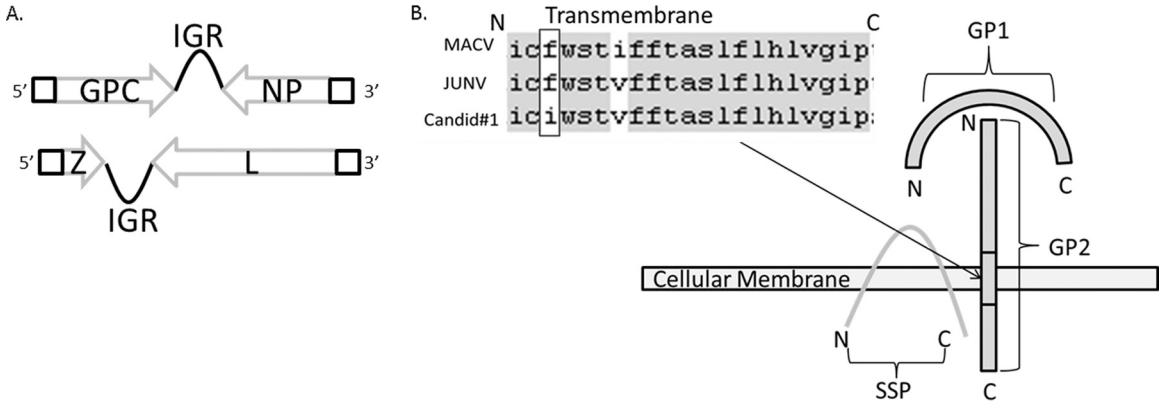

FIG 1 Schematic representation of arenavirus genome and processed glycoprotein. (A) Schematic of the small and large segments of Machupo virus with each gene separated by the IGR. Each open reading frame (ORF) is preceded by an untranslated region, denoted by the bold squares, which plays a pivotal role in secondary structure and polymerase binding. (B) Sequence analysis of the glycoprotein membrane region of MACV, JUNV-Romero, and Candid#1. There is a single amino acid difference of an isoleucine versus a valine between MACV and JUNV-Romero/Candid#1 at amino acid residues 442 and 431, respectively. The box around the residues identifies phenylalanines that are identical in MACV and JUNV but different in Candid#1. Sequence analysis is based upon published online sequences (GenBank accession no. JN794584.1, AY619640, and AY746353.1). The schematic identifies the transmembrane region, which spans the region of GP2 within the cellular membrane.

[ANOVA]) weight loss at 10, 12, 14, and 15 days postinfection (dpi) was identified in rMACV-challenged animals compared to animals challenged with rMACV-F438I (Fig. 3A). Hypothermia was observed in animals 1 to 2 days prior to death (Fig. 3B).

Starting at ~20 dpi, neurological impairment and clinical disease were identified in all rMACV-infected mice. Interestingly, two of the seven animals challenged with rMACV-F438I developed disease symptoms, including scruffed fur and hunched pos-

A.

Virus 5′ to 3′	F438I Mutation nt1400	Silent Marker 1 nt1447	Silent Marker 2 nt808
MACV	uauuuguuucugg	ucuagucggaau	aguuugaccaugu
rMACV	uauuuguuucugg	ucuaguUggaau	aguuugaccaugu
rMACV-F438I	uauuuguAucugg	ucuaguUggaau	aguuugaUcaugu

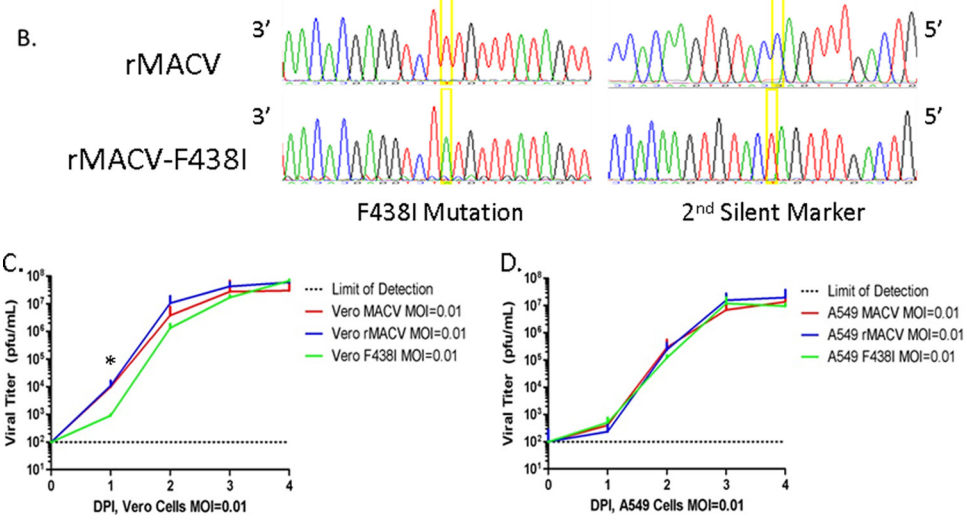

FIG 2 Rescue and *in vitro* analysis of rMACV-F438I. (A) The mutation to generate the F438I amino acid change was accomplished through target PCR mutagenesis by changing the adenosine to a uracil at nt 1400. The addition of a silent mutation at nt 1407 distinguishes all rMACVs from the wild-type virus. The addition of the second marker distinguishes rMACV-F438I from rMACV. Differences between sequences are denoted by a capital letter. (B) Chromatography sequence analysis of viral cDNA confirms the presence of the F438I mutation and both silent markers. (C) TCSs from cells infected at an MOI of 0.01 with MACV, rMACV, or rMACV-F438I in triplicate ($n = 3$) were plaque analyzed to calculate viral titers from 0 to 4 dpi. TCSs collected from IFN-incompetent Vero cells showed similar growth of all three viruses. By 3 dpi, all three viruses reached comparable viral peaks, which were maintained at 4 dpi. (D) TCS collected from IFN-competent A549 cells had no significant difference in viral titers between all three viruses ($P > 0.05$, two-way ANOVA, $n = 3$).

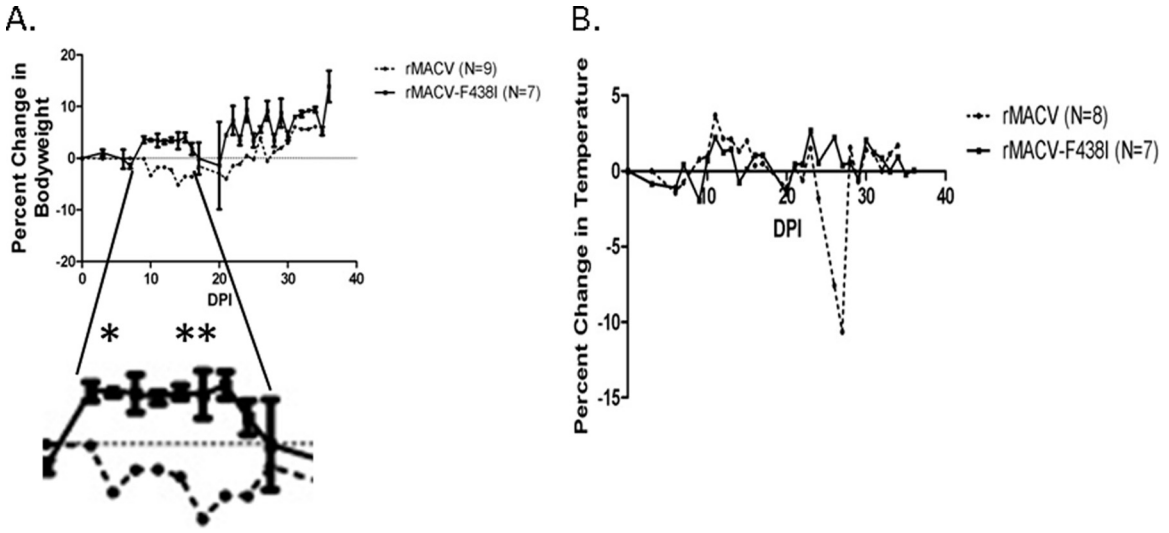

FIG 3 Change in weight and temperature of rMACV and rMACV-F438I-infected mice. (A) Daily collection of weight data from mice infected intraperitoneally with 10,000 PFU of MACV identified a significant difference in weight change during the period from 10 to 15 dpi between the rMACV-F438I- and the rMACV-challenged mice. The only period of significant weight loss identified in the rMACV-F438I-infected mice was 1 to 2 days prior to the two mice succumbing to disease. Asterisks denote significant differences at 9, 13, and 14 dpi ($P < 0.05$, two-way ANOVA). (B) Daily collection of temperature data from infected mice identified no development of febrile disease throughout the study in either group of mice. Hypothermia was commonly identified in rMACV-infected mice 1 to 2 days prior to death.

tures at 19 and 20 dpi, which progressed to rear limb paralysis by 20 and 21 dpi, at which point both animals were euthanized following IACUC protocol requirements. The remaining five animals did not show any observable symptoms. All animals challenged with rMACV developed severe disease starting at 21 dpi and succumbed to disease or were euthanized with a mean time to death (MTD) at 27 dpi. Challenge with rMACV-F438I resulted in a significant reduction in mortality compared to that of rMACV-infected animals ($P = 0.024$, log rank test) (Fig. 4A), suggesting attenuation of this virus.

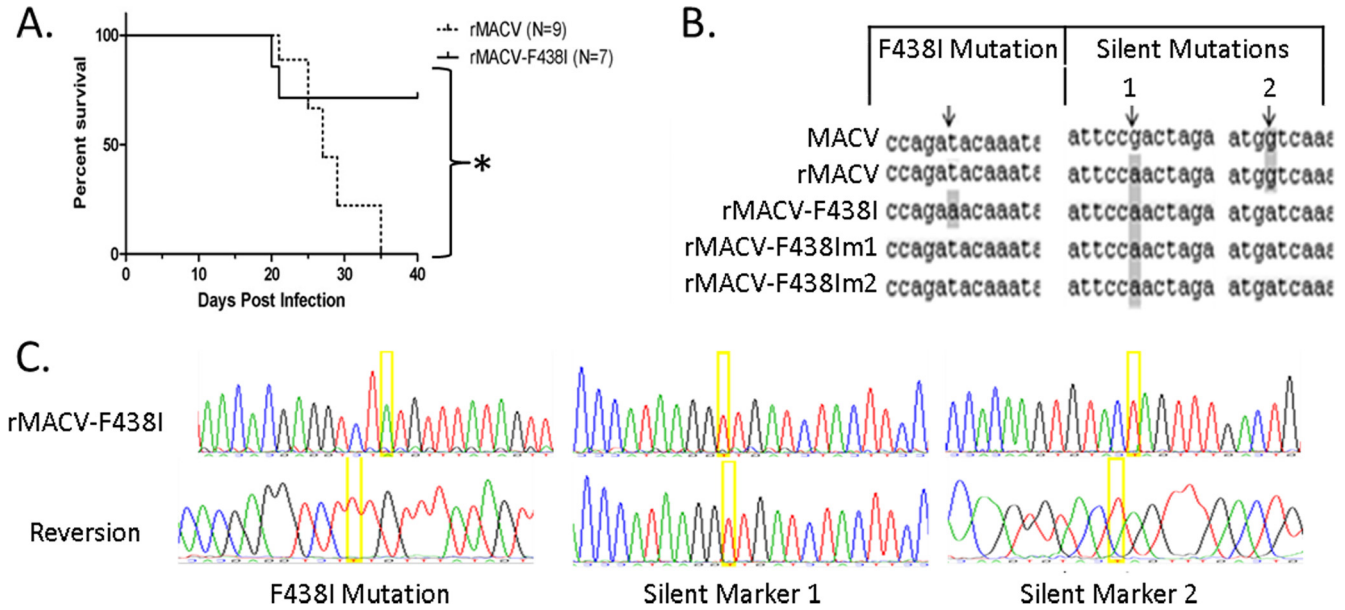

FIG 4 Survival curves of rMACV- and rMACV-F438I-infected mice and sequencing identifying reversion of rMACV-F438I. (A) All mice challenged with rMACV succumbed to disease with an MTD of ~27 dpi, while only 28% of animals challenged with rMACV-F438I succumbed to disease. Mice that succumbed following rMACV-F438I challenge had an MTD of 20.5 dpi. Asterisks denote a significant difference in survival between the two viruses ($P < 0.05$, Mantel Cox). (B) Graphical sequence analysis and comparison of cDNA generated from MACV, rMACV, and rMACV-F438I isolates. rMACV and MACV maintain the wild-type genotype at the nucleotide coding for F438. The nucleotide change coding for I438 is present in the stock of rMACV-F438I, while a reversion has occurred in the viral RNA isolated from mice 1 and 2. The presence of the second silent marker confirms that both animals were challenged with rMACV-F438I. (C) Chromatography analysis of sequenced viral cDNA confirming the reversion of the mutation in the consensus sequence and the presence of both silent markers.

To investigate if any mutation could account for the differential outcome observed in rMACV-F438I-infected mice, we purified viral RNA from the homogenate of the brains, lungs, kidneys, and spleens of the mice. Amplification of viral RNA into cDNA was accomplished utilizing primers designed previously (36). Whole-genome sequencing of viral RNA purified from the lung, kidney, and brain homogenates of both sick mice uniformly identified an A-to-U reverse mutation at nt 1400 of the S segment, resulting in a reversion to the wild-type GPC F438 genotype (Fig. 4B and C). As rMACV contains only one of the silent mutation markers, the presence of both silent genetic markers in these two rMACV-F438I isolates further excluded a possibility of cross-contamination with rMACV and confirmed that they were indeed the MACV-F438I revertants.

The similar growth curves of rMACV-F438I, rMACV, and MACV, as well as the similar stock virus titers between rescued rMACV-F438I and rMACV, indicated that the single mutation did not noticeably impact virus replication in cell culture. This result is comparable to the reports of rJUNV and rJUNV-F427I viral growth kinetics as reported previously but not that of LASV (33).

To identify if the single mutant virus is genetically stable *in vitro*, we serially passaged rMACV-F438I in Vero cells in duplicate. The serial passage was performed at an estimated MOI of 0.01 based on Fig. 2C. At each passage, virus was collected at 3 or 4 dpi followed by infection of fresh Vero cells for five passages. Viral RNAs were prepared from cellular lysate and supernatant and sequenced as described previously (36). Following partial GPC sequencing of all passaged viruses, we observed that the virus maintained the single mutation for the GPC I438 residue as well as both silent markers. No additional mutations were detected in the fifth passage of the virus following whole-genome sequencing, demonstrating the genetic stability of the rMACV-F438I virus *in vitro*.

Challenge of IFN-αβ/γ $R^{-/-}$ mice with rMACV resulted in a biphasic disease, clinical signs, and MTD similar to what our laboratory reported previously when characterizing the model (36). Of the seven animals challenged with rMACV-F438I, two developed severe disease and succumbed to disease. The other five had no identifiable disease throughout the study. The diverse outcomes from these animals are interesting, especially as the two animals had such a rapid decline in health. A significant difference in the mortality rates between rMACV- and rMACV-F438I-infected animals indicated that the single mutation in the GPC transmembrane region could lead to MACV attenuation, the same as JUNV.

Based on the sequence analysis of virus collected from the two animals that succumbed to rMACV-F438I challenge, the phenotypic reversion of rMACV-F438I to the virulent infection was determined by the genotypic reversion of the attenuating GPC mutation at amino acid 438 to the wild type. This reversion was confirmed in all organs examined from both lethally infected mice. The apparent instability of the single mutation *in vivo* leads us to presume that additional mutations are necessary for maintaining attenuation. Introduction of the single GPC F427I mutation in mouse-pathogenic XJ13 JUNV further reduced the virulence; however, no previous reports exist that describe a reversion to wild-type sequence. Taken together with the results from passaging of rMACV-F438I, the single mutant attenuated MACV-F438I strain is genetically stable *in vitro* but not *in vivo*, and hence, the single mutant strain is not sufficient in and of itself as a vaccine candidate.

In summary, in this article we have identified that a single mutation in the glycoprotein transmembrane region of MACV does not change its replication *in vitro*, while it significantly attenuates the virus *in vivo*. This is the first report of a rationally attenuated MACV and provides further evidence of the utility of the reverse-genetics system and mouse model our laboratory has developed to study MACV. The significant role that the F438I mutation plays in attenuating MACV and JUNV leads us to believe that further elucidation of its role will allow for future development of rationally designed vaccines against New World hemorrhagic arenaviruses.

Nucleotide sequence accession numbers. The reference sequences for MACV S and L were submitted to GenBank under accession numbers KM198593 and KM198592.

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