Partial deletion of the L-segment intergenic region produces an attenuated Machupo virus that protects guinea pigs against lethal Guanarito virus infection

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ABSTRACT

Machupo virus strain Carvallo is historically lethal in guinea pigs. We identified a variant termed Car\textsuperscript{91} that failed to produce even mild disease in infected animals. Here, the nature of this attenuation was explored in detail. Car\textsuperscript{91} was serologically indistinguishable from a lower passage isolate termed Car\textsuperscript{68}, however Car\textsuperscript{91} replicated less efficiently in three cell lines. Sequencing analysis revealed Car\textsuperscript{91} had a 35 nucleotide deletion in the L-segment non-coding intergenic region. Contrary to Car\textsuperscript{91}, Car\textsuperscript{68} produced a lethal infection in guinea pigs with a mortality rate of 63%. The ability of non-virulent Car\textsuperscript{91} to protect guinea pigs against a related arenavirus, Guanarito virus, was investigated. All naïve animals succumbed to infection, however; 88% of Car\textsuperscript{91}-infected guinea pigs were protected. Our findings suggest that partial deletion of the arenavirus IGR is a viable means of producing attenuated arenaviruses that can function as broad spectrum arenavirus vaccines.

Keywords: Arenavirus, Machupo virus, Guanarito virus, intergenic region, live-attenuated vaccines, cross-protection
**BACKGROUND**

Arenaviruses are enveloped ambisense single-stranded RNA viruses with two segments, small (S) and large (L), encoding a 10.7 Kb genome expressing five distinct proteins [1-3]. The L-segment encodes the matrix ring finger Z protein [4] and the polymerase L protein (L-segment) [5]. The S-segment encodes the nucleoprotein (NP) and the glycoprotein precursor (GPC) [6]. GPC is cleaved into two glycoproteins, GP1 and GP2 by the cellular protease S1P [7]. Each RNA segment encodes two ORFs and also contains noncoding regions including 5’ and 3’ untranslated regions (UTRs) and a non-coding intergenic region (IGR) [3, 8, 9]. Arenaviruses are divided into the Old World (OW) and New World (NW) complexes based on geographical distribution and serology [2]. Machupo virus (MACV) is a member of the NW complex of arenaviruses and is the causative agent of Bolivian hemorrhagic fever (HF) [2]. Human infections generally result from exposure to chronically infected rodents (*Calomys callosus*) [10], but human-to-human spread has been reported [11]. Bolivian HF is a febrile illness often associated with vascular leakage and occasional concomitant neurological manifestations [11, 12]. Infection can result in a systemic inflammatory response syndrome leading to multiple organ failure and death. Other NW arenaviruses, including Junin virus (JUNV) and Guanarito virus (GTOV), also cause HF in South America [2, 13].

No FDA licensed countermeasures exist to treat arenaviruses. Active and passive vaccine strategies, as well as small molecule inhibitors have been shown to be effective at reducing lethality in humans [14-16]. An attenuated live-virus vaccine, termed Candid#1, is currently used in populations at high risk to JUNV infection in Argentina [17-20]. Implementation of this vaccine in endemic regions reduced fatality rates substantially. Some evidence based on studies in guinea pigs and non-human primates (NHPs) suggest that Candid#1 can cross-protect against
MACV [21]; however, these findings have not been validated in humans. Candid#1 was produced by passage of the virulent XJ strain twice in guinea pigs, 44 times in mouse brains and finally several passages in fetal rhesus lung diploid cells (FRhL-2) [20, 22-24]. This process produced a strain attenuated in humans, non-human primates, guinea pigs, and mice that also lacked neurotropism in animal models. The exact nature of the attenuation is enigmatic and recent evidence suggests that a single amino acid change in the GP2 transmembrane region restores virulence in neonatal mice [22]. Other vaccine strategies have included the use of Tacaribe virus (TACV), an arenavirus serologically related to JUNV and MACV that is not known to be pathogenic in humans. In animal models, TACV functions as a vaccine against JUNV [25, 26]. However the underlying mechanism(s) attenuating TACV in humans is unclear.

Glycoprotein-targeting vaccines based on modified vaccine Ankara (MVA) or Venezuelan equine encephalitis replicon (VRPs) vectored systems protect against lethal infection by JUNV in guinea pig models [27, 28]. However, because of heterogeneity in the glycoproteins [6, 25, 29], it is unclear if these vaccines will provide sufficient cross-protection against other arenaviruses. Thus, strategies aimed at producing safe and broadly protective NW arenavirus vaccines are desired.

MACV strain Carvallo is the prototypical MACV strain first isolated in 1963 [30, 31]. Several studies report this strain is pathogenic in guinea pigs, with lethality upwards of 60% [25, 32]. We previously reported that a MACV strain Carvallo variant (Car⁹¹) does not cause lethal disease in guinea pigs [33]. To reconcile this discrepancy, we compared the attenuated strain with an earlier isolate of strain Carvallo (Car⁶⁸) by evaluating the genetic basis for this attenuation and exploring the virulence of these two Carvallo isolates in guinea pigs, the small
animal model for arenaviruses [31]. We also investigated the feasibility of using the attenuated Car$^{91}$ strain as an arenavirus vaccine.

METHODS

Viruses and cells. GTOV strain INH95551, MACV strain Chicava and two MACV strains of Carvallo from passages dated 1968 (Car$^{68}$) and 1991 (Car$^{91}$) were propagated as previously reported [33]. 239T cells and 104CL guinea pig fibroblasts (ATCC) were maintained in MEM or RPMI containing 10% heat-inactivated fetal bovine serum (FBS), 1% antibiotics (100 U/ml penicillin, 100 µg/ml of streptomycin, respectively. Human umbilical vein cells (HUVECs) (Lonza) were maintained in endothelial growth medium.

Plaque reduction and neutralization tests (PRNTs). PRNTs were performed as previously described [34] using rabbit antiserum targeting MACV glycoprotein [33] or guinea pig serum (this study) serially diluted two-fold starting at 1:40. Percent neutralization was calculated relative to the number of plaques in the presence of negative control serum. Titers represent the reciprocal of the highest dilution resulting in a 50% reduction in the number of plaques. Data were plotted using Graphpad Prism software.

Growth kinetics. Vero, 104CL and HUVECs were seeded at a density of 1 x $10^5$ cells per well in 24-well plates and infected at an MOI of 0.1 with the indicated viruses diluted in culture medium. Virus growth at 24, 48 and 72 h was determined by plaque assay on Vero cell monolayers. All samples were run in two independent replicates and plotted as the mean +/- standard deviation (SD) using Graphpad Prism software.
Particle-to-PFU ratio. Particle counts were determined with a Virocyt machine (Virocyt, Boulder, CO) using the manufacture’s protocol. The particle-to-pfu ratios were determined by dividing particle counts by the amount of infectious virus. Four independent virus preparations per strain were used in the calculations.

Genome sequencing. RNA was extracted from Trizol homogenates of MACV, converted to cDNA, and subjected to sequence-independent, single primer amplification (SISPA) [35]. The products of these reactions were used to generate libraries that were sequenced on an Illumina MiSeq. Sequencing reads were assembled using DNAStar SeqMan NGen. Predicted secondary structures of the IGRs were determined using DNAStar Genequest.

Pseudovirion neutralization assay (PsVNA) and ELISA. The pseudovirion neutralization assay (PsVNA) has been described in detail elsewhere [33, 36]. Briefly, a vesicular stomatitis virus backbone with a luciferase reporter gene (PsV) was used to produce particles decorated with glycoproteins from MACV, JUNV and GTOV. These particles were subsequently incubated with the indicated serially diluted sera in triplicate and the geometric mean PsVNA80 titers (GMT) plotted. The use of PsV as solid phase antigen in ELISAs has been previously described in detail [33].

Challenge of Hartley guinea pigs. Female Hartley guinea pigs (300-400g) were implanted with IPTT-3000 identification chips to monitor temperature (BMDS INC; Seaford, DE). Animals were challenged with the indicated MACV strains (1,000 pfu) or GTOV (2,000 pfu) diluted in a total volume of 0.5 ml PBS by intraperitoneal (i.p.) injection. Animals were weighed and
monitored for fever. All animal studies were conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council [37]. The facilities where this research was conducted are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Animals meeting criteria were humanely euthanized.

**Statistical analysis.** Two-way ANOVA with the Bonferroni correction was used to analyze both weight and viral replication (*in vitro*). Log-rank test was performed for statistical analysis of survival. The statistical significance of PRNTs was determined using an unpaired two-tailed Student’s *t* test. Significance levels were set at a *p* value less than 0.05. All analyses were performed using Prism software.

**RESULTS**

**In vitro characterization of two MACV strain Carvallo passages and strain Chicava.** We previously reported that MACV strain Car91 does not produce acute disease in Hartley guinea pigs ([33] and SFig.1). Because the failure of Car91 to cause lethality was unexpected, we produced another stock of virus derived from an early passage of strain Carvallo (1968) and designated it Car68. We then compared growth characteristics of both viruses. Plaque morphology on Vero cells was similar between Car91 and Car68 (*data not shown*). By examining the particle-to-pfu ratio, we determined that Car91 had more defective particles compared to Car68 and MACV strain Chic (*Fig. 1A*). Car91 had the highest particle-to-pfu ratio with a geometric mean of 369, compared to Car68 and Chic whose GMT ratios were 26 and 13, respectively.
However, these differences were not statistically significant (T-test and one-way ANOVA; p>0.05).

The growth kinetics of all three strains was investigated in Vero cells, 104CL guinea pig fibroblasts and HUVECs. Cells were infected with Car\textsuperscript{91}, Car\textsuperscript{68} or Chic and replication was assayed at 24, 48 and 72 h post-infection (hpi) (Fig. 1B). After 24 h, Car\textsuperscript{68} grew to the highest levels in both Vero and HUVEC. 24 h growth for all three viruses in 104CL cells was markedly lower than Vero and HUVECs, however Car\textsuperscript{91} titers were the lowest in these cells. At 48 hpi, Car\textsuperscript{91} replication was still reduced compared to the other viruses in 104Cl and Vero cells, however in HUVECs Car\textsuperscript{91} and Car\textsuperscript{68} had similar titers. After 72 h, Chic had the highest titers in Vero and HUVECS. Titers of Car\textsuperscript{68} and Chic were similar in 104CL cells. Overall, Car\textsuperscript{91} replicated the poorest in all cell types with titers several fold lower than those of Car\textsuperscript{68} and Chic. The replication differences between Car\textsuperscript{91} and Car\textsuperscript{68} were statistically significant (two-way ANOVA; p<0.05) at 72 h in HUVECs and 104CL cells, but not in Vero cells. Growth titers were also significant different between Car\textsuperscript{91} and Chic at 72 hpi in all cell types (two-way ANOVA; p<0.05). Together these findings indicated that MACV strain Car\textsuperscript{91} does not replicate as efficiently in cell culture compared to other strains of MACV, including an older isolate of Carvallo from 1968.

Evaluating differences in neutralizing antibody titers is a stringent means of determining if NW arenavirus strains are highly homologous [29, 38]. Therefore, we confirmed that Car\textsuperscript{91} and Car\textsuperscript{68} were serologically identical by determining the PRNTs of neutralizing rabbit antisera targeting the MACV strain Carvallo GP1 and GP2 proteins against these two isolates [33]. We also compared the ability of this serum to neutralize the heterologous strain Chic. As expect, titers against heterologous strain Chic were markedly reduced and exhibited a ~10-fold reduction.
comparing to Car\textsuperscript{91} and Car\textsuperscript{68} (Fig. 1C). The PRNT50 values of Car\textsuperscript{68} to Chic were statistically significant (T-test; \(p=0.0481\)), but not the PRNT80 values (T-test; \(p=0.1168\)). In contrast, titers between Car\textsuperscript{91} and Car\textsuperscript{68} were not statistically different (Car\textsuperscript{91} versus Car\textsuperscript{68}; T-test; PRNT50 \(p=0.3436\) and PRNT80 \(p=0.9130\)) indicating these two isolates are serologically indistinguishable.

**MACV strain Car\textsuperscript{91} is missing a region of the L segment IGR.** The genomes of Car\textsuperscript{91} and Car\textsuperscript{68} were sequenced. Sequencing revealed five changes between strains Car\textsuperscript{68} and Car\textsuperscript{91} in the L-segment (Fig 2A). Three changes resulted in undisruptive silent nucleotide (nt) substitutions in the polymerase protein sequence. Another nt change in the Car\textsuperscript{91} IGR at position 399 (C\(\rightarrow\)T) was detected that matched the reference strain Carvallo sequence (Genbank accession #NC005079). We also identified a 35 nt deletion in the IGR of strain Car\textsuperscript{91} (Fig. 2B). No changes in the S segment were identified between Car\textsuperscript{91} and Car\textsuperscript{68}. These findings demonstrated that Car\textsuperscript{91} is missing a significant part of the L-segment IGR relative to the earlier passaged Car\textsuperscript{68}. This deletion resulted in a predicted IGR structure with a \(\Delta G\) value of -16.2 kcal/mol compared to -51.4 kcal/mol of Car\textsuperscript{68} (Fig 2C). Thus, the Car\textsuperscript{68} structure is predicted to be more thermodynamically stable compared to Car\textsuperscript{91} by 3.2-fold. These findings suggest attenuation of Car\textsuperscript{91} is related to the IGR deletion.

**MACV strain Car\textsuperscript{68} and Chic are lethal in guinea pigs while strain Car\textsuperscript{91} is attenuated.** The virulence of strain Car\textsuperscript{68} was examined in Hartley guinea pigs to determine if, contrary to Car\textsuperscript{91}, this isolate produced acute disease. Animals were also infected with Chic, a strain known to cause lethal disease in this model [39]. Groups of eight animals were infected with the indicated strains and survival, weight and fever were monitored for 30 days (Fig. 3). All animals infected with strain Chic begin to lose weight between days 8-20 (Fig. 3B), but only one animal...
developed high fever (>41.0°C) (Fig. 3C). Chic-infected animals succumbed to infection by day 24. Animals infected with Car$^{68}$ displayed weight loss between days 9-21, but none of the animals developed high fever (>41.0 °C). Car$^{68}$ also produced a lethal disease in guinea pigs; however three animals survived infection (~63% mortality rate). Distinct from Chic, three Car$^{68}$ infected animals developed paralysis starting with the hind-limb and were euthanized on day 21. The three surviving Car$^{68}$ infected animals began to rapidly increase in weight after a period of weight loss, and by day 30 they exceeded their starting weight by ~3-20%. The mean time to death (MTD) for Car$^{68}$ and Chic was 23.5 and 22 days, respectively. Confirming our earlier observations (SFig. 1), animals infected with Car$^{91}$ survived infection without displaying signs of disease (Fig. 3A). Survival differences between Car$^{68}$ and Chic were not significant (log-rank; p=0.1331) however, the survival of Car$^{91}$ versus Car$^{68}$ were highly significant (log-rank; p=0.0082). Additionally, weight loss between Car$^{68}$ and Chic were significant compared to Car$^{91}$ for several days (Two-way ANOVA; p<0.05). Viremia was detected in all four Chic infected animals that were euthanized due to disease severity with GMT titers of 1088 pfu/ml (Fig. 4A). Only one Car$^{68}$ animal had detectable viremia (166 pfu/ml), and viremia was undetected in the Car$^{91}$ infected group. These results demonstrated that contrary to Car$^{91}$, the Car$^{68}$ isolate can produce a severe and lethal disease in guinea pigs.

We evaluated the serum from Car$^{91}$-challenged guinea pigs for the presence of binding and neutralization antibodies 30 days post-challenge. ELISA titers were determined using VSVΔG particles pseudotyped with glycoproteins from the MACV strain Carvallo as antigen. Six of eight guinea pigs had detectable antibodies against MACV glycoprotein with a log$_{10}$ GMT of 2.8 (Fig. 4B). MACV neutralizing antibody was detected in all but two infected animals with...
PRNT50 and PRNT80 GMTs of 269 and 59.5, respectively (Fig. 4C). The same two guinea pigs had no detectable PRNT50 or ELISA titers.

**MACV strain Car⁹¹ protects guinea pigs against lethal infection by GTOV.** Because strain Car⁹¹ was highly attenuated in guinea pigs but produced detectable immune responses in 10/12 guinea pigs (SFig. 1C and Fig. 4BC), we hypothesized that it might function as an attenuated vaccine. Therefore, we examined the ability of Car⁹¹ to protect guinea pigs against GTOV, a distantly related NW arenavirus species [40]. Eight guinea pigs were challenged with GTOV 45 days after exposure to Car⁹¹ (Fig. 5). As a control for acute infection, a group of six weight-matched naïve guinea pigs were also infected with GTOV. Animals were monitored for survival, weight loss and fever over 25 days (Fig. 5A-C). Consistent with previous findings [33, 41], control animals began to lose weight starting around day 6 with concomitant fever. All control animals succumbed to infection with a MTD of 16 days. All but one animal previously exposed to Car⁹¹ survived infection. The single non-survivor succumbed to disease on day 17 after a period of weight loss and mild fever (~40.3°C).

The presence of binding antibody against MACV, JUNV and GTOV were evaluated by ELISA using sera from Car⁹¹-exposed (vaccinated) guinea pigs collected prior to and 30 days after GTOV challenge (Fig. 6A). Prior to GTOV challenge, six of eight animals infected with MACV strain Car⁹¹ had detectable antibodies against MACV with a log₁₀ GMT of 2.5. These responses increased following GTOV challenge to 3.6, but this was not significant (T-test; p=0.1461). Antibody titers against GTOV prior to GTOV challenge were low or below detection. However, antibody titers against GTOV rose significantly (T-test; p=0.0002) after GTOV challenge to a log₁₀ GMT of 2.5. Antibody titers against JUNV were also detected prior to GTOV challenge (log₁₀ GMT 2.0) in all but one animal and these responses increased to...
statistically significant levels (T-test; \( p=0.0242 \)) with a \( \log_{10} \) GMT titers of 3.0 after GTOV challenge. Animal#4, which succumbed to GTOV infection despite receiving Car\(^91\), had undetectable ELISA tiers against MACV, GTOV and JUNV and an undetectable PRNT50 titer against MACV. Animal#1 survived GTOV challenge despite having no detectable humoral response against GTOV and MACV, and a low response against JUNV ELISA antigen.

The PRNT titers against MACV significantly increased subsequent to GTOV challenge, with GMT PRNT80 titers rising from 59 to 320 (T-test; \( p=0.0112 \)) (Fig. 6B). Despite the presence of MACV, GTOV and JUNV IgG antibody in all animals surviving GTOV challenge, we did not detect any neutralizing activity against GTOV or JUNV (Fig. 6C). These findings demonstrated that guinea pigs exposed to MACV strain Car\(^91\) are protected against GTOV in the absence of a detectable neutralizing antibody response against the challenge virus.

DISCUSSION

The IGR is situated between each encoded ORF [8], and plays important roles in transcription and production of infectious progeny virions [42]. Mechanistically, IGRs fold into single or double stem-loop structures and are essential for transcription termination. Because the tertiary structure of the IGR is critical for mRNA transcription termination, modifications can significantly impact the efficiency of replication by disrupting protein synthesis. For example, truncation of the Lujo virus (LUJV) L-segment IGR produces a virus that replicates much less efficiently \textit{in vitro} due to inefficient gene transcription [43]. Our findings strongly indicate that spontaneous deletion of the L-segment IGR is responsible for the loss of virulence of MACV strain Car\(^91\) in guinea pigs. Car\(^91\) had reduced replication fitness \textit{in vitro}, however it is unclear if this causes its attenuation \textit{in vivo}. Reduction in the size of the IGR may impact innate immune
signaling pathways within infected cells and this may play a role in attenuation. Future studies will be needed to fully elucidate the mechanics of how the partial IGR deletion produces an attenuated virus. It is unclear how this mutation arose during viral passage. Some work has shown that MACV can be attenuated by cell culture passage [20], but these studies did not report if attenuation involved IGR modification.

Recent work has focused on exploiting alterations in the IGR as a means of producing rational whole-virus vaccines against arenaviruses. Addition of synthetic (non-viral S-IGR like) sequence or swapping the IGRs of the L- and S-segments produces attenuated viruses that can protect mice against secondary challenge with wild-type LCMV [44, 45]. Our work demonstrates that partial deletion of the IGR can also produce an attenuated virus that functions as a vaccine. Whether substitution of the IGR, incorporation of a synthetic IGR, or deletion of the IGR is the best approach in live-attenuated arenavirus vaccine development remains to be determined. One advantage to deletion of multiple nucleotides is that reversion to wild type is improbable. Work involving the arenavirus IGR as a vaccine strategy has focused on OW arenaviruses, specifically LCMV. Our study advances these vaccine strategies by demonstrating that alteration of the IGRs is a powerful means of producing an attenuated live-virus vaccine against NW arenaviruses.

A major goal of arenavirus vaccine development is a pan-arenavirus vaccine that protects against heterologous species either within the OW and NW complexes or even more broadly. Previous work has shown that JUNV, MACV and TACV can cross-protect animals against each other [21, 25]. To more thoroughly gauge the level of cross-protection against NW arenavirus, we purposely challenged MACV Car91-vaccinated guinea pigs with the more serologically distant GTOV [33, 40]. The single animal succumbing to GTOV challenge failed to produce detectable antibodies against even MACV. The dose of Candid#1 in humans is 40,000 pfu [17],

whereas the dose used here was 1,000 pfu. We predict a higher dose of Car$^{91}$ would have elicited more robust and protective immune responses. Curiously, neutralizing antibody against GTOV was not detected in any animal even after GTOV challenge. Other studies demonstrate that TACV protects against JUNV infection in the absence of neutralizing antibody responses targeting the challenge virus [25]. These findings suggest that non-neutralizing antibody and/or CD8-mediated T-cell responses may play a role in protection. Although, more work will be needed to fully address the correlates of protection, our findings clearly indicate that modification of the IGR can produce cross-protective immune responses against distantly related arenaviruses.

Within the NW complex are several other arenaviruses that cause HF disease in humans, including JUNV and GTOV [13]. Other NW human pathogenic HF arenaviruses, have more recently emerged, including Sabia virus, Chapare virus and Whitewater Arroyo virus [2, 13, 46-48]. The perpetuation of arenaviruses within rodent populations [31], combined with the recent emergence of novel human arenaviruses capable of causing severe human disease, indicates this is an important family of emerging and re-emerging human pathogens. Our study supports the use of live-attenuated vaccine strategies aimed at protecting against multiple NW arenaviruses. Overall these findings will help guide the development of rationally designed attenuated pan-arenavirus vaccines.

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FOOTNOTES

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Potential conflicts of interest. J.W.G and J.W.H. have applied for a patent regarding the use of partially deleted IGR regions as live-attenuated NW arenavirus vaccines.
REFERENCES


FIGURE LEGENDS

**Figure 1. In vitro characterization of Car⁹¹, Car⁶⁸ and Chic.** A. Particle-to-pfu ratios for each of the indicated MACV strains were determined using a ViroCyt system. Four virus preparations for each virus were tested in triplicate. The geometric means are indicated by the solid line. B. The indicated cell types were infected with Car⁹¹ (circles), Car⁶⁸ (squares) or Chic (triangles) at an MOI of 0.1 and replication was quantitated at 24, 48 and 72 hpi by plaque assay. All samples were titered in duplicate and the mean +/- SD were graphed. C. Anti-glycoprotein antisera from four rabbits vaccinated by DNA vaccination with MACV strain Carvallo GPC [33] was tested for capacity to neutralize Car⁶⁸, Car⁹¹ and Chic. PRNT50 and PRNT80 titers were plotted. The dashed line denotes the limit of detection. The solid line represents the GMT values. For all panels, asterisks denote statistical significance.

**Figure 2. Sequence of MACV strain Car⁹¹ and Car⁶⁸.** A. Predicted L-segment amino acid changes between Car⁹¹ and Car⁶⁸. B. Alignment of the Car⁹¹ and Car⁶⁸ IGR. The underlined region denotes the region deleted in Car⁹¹. Also underlined is the single nucleotide change at position 399. C. Predicted hairpin tertiary structure of the Car⁹¹ and Car⁶⁸ L-segment IGR.

**Figure 3. Infection of Hartley guinea pigs with MACV strain Car⁶⁸, Car⁹¹ and Chicava.** A. Guinea pigs were challenged with MACV strains Car⁹¹, Car⁶⁸ and Chic. Survival was monitored for 30 days post-infection. Asterisks denote statistical significance.


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B. Percent weight loss for individual guinea pigs in the indicated groups was plotted based on day 0 starting weight. Animals succumbing to infection are shown in red. C. Group temperatures were plotted. Normal temperature range is shaded in grey. For visualization, data for Car\textsuperscript{91} is depicted in orange.

**Figure 4. Evaluation of serum from infected guinea pigs.** A. Serum viremia was determined on Vero cell monolayers using guinea pig serum taken from survivors on day 30 (black) or when animals were euthanized (red). The solid line represents the GMT values. B. Antibody binding ELISA titers were determined by incubating sera from Car\textsuperscript{91} infected animals with PsVs pseudotyped with GPC from Strain Carvallo. Antiserum samples were serially diluted prior to incubation. The dashed line denotes the limit of detection. The solid line represents the GMT values. C. Titer of neutralizing antibody in Car\textsuperscript{91} infected animals was determined and PRNT\textsubscript{50} and PRNT\textsubscript{80} titers were plotted. The dashed line denotes the limit of detection. The solid line represents the GMT values.

**Figure 5. Protective efficacy of MACV strain Car\textsuperscript{91} against lethal GTOV challenge in guinea pigs.** A. Survival plot of naïve (red squares) and Car\textsuperscript{91}-vaccinated (black circles) guinea pigs infected by the i.p. route with 2,000 pfu GTOV. Survival was plotted for 30 day post-infection. B. Percent loss from starting weight was plotted for each group as described above. C. Temperature was monitored as in Fig. 3.

**Figure 6. Binding and neutralizing antibody responses in guinea pigs infected with GTOV.**
A. Antibody binding titers were determined by coating 96-well plates with the indicated PsVs...
and incubating them with serially diluted antiserum samples from before GTOV challenge (circles/PRE) or after GTOV challenge (squares/POST). The dashed line denotes the limits of detection. The red circle denotes the single animal (Animal #4) that succumbed to infection. The blue symbols denote the same animal before and after GTOV challenge. Note that titers against MACV prior to GTOV challenge are also depicted in Fig. 4B. B. PRNT80 titers against Car$^{68}$ prior to and after challenge with GTOV were determined as above. Note that PRNT80 titers prior to GTOV challenge are also depicted in Fig. 4C. C. PRNT50 titers against JUNV, MACV and GTOV were determined as in Fig. 2. Titers were determined as described above. The dashed line indicates the limits of detection. For all panels, asterisks denote statistical significance.
Supplemental material

MACV strain Carvallo is not lethal in Hartley guinea pigs. MACV strain Car\textsuperscript{91} was tested for its ability to produce acute disease in Hartley guinea pigs. Six guinea pigs were infected with 2000 pfu of strain Car\textsuperscript{91} and monitored for weight (SFig. 1A) and temperature (SFig. 1B) for 28 days post infection. All animals gained weight over the course of the study and by day 28 was >30\% higher than the starting weights. Animals did not exhibit signs of infection, such as lethargy and no animal produced a fever. Serum from infected animals was tested for MACV neutralizing antibody using VSV particles pseudotyped with the MACV strain Carvallo glycoprotein molecules. Each guinea pig produced a neutralizing antibody response with the geometric mean titer (GMT) PsVNA80 of 177 (SFig. 1C). These results indicated that contrary to published reports \cite{25,32}, MACV strain Carvallo failed to produce acute disease in infected guinea pigs. However, the virus was able to elicit a neutralizing antibody response in inoculated animals.

Supplemental Figure 1. Infection of Hartley guinea pigs with MACV strain Carvallo. A. Guinea pigs were infected with 2000 pfu MACV strain Car\textsuperscript{91} and weights based on day 0 starting weight were graphed. B. Percent change in temperature relative to day 0 was plotted. C. Serum from guinea pigs was incubated with VSV\textDelta G particles pseudotyped with MACV strain Carvallo glycoprotein and the PsVNA80 titers were graphed.
### A

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*Reference Carvallo strains accession #NC005079

### B

**L-segment IGR**

Car\(^{91}\): CATCCCCAAAACCTCGGTTCCACAAGACCCCCGGGGGGGGGGGGGGGGGGGGGGGGGATCCCCGGGGGTCTTGGGCTGCG...

Car\(^{68}\): CATCCCCAAAACCTCGGTTCCACAAGACCCCCGGGGGGGGGGGGGGGGGGGGGGGGGATCCCCGGGGGTCTTGGGCTGCG...

### C

**Car\(^{91}\)**

\[ \Delta G = -16.2 \text{ kcal/mol} \]

**Car\(^{68}\)**

\[ \Delta G = -51.4 \text{ kcal/mol} \]
A

B

C
SupFIG. 1