Identification and Characterization of a Ross River Virus Variant That Grows Persistently in Macrophage, Shows Altered Disease Kinetics in a Mouse Model and Exhibits Resistance to Type I Interferon

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Running title: Ross River virus macrophage persistent variant exhibits enhanced virulence

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Abstract

Alphaviruses such as chikungunya virus, o’nyong-nyong virus and Ross River virus (RRV) cause outbreaks of human rheumatic disease worldwide. RRV is a positive sense single stranded RNA virus endemic to Australia and Papua New Guinea. In this study, we sought to establish an in vitro model of RRV evolution in response to cellular antiviral defence mechanisms. RRV was able to establish persistent infection in activated macrophages, and a small plaque variant (RRV<sub>PERS</sub>) was isolated after several weeks of culture. Nucleotide sequence analysis of RRV<sub>PERS</sub> found several nucleotide differences in the non-structural protein (nsP) region of the RRV<sub>PERS</sub> genome. A point mutation was also detected in the E2 gene. When compared to the parent virus (RRV-T48), RRV<sub>PERS</sub> showed significantly enhanced resistance to IFN-β-stimulated antiviral activity. RRV<sub>PERS</sub> infection of RAW 264.7 macrophages induced lower levels of IFN-β expression and production when compared to infection with RRV-T48. RRV<sub>PERS</sub> was also able to inhibit type I IFN signaling. Mice infected with RRV<sub>PERS</sub> exhibited significantly enhanced disease severity and mortality than mice infected with RRV-T48. These results provide strong evidence that the cellular antiviral response can direct selective pressure for viral sequence evolution that impacts on virus fitness and sensitivity to IFN-α/β.

Keywords: Ross River virus, small plaque variant, viral persistence, alphavirus, immune evasion, interferon
Introduction

Arthritogenic alphaviruses are distributed globally and are maintained in nature by cycles of transmission between haematophagous arthropods (for example, mosquitoes) and enzootic vertebrate hosts (mammals, marsupials or birds) (33, 53). Nearly all symptomatic infections with these alphaviruses manifest with joint symptoms (arthritis and arthralgia), with myalgia, rash and lethargy also common. Ross River virus (RRV) is an Australian alphavirus associated with chronic polyarthritis and causes up to 8,000 cases annually in Australia with the number of cases reported in new localities increasing (42, 55). During the late 1970s and early 1980s, a number of South Pacific island nations experienced a major outbreak of RRV disease (RRVD) affecting more than 50,000 people (17). More recently, a related virus, chikungunya virus, caused similar rheumatic disease in one third of the population of Réunion Island in the Western Indian Ocean and an estimated 1.39 million cases in India (22, 23, 37, 52). This outbreak was also associated for the first time with some severe clinical manifestation and mortality (37).

In RRV-infected patients, the disease is usually severe at onset with a gradual resolution over three to six months (40). Alphavirus-induced rheumatic disease is thought to have a substantial immunopathological component. For example, in animal models tissue damage results from the induction of pro-inflammatory cytokines and chemokines and recruitment of macrophages in response to infection (29, 43, 55). RRV infects and replicates in human and mouse macrophages, and infection of the mouse macrophage cell line RAW 264.7 results in the establishment of a persistent productive infection (31, 36, 60), which has been regarded as a model of how RRV persists in synovial tissues and produces arthritis/arthralgia (54, 55). Persistence of RRV occurs in RAW 264.7 cells despite the ability of these cells to produce IFN-β (30, 36), which is known to effectively control alphavirus replication (44).
There is considerable evidence that RRV can persist and establish chronic infection. For example, Soden et al. (49) showed genetic evidence of RRV in synovial tissue of two patients from a cohort of twelve, five weeks after initial symptoms of RRV infection were reported. We have also obtained evidence for chronic infection in a mouse model of RRV infection, with RRV RNA being detected in the ankle joints of mice three months after infection (N. Rulli and S. Mahalingam, unpublished data). A number of cell culture models of RRV persistence have also been described. Journeaux et al. (21) established long-term infection (up to 35 days) of primary human synovial cell cultures (that were found to contain “macrophage-like cells”). Small plaque variants developed in these cultures, suggesting that RRV underwent mutation and adaptation to the culture conditions. We have previously described persistent RRV infection in macrophages for periods up to 180 days (60). We have also recently observed persistent infection in murine and human osteoblast cultures, suggesting that these cells may also be persistently infected in vivo (N. Rulli, R. Li, P. Smith, A. Choo, C. Musso, YC. Su, B. Lidbury and S. Mahalingam, unpublished data). Long-term persistence of antigen from the related alphavirus chikungunya has recently been reported in perivascular synovial macrophages in one chronic patient 18 months after initial infection (19). In a macaque model, Labadie et al observed long-term chikungunya virus infection in joints, muscles, lymphoid organs, and liver, which may explain the long-lasting disease symptoms observed in humans (26). In addition, the authors identified macrophages as the main cellular reservoirs during the late stages of chikungunya virus infection in vivo (26).

Here we describe the generation of a small plaque mutant of RRV (RRV_PERS) derived from persistently infected RAW 264.7 macrophages that had been stimulated with LPS to induce an antiviral phenotype, including the production of IFN-α/β (30, 34). RRV_PERS displayed significantly increased resistance to IFN-β-induced antiviral activity compared to the parental
RRV-T48 virus. RRV$_{PERS}$ also induced lower levels of type I IFN compared to RRV-T48 and was able to inhibit type I IFN signaling. RRV$_{PERS}$ infection of mice resulted in increased morbidity, mortality and disease kinetics. Sequencing of RRV$_{PERS}$ identified 12 substitutions in the E2 and nsP regions of the RRV$_{PERS}$ genome. The study provides the evidence for an arthrogenic alphavirus that specifically targets host IFN-α/β responses leading to increased pathogenicity. RRV$_{PERS}$ appears to have evolved specific strategies to counteract the IFN-α/β induced antiviral responses, and we speculate that a similar evolution may occur in vivo.

**Materials and Methods**

**Ross River Virus and Macrophage cell line.** Ross River virus (RRV) derived from an infectious clone of strain T48 (originally designated RR64) (25) was used to initially infect the mouse macrophage cell line RAW 264.7 (ATCC TIB-71) cultured at $5.0 \times 10^5$ cells per well/ml in 24-well trays (Nunc, Roskilde Denmark). To produce infectious virus, pRR64 was linearised by Sac I digestion, transcribed *in vitro* from the cDNA using SP6 RNA polymerase, and the infectious RNA transfected into BHK-21 cells as previously described (25). Viral stocks were propagated in Vero cells (ATCC CCL-81), as previously described (28), and no virus stock exceeded two Vero cell passages prior to experimental use. Viral titers were determined by plaque assay on Vero cells (see details below).

RAW 264.7 cells were maintained in EMEM (Thermo-TRACE, Melbourne) supplemented with 5% heat-inactivated fetal calf serum (HI-FCS) (Thermo-TRACE), 1% Penicillin/Streptomycin 1-1.5% sodium bicarbonate and 2.0 mM L-Glutamine (Thermo-TRACE). Following RRV infection, 5.0 ng/ml lipopolysaccharide (LPS, *E.coli* serotype 0111:B4; Sigma) was added to the cultures, and this LPS concentration maintained over the entire experimental...
RAW 264.7 cells were infected with RRV at a multiplicity of infection (MOI) of 0.1 (in PBS + 1% HI-FCS) for one hour at 37°C. The virus inoculum was removed and 1.0 ml of fresh EMEM-FCS added to each well, after which the infected cultures were further incubated at 37°C (5.0% CO₂, 95% humidity). Culture supernatants were collected at several time points (days 1, 2, 5, 14 and 21) for RRV plaque assay. Fresh and warmed EMEM-FCS (500 µL per well) was added to each well after sample collection.

Detection and purification of small plaque RRV. The parent RRV-T48 plaques are visible on Vero cell monolayers by day 2 post-infection. To visualise the small plaques by eye, an extra day of incubation was required at room temperature. At day 14 post-infection (approximately 90% small plaques), small plaques were purified by picking individual isolated small plaques from the stained Vero cell monolayer. An isolated large plaque was also purified from the same culture. Infected Vero monolayers were incubated under a semi-solid agar overlay (complete M199 [Thermo-TRACE] or DMEM supplemented with 2.0% FCS, 0.02% DEAE-Dextran, 4.0% heat-inactivated new born serum [Thermo-TRACE] and 1.0% agar [Bacto Laboratories Pty. Ltd., Liverpool, NSW, Australia], penicillin/streptomycin, l-glutamine and sodium bicarbonate at the final concentrations described above) and at day 2 or 3 of incubation 0.02% neutral red in phosphate-buffered saline (PBS, pH 7.4) supplemented with 2.0% penicillin/streptomycin and 0.7% agar was added to the agar overlay, and the plaque assays incubated for a further 1-2 hours at 37°C. The agar plugs were carefully removed and individual plaques were picked by holding a pipette tip directly on top of the plaque and gently pipetting 10 µL of complete EMEM-FCS five times onto the plaque. After the final wash, the 10µL sample was diluted in 1.0 mL of complete
EMEM-FCS and added to a fresh monolayer of confluent Vero cells in a 25cm³ flask (Corning, NY, USA) and the culture incubated at 37°C (5.0% CO₂, 95% humidity). As soon as cytopathic effect was observed in the Vero cell monolayer (2-3 days post-infection), the infectious supernatants were collected and centrifuged to remove cell debris (400 g). Plaque assays were performed to confirm the small plaque phenotype in fresh Vero cell cultures, and to estimate the virus concentration (Log₁₀ PFU/mL). This plaque purification procedure was then repeated on isolated plaques found on the fresh Vero cell monolayers. After the second round of purification, stocks of small and large plaque (parent) virus were grown in fresh Vero cell cultures, as described above; these stocks were used for all subsequent experiments and nucleotide sequence analyses. The small plaque RRV was designated RRVₚₑʳₛ. These studies are covered by a license from the Australian Government’s Office of Gene Technology Regulator (licence number DNIR 389/2006).

**RRV neutralisation by polyclonal anti-RRV sera and anti-RRV-E2 monoclonal antibodies.**

Murine anti-RRV polyclonal serum was added to Hanks balanced salt solution (HBSS [pH 7.2], Thermo-TRACE, Melbourne) containing 2% bovine serum albumin (w/v, BSA, Sigma, MO), to a final dilution of 5.0 x 10⁻¹. Serial 10-fold dilutions of this polyclonal serum were then prepared. An equal volume of HBSS-BSA containing 200 PFU of RRV-T48 or RRVₚₑʳₛ was added to the diluted antibody. The antibody:virus preparations were incubated at 37°C for 1 hour, after which 100 µL was plated onto confluent Vero cell monolayers and incubated at 37°C (5% CO₂, 95% humidity) for 48 hours. The monolayers were stained for plaque enumeration exactly as described above.

Reaction of RRVₚₑʳₛ or RRV-T48 with E2 specific monoclonal antibodies (Mabs) was
performed exactly as described above for polyclonal sera. Mabs designated 10C9, 3C4 and E7 were generously provided by Dr Ron Weir (Australian National University) and Dr Roy Hall (University of Queensland) from a previously described antibody panel (6).

**RNA extraction and isolation of RRV genomic cDNA by Reverse Transcriptase (RT)-PCR.**

Vero cell cultured in 25cm³ flasks were infected with 0.1 MOI of RRV<sub>PERS</sub>. As soon as cytopathic effect was observed (36 hours post-infection), culture medium was removed and cells treated with 200 µL of acidified (pH 4.5) guanidinium iso-thiocyanate, phenol, sodium acetate (buffer) following the protocol of Chomczynski and Sacchi (7). To isolate RRV-specific RNA from the total RNA collected, reverse transcription (SuperScript™ Preamplification System for First Strand cDNA Synthesis; Invitrogen, Carlsbad CA USA), was performed using either RRV-E2 specific antisense primer P2 or P5 (Table 1). Standard PCR was then performed with the same antisense primer as used for reverse transcription, and primer P1 (sense primer, see Table 1). Taq DNA polymerase (Taq), deoxyribose nucleoside triphosphates (dNTP)s, buffers and magnesium chloride (MgCl₂) were supplied by QIAGEN (Taq PCR Core Kit, catalogue no. 201223) (Hilden, Germany) and RRV-E2 primers synthesised by GeneWorks (Adelaide, South Australia).

To specifically amplify the entire RRV-E2 cDNA, the following cycling and temperature protocol was used: Denaturation at 94°C for 2 minutes (1 cycle), followed by sequential denaturation, primer annealing and strand elongation by Taq cycles of; 94°C [30 seconds] + 60°C [40 seconds] + 72°C [50 seconds] (35 cycles) and 72°C [5 minutes] (1 cycle). For the primer sets used to amplify the entire RRV-E2 (primers P1 + P2 or P1 + P5, Table 1) this protocol was performed with equal efficiency.

Amplification of RRV nsP 1-4 was achieved after the extraction of total RNA from...
infected Vero cells, as described above, followed by RT using Oligo dT or gene specific primer (Table 2) and Superscript First-strand Synthesis System (Invitrogen) to produce a single-stranded cDNA of the RRV-nsP sequence. The PCR amplification of nsP fragments utilised Proof Start DNA polymerase (QIAGEN) and reaction conditions were as recommended by the manufacturers. Samples were amplified through 45 cycles of primer annealing for 1 minute at 58°C, elongation for 1 minute at 72°C and denaturation for 1 minute at 94°C. The primers used for reverse transcription and subsequent cDNA amplification of the viral nsP (Table 2) were designed on the basis of the complete genomic sequence of the RRV strain 48 (T48) (10). Prior to sequencing, the quality of the PCR products were checked by agarose gel electrophoresis and ethidium bromide staining.

Nucleotide sequencing of the RRV_pers E2 and nsP genes. ABI Prism® BigDye™ Terminator cycle sequencing chemistries (PE Applied Biosystems, CA) were used to generate the specific RRV-E2 and RRV-nsP sequences prior to gel analysis, as described below. Thermal cycling protocols and conditions were run according to the manufacturer’s recommendations.

RRV-E2 gene sequencing: Template cDNA was included in the sequencing reaction at 0.5 µg per reaction. The entire RRV-E2 cDNA sequence was obtained using the primers listed in Table 1. Further confirmation of the single base change found for RRV_pers was demonstrated by m13 sequencing of the RRV-E2 cDNA after cloning into pUC-19. Briefly, the entire RRV-E2 cDNA was cloned into the EcoRI site of pUC-19 at a 3:1 insert to vector ratio with T4 DNA ligase (Promega Corp). To facilitate cloning, the ends of the RRV-E2 cDNA were modified during PCR amplification to incorporate EcoRI sites; this was achieved by the addition of GCGCGAATTTC to the 5’ end of primers P1 and P2 (Table 1) (44). Amplification and sequencing were repeated to confirm base changes. After the above preparations, gel analysis of
the RRV-E2 gene sequence was performed through the DNA Sequencing Analysis Facility at the University of New South Wales, Australia.

**nsP gene sequencing:** PCR products were cut from the 1% agarose gel and purified using a minicolumn system (Millipore). PCR sense and antisense primers (Table 2) were used as sequencing primers to ensure full coverage of RRV *nsP* 1-4 genes. Amplification and sequencing were repeated to confirm base changes. Thermal cycle sequencing reactions and gel analyses were performed at the Australian National University (ANU) Sequencing Core Facility.

**Growth kinetics of RRV.** Vero, HEp-2 (human laryngeal epithelial cell line) and RAW 264.7 cells were cultured in EMEM-FCS at 37°C (5.0% CO2, 95% humidity). Cells were infected with 0.1 MOI of RRV-T48 or RRV*PERS*. Culture supernatants were collected at 12, 24 and 48 hours post infection and viral growth assessed by plaque assay on Vero cell monolayers.

In a separate experiment, RAW 264.7 cells were pre-treated with media containing anti-murine IFN-α and anti-murine IFN-γ (10⁴ unit/ml; both from R&D Systems) for 1 h at 37 °C. The treated cells were then infected with 0.1 MOI of RRV-T48 or RRV*PERS* for 1 h at 37 °C. Cells were then washed and fresh media containing 10⁴ unit/ml of anti-murine IFN-α and anti-murine IFN-γ antibodies was added to the cells. Supernatants were collected at 12, 24 and 48 hours post infection and viral growth assessed by plaque assay on Vero cell monolayers.

**Determination of RRV infectivity by immunofluorescence assays (IFA).** The percentage of infected cells was determined by IFA assays at 6, 12 and 24 hrs postinfection as described previously (30). Briefly, confluent virus-infected and non-infected control cells in glass chamber slides were fixed for 1 min with 1:1 ratio of acetone-methanol followed by overnight
incubation at 4°C in PBS. The cells were then incubated at 37°C for 2 hrs with mouse anti-
RRV hyperimmune ascitic fluid diluted (10^3) in PBS containing 1% HI-FCS followed by three
washes with sterile PBS. This was followed by incubating the cells with fluorescein
isothiocyanate (FITC)-conjugated sheep anti-mouse IgG antibody (Silenius, Melbourne,
Australia) in PBS (1% HI-FCS) for 1 h at 37°C. Cells were then washed three times with
sterile PBS. Cells were counted with a Leica fluorescent microscope.

IFN-β treatment of RAW 264.7 cells. RAW 264.7 cells were cultured in EMEM-FCS
containing 10, 50 or 100 IU/ml of recombinant mouse IFN-β (Cat. No. PMC4024, Invitrogen,
Australia) for 24 hrs prior to infection with 0.1 MOI of parent RRV-T48 or RRV_PERS.
Supernatants were collected 12 hrs p.i. and titrated by plaque assay on Vero cell monolayers for
determination of virus titers.

Plasmids and DNA transfection for luciferase assay. Luciferase reporter plasmid constructs
pIFN-β (-125/+72) Lucter and pISRE (9-27) Lucter were used in experiments involving
transfection of RAW 264.7 cells and Vero cells, respectively. Plasmid DNA was transfected
using GeneJammer transfection reagent, following the manufacturer’s protocol (Stratagene). For
RAW 264.7 cell experiments, cells were transfected with 2 μg of pIFN−β (-125/+72) Lucter,
together with an equal amount of pCMV-βGal plasmid (Promega) as an internal control. Cells
were cultured for a further 24 h and then were infected with 0.1 MOI of RRV-T48 or RRV_PERS.
Cells were harvested at 12 hours post-infection and processed for measurement of luciferase
activity (Luciferase Reporter Assay Kit, Promega) and β-galactosidase activity (β-galactosidase
Reporter Assay Kit, Promega) according to the manufacturer’s instructions. Luciferase activity
was read on an Ascent Luminoskan (Pathtech) and β-galactosidase activity was measured on a UV-visible microplate reader at 495 nm (Bio-Rad). For Vero cell experiments, cells were transfected with 2 µg of pISRE (9-27) Lucter and 2 µg pCMV-βGal (Promega). Cells were then infected with 5 MOI of RRV-T48 or RRV_{PERS}. Twelve hours later, IFN-β (100 IU/ml) was added to cell culture and luciferase expression measured at 6 hours following incubation.

Quantitative real-time PCR. Total RNA was isolated from RAW 264.7 cells infected with 0.1 MOI of RRV-T48 or RRV_{PERS} using TRIzol (Invitrogen Life Technologies). Real-time PCR for IFN-β was performed on the Rotor-Gene RG-3000 (Corbett-Research, Australia), using Quantitect Primer Assay kits (Qiagen, Germany) based on quantification of the SYBR Green I fluorescent dye. Data normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

For viral quantitation by real-time PCR, the method of Shabman et al was used (47). Briefly, RNA was purified from infected culture media using a MagMAX™ Viral RNA Isolatation Kit (Ambion, cat# AM1939). Virion RNA was reversed transcribed using leukemia virus reverse transcriptase (invitrogen) and the cDNA was used for quantitative real-time PCR. TaqMan primer-probe specific for the NSP3 region of RRV were used as described previously (47). A DNA standard curve for RRV genome was generated to ensure optimal primer-probe efficiency and assign relative genome numbers to compare samples.

Semiquantitative RT-PCR analysis of RRV E2 gene. Total RNA was extracted from RAW 264.7 cells infected with 0.1 MOI of RRV-T48 or RRV_{PERS} using TRIzol (Invitrogen Life Technologies). Primer and probe sequences for RRV E2 and HPRT have been described (34, 60). The cycle numbers used for amplification of each gene product is in the linear phase of
amplification: E2, 25 cycles and hypoxanthine phosphoribosyltransferase (HPRT), 23 cycles. The amplified DNA was analyzed by gel electrophoresis, southern blotting and detected using the ECL detection system as recommended by the manufacturer (Amersham). PCR amplification with the HPRT reference gene was performed to assess variations in cDNA or total RNA loading between samples. Relative transcript levels were quantified in arbitrary units using ImageJ (Image Processing and Analysis in Java; http://rsb.info.nih.gov/ij/).

**IFN-β ELISA.** The concentration of IFN-β in samples was determined by ELISA (Biomedical Laboratories), according to the manufacturer’s instructions.
Western blot analysis. Total protein was obtained from RAW 264.7 cells stimulated with 50 IU/ml IFN-β for 24 hours prior to RRV infection (MOI = 0.1) and cell lysates analyzed on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot performed using a polyclonal antibody against RRV E2 glycoprotein (kindly provided by L. Hueston, Westmead Hospital, Sydney, Australia). Fluorescence was detected by ECL Plus immunofluorescence (Amersham). In a separate experiment HEp-2 cells were infected for 12 hrs with 5 MOI of parent RRV-T48 or RRV<sub>PERS</sub> followed by IFN-β treatment (100 IU/ml) for 30 mins or left untreated. Proteins were analyzed from whole-cell protein extracts by immunoblotting as previously described (48). The following primary antibodies recognizing non-phosphorylated and phosphorylated (P) forms of STAT-1 and STAT-2, α-actin (loading control) and secondary antibody were purchased from the indicated manufacturers: anti-STAT-1 (sc-345), anti-P-STAT-1 (sc-135648), anti-STAT-2 (sc-476), anti-P-STAT-2 (sc-21689), anti-α-actin from Santa Cruz Biotechnology; peroxidase-conjugated goat anti-rabbit antibody from Jackson Immunoresearch.

Outbred mouse mortality and morbidity studies. Mice were obtained from the Animal Resources Centre, Canning Vale, Western Australia. Fourteen day old Swiss outbred mice were inoculated intra-peritoneally (i.p.) with 100 µL PBS - 1% FCS containing various doses (from $10^4$ – $10^6$ PFU) of plaque-purified parent RRV (T48) or purified RRV<sub>PERS</sub>. Plaque assays on T48 and RRV<sub>PERS</sub> stocks used in mouse experiments were performed in parallel using the same batch of Vero cells. Both parent RRV and RRV<sub>PERS</sub> showed stock titers of > $10^6$ PFU/ml. In addition, to reflect equal particle doses specific infectivity of the RNA of each virus was determined by the overlay of agarose on transfected BHK-21 cells and the enumeration of plaques from each virus. The specific infectivity of the RRV-T48 and RRV<sub>PERS</sub> was found to be similar (RRV-T48...
1.8 \times 10^5 \text{ and } \text{RRV}_{\text{PERS}} = 1.9 \times 10^5 \text{ PFU per microgram of RNA). Experiments were performed to compare RRV}_{\text{PERS}} \text{ and parent RRV titers in mouse serum at 24-hours post-infection (i.p.) with } 10^4 \text{ PFU RRV. Control mice inoculated i.p. with PBS alone showed no mortality or disease symptoms over the experimental period. All experiments were approved by the Animal Ethics Committee of the University of Canberra and Australian National University. In a separate experiment, 5 week old Swiss outbred mice were inoculated in the right hind-leg footpad with } 10^4 \text{ PFU RRV}_{\text{PERS}} \text{ or RRV-T48. At 1 day post-infection mice were sacrificed and popliteal lymph nodes collected and homogenized for IFN-\beta ELISA analysis.}

Outbred mouse RRVD studies. The experimental approach used for murine RRV infection and monitoring of clinical disease was as described previously by Lidbury et al. (28, 29). Eighteen day old mice were inoculated subcutaneously in the pectoral area with } 10^4 \text{ PFU of RRV diluted in PBS (pH 7.2) in a 20 \mu l volume. Mock-inoculated animals were injected with the diluent alone. Mice were scored for disease symptoms every 24 hours. Signs of disease were determined by assessing grip strength and altered gait. Mice were scored as follows: 0, no disease; 1, ruffled fur; 2, very mild hind limb weakness; 3, mild hind limb weakness; 4, moderate hind limb weakness; 5, severe hind limb weakness/dragging; 6, complete loss of hind limb function; 7, moribund; 8 dead. The experiments were approved by the Animal Ethics Committee of the University of Wollongong.

Statistical analysis. The significance of differences between experimental groups was analysed by one-way ANOVA followed by Bonferroni test. Values were reported as the mean ± SEM. For disease scores, data were analyzed by the Mann-Whitney test. For survival studies, survival curves were analyzed by using the log-rank test. Statistical analyses were performed using
Results

Kinetics of small plaque RRV growth. To study the interplay between virus and host antiviral pathways, we established persistent RRV infection in macrophages that had been treated with LPS to stimulate antiviral activity. In LPS-treated RAW 264.7 cell cultures, small plaque mutants of RRV first emerged at day 5 post-infection, and co-existed with large (parent RRV) plaques. By day 14 post-infection, 90% of plaques visible on Vero cell monolayers were of the small phenotype (Table 3). In cultures treated with polymyxin B sulphate (to remove LPS) at the same time as RRV infection, the appearance of small plaques was delayed until day 14 post-infection (data not shown). RRV<PERS> maintained a small plaque phenotype after several rounds of plaque purification and growth in fresh Vero cell cultures (Fig 1A).

To determine whether there were differences in the growth of RRV-T48 and RRV<PERS>, kinetics of virus growth was compared in Vero, HEp-2 and RAW 264.7 cells. There were no significant differences in growth kinetics observed in Vero cells (Fig 1B) (which do not make IFN-α/β) and in HEp-2 cells (Fig 1C). In contrast, in RAW 264.7, growth kinetics for RRV-T48 and RRV<PERS> differed, with RRV<PERS> producing >1 log<sub>10</sub> more virus at 24 and 48 hrs post-infection (Fig 1D). Importantly, growth kinetics for RRV-T48 and RRV<PERS> were not significantly different in RAW 264.7 cells pre-treated with anti-murine IFN-α and anti-murine IFN-β antibodies (Fig 1E). These findings suggest that the enhanced growth of RRV<PERS> in RAW 264.7 cells involves the modulation of the IFN-α/β response.
Characterisation of RRV<sub>PERS</sub> using monoclonal antibodies. Using polyclonal anti-RRV antibodies, plaque inhibition assays (PIA) showed an identical 50% neutralisation endpoint for small plaque RRV in comparison with the parent RRV-T48 virus (1.9 x 10<sup>-5</sup> versus 1.5 x 10<sup>-5</sup>), confirming the identity of the small plaque virus as RRV (Table 4).

PIA analysis using three monoclonal antibodies (Mab) specific to viral E2 protein revealed differences in neutralisation. For two of the three Mabs tested (E7 and 3C4), 50% plaque neutralisation antibody titers were significantly lower for the RRV<sub>PERS</sub> compared to the parent RRV-T48 virus (Table 4). The relative resistance of RRV<sub>PERS</sub> to neutralisation by Mabs E7 and 3C4 suggested an alteration in the RRV-E2 sequence in the small plaque variant.

Nucleotide sequence of RRV<sub>PERS</sub>. The E2 gene in RRV<sub>PERS</sub> and RRV-T48 E2 was sequenced using RRV-E2 specific primers (Table 1) that covered the entire 1266 base pair sequence. E2 PCR products were also cloned into the EcoRI site of pUC-19, and forward and reverse m13 nucleotide sequencing performed. Analysis of the E2 sequences revealed a single nucleotide change at position 347 (5´→3´) of the RRV E2 gene sequence (RRV-T48 [GA<sub>G</sub>], small plaque RRV [GU<sub>G</sub>]). This genetic alteration resulted in a non-conservative change from the acidic (negatively charged) glutamic acid residue at amino acid position 116 of the parent virus E2 protein, to a non-polar hydrophobic valine residue in RRV<sub>PERS</sub> (Table 5). Sequence analysis of the UTR regions and other genes encoding RRV structural proteins outside of the E2 gene (e.g. 6K, E1 and E3) did not show any differences between RRV<sub>PERS</sub> and RRV-T48 (data not shown).

The complete nsP region of RRV<sub>PERS</sub> was sequenced and compared with the parent strain virus RRV-T48 (NCBI accession number DQ226993). Sequence analysis was started from 21 nucleotides 5´ of the polyprotein start codon (AUG) (position 59 in full length RRV sequence) and finished at the nsP4 stop codon (UAA - position 7523). The genomic organisation was the
same for RRV\textsubscript{PERS} and RRV-T48. Four silent mutations and eleven mutations resulting in single AA substitutions were identified in RRV\textsubscript{PERS} \textit{nsP}\textsubscript{1-4} (Table 5). No base deletion or nonsense mutations were found.

RRV T48 and RRV\textsubscript{PERS} \textit{nsP} thus showed an average amino acid identity of 99%. Eighty-three percent (83%) of the AA substitutions are within the \textit{nsP}\textsubscript{1} and \textit{nsP}\textsubscript{2} regions. The majority of AA changes were non-conservative. Interestingly, a non-conservative change of arginine (basic) to proline (hydrophobic) was present near the centre of \textit{nsP}\textsubscript{1} protein (Pro-303), a region that is highly conserved among alphaviruses (10).

\textbf{RRV\textsubscript{PERS} induces less IFN-\beta mRNA expression and protein production.} To determine whether RRV-T48 and RRV\textsubscript{PERS} differentially affect IFN-\beta production, the levels of IFN-\beta mRNA were measured in RAW 264.7 cells infected with each virus. The increase in IFN-\beta mRNA expression in response to RRV\textsubscript{PERS} infection was significantly less than in cells infected with RRV-T48 (Fig. 2A). This reduction in IFN-\beta mRNA expression occurred despite the fact that RRV\textsubscript{PERS}-infected cells contained about 4-fold more genomic RNA than RRV-T48-infected cells as determined by semi-quantitative RT-PCR analysis of RRV-E2 gene expression (Fig. 2B, C). Using a reporter plasmid encoding luciferase under the control of the IFN-\beta promoter, RRV\textsubscript{PERS} infected cells also showed a 2-fold reduction in luciferase activity compared to RRV-T48 infected cells (Fig. 2D). ELISA analysis confirmed these observations, with 2.5-fold more IFN-\beta protein in cells infected with RRV-T48 than in cells infected with RRV\textsubscript{PERS} (Fig. 2E).

These observations clearly show that infection of RAW 264.7 cells with RRV\textsubscript{PERS} induced less IFN-\beta than infection with RRV-T48, and suggest that the increased replication of RRV\textsubscript{PERS} in RAW 264.7 cells (Fig. 1D) may due to lower levels of IFN-\alpha/\beta induction.
In addition, we sought to exclude the possibility that reduced IFN-α/β production in cells infected with RRV_PERS was due to differences in the number of cells infected early during infection. The percentage of cells infected with each virus at 6, 12 and 24 hours post-infection was measured. At 6 hours post-infection, the percentage of cells infected with each virus was found to be similar (Fig 2F). However, at 12 and 24 hours post-infection, a higher percentage of RRV-positive cells was detected in cultures infected with RRV_PERS than in cultures infected with RRV-T48 (Fig 2F). This result suggests that the differences in the induction of IFN-α/β between RRV-T48 and RRV_PERS were not due to differences in the percentage of cells infected at early time points after infection.

RRV_PERS exhibits higher resistance to IFN-β-induced antiviral activity. We next investigated the effects of IFN-β treatment on the replication of RRV_PERS and RRV-T48 in RAW 264.7 cells. RAW 264.7 cells were treated with 10, 50 or 100 IU IFN-β and infected 24 hours later with RRV_PERS or RRV-T48. The amount of virus in the cultures was determined by plaque assay, real time PCR and Western blotting. RRV_PERS showed increased resistance to IFN-β-mediated antiviral activity, with RRV_PERS infected cells generating substantially higher viral load than cells infected with the parent RRV-T48 virus (Figs 3A-D). Cells were also collected for Western blotting to analyze RRV E2 glycoprotein levels. Qualitative analysis reveal that at 24 h post infection RRV E2 levels were higher in RRV_PERS infected cells compared to those infected with RRV-T48 (Fig 3E). These results indicate that RRV_PERS has increased resistance to the antiviral activity of IFN-β.

Infection by RRV_PERS shows inhibitory effects on type I IFN signaling. To determine
whether the resistance of RRV<br>PERS to IFN-β treatment is mediated by the inhibition of type I IFN signaling, a plasmid encoding the luciferase reporter gene under the control of type I IFN (ISRE)-responsive element was used. These studies were carried out with Vero cells, which are able to respond to IFN-α/β but do not produce these factors themselves. Induction of luciferase can thus be attributed exclusively to exogenously added IFN-β. Vero cells were transfected with pISRE-luc plasmid followed by infection with RRV-T48 or RRV<br>PERS at an MOI of 5. IFN-β (100 IU/ml) was added 12 h later and luciferase expression measured 6 hours later. Luciferase expression from the ISRE promoter in response to treatment with IFN-β was lower in cells infected with RRV<br>PERS than those infected with RRV-T48 (Fig 4A), suggesting that RRV<br>PERS is able to interfere with the IFN-α/β receptor signaling pathway to a greater extent than wild type virus.

To further analyse IFN-α/β receptor signaling, we determined the level of STAT-1 and STAT-2 phosphorylation in HEp-2 cells infected with 5 MOI RRV-T48 or RRV<br>PERS for 12 h followed by IFN-β (100 IU/ml) treatment for 30 mins. Treatment with IFN-β resulted in high levels of STAT-1 phosphorylation, but this was markedly reduced in cells infected with RRV<br>PERS compared to cells infected with RRV-T48 (Fig 4B, C). There were no detectable difference in phosphorylated STAT-2, total STAT-1, total STAT-2 and α-actin in cells infected with either of the viruses (Fig 4B, C). Similar results were obtained when the same experiment was performed in Vero cells (data not shown). *Uninfected cells treated with IFN-β showed comparable levels of phosphorylated STAT-1 and STAT-2 to cells infected with RRV-T48 and treated with IFN-β (Fig 4C).* These results suggest that RRV<br>PERS and not RRV-T48 can interfere with the IFN-α/β receptor signaling pathway at the level, or upstream, of STAT-1 phosphorylation. The observation that the inhibition of STAT-1 phosphorylation was not associated with detectable
changes in STAT-2 phosphorylation, total STAT-1, total STAT-2 and α-actin levels suggests that shut down of host protein synthesis was not responsible for this inhibition.

Infection of outbred mice with RRV<sub>PERS</sub> results in enhanced mortality associated with high virus titers and reduced IFN-β. To determine whether the altered in vitro phenotype of RRV<sub>PERS</sub> resulted in increased pathogenicity in vivo, we undertook infection studies in Swiss outbred mice, which have been used extensively in RRV pathogenesis studies (58, 59). Following infection of Swiss mice with a dose range of 10<sup>4</sup> - 10<sup>6</sup> PFU RRV (i.p.), markedly enhanced mortality was observed in mice infected with RRV<sub>PERS</sub> compared to RRV-T48 (Fig. 45A, B, C). Serum RRV titers were 1.5 log<sub>10</sub> higher in RRV<sub>PERS</sub>-infected mice compared to RRV-T48-infected animals (p < 0.05) (Fig. 5D), suggesting that the increased mortality in mice infected with RRV<sub>PERS</sub> was related to enhanced viral replication.

To determine whether RRV<sub>PERS</sub> induces less IFN-β in vivo, lymph nodes from 5 week old Swiss outbred mice at one-day following infection with RRV-T48 or RRV<sub>PERS</sub> were homogenized and analyzed for the presence of IFN-β by ELISA. Nearly 50% less IFN-β was detected in the lymph nodes of mice infected with RRV<sub>PERS</sub> than in mice infected with the RRV-T48 (Fig. 5E).

Infection of Swiss outbred mice with RRV<sub>PERS</sub> results in enhanced severity of hind limb disease and myositis compared to parent virus. We recently demonstrated that Swiss outbred mice infected with RRV developed severe disease characterized by loss of hind limb gripping ability and altered gait. These disease signs correlated with inflammation of joint and skeletal muscle tissue (29, 38). Since RRV<sub>PERS</sub> has enhanced virulence in vivo (Fig 5), we examined its
ability to induced arthritis and myositis in Swiss outbred mice. Eighteen day-old mice were infected with $10^4$ PFU RRV \textsubscript{PERS} or RRV-T48 and mice were monitored for the development of disease signs. Infection of mice with RRV \textsubscript{PERS} resulted in more severe disease than infection with RRV T48 (Fig. 6A). Mice infected with RRV \textsubscript{PERS} reached a level of disease requiring euthanasia by day 10 post-infection.

To compare muscle tissue inflammation and pathology in mice infected with RRV \textsubscript{PERS} and RRV-T48, histological analysis of skeletal muscle was performed. In RRV \textsubscript{PERS}-infected mice, severe inflammation and tissue damage was observed in muscle tissue at 5 days post-infection (Fig. 6B, panels c and f). Inflammatory infiltrates were also observed in quadriceps skeletal muscle of RRV-T48-infected mice, however, the level of inflammation was not as severe and tissue damage was not apparent at this time point (Fig. 6B, b, e).

**Discussion**

In the present study, we characterized an RRV variant (RRV \textsubscript{PERS}) that had been selected during persistent infection of a mouse macrophage cell line under antiviral conditions. RRV \textsubscript{PERS} formed small plaques and possessed mutations in the structural and \textit{nsP} regions. RRV \textsubscript{PERS} induced lower levels of type I IFN and showed enhanced resistance to antiviral stimuli associated with inhibition of IFN-\textit{α/β} signaling. In association with these changes, RRV \textsubscript{PERS} was highly pathogenic, with increased disease severity and mortality following infection of mice.

Significant insights into the selection of an IFN-resistant viral phenotype have come through the study of human Hepatitis C virus (HCV), which successfully persists in some patients (and cell cultures) for very long periods. The treatment of HCV patients with IFN is often stymied by the eventual development of IFN-resistance by the virus. It has been suggested that exogenous IFN treatment could lead to selective pressure on the virus, and the development...
of mutant strains able to resist IFN-mediated host defence (16, 27, 56). Sumpter et al. (56) found that persistent growth of HCV in cell culture was associated with genetic variation of viral NS5A, NS3 and NS4A, which endowed the virus with the ability to disrupt IRF-1 and IRF-3 signaling following IFN stimulation. HCV proteins have been linked to the attenuation of signaling via the IFN-α/β receptor (18) and HCV variant E2 and NS5A proteins have been demonstrated to bind PKR and interfere with IRF-1 stimulation (41, 57). The ability of HCV to modulate the activity of type 1 IFN may contribute to its ability to persist in the host. In the present study, we observed that small plaque variants also arose in unstimulated RAW 264.7 cells, but at a much slower rate than in cultures stimulated with LPS. This observation is consistent with the model that has been proposed for HCV infection (56), in which exogenous IFN can drive the evolution of strains resistant to IFN-mediated antiviral activity.

Alphaviruses efficiently induce IFN-α/β and are also generally highly sensitive to the antiviral effects of IFN-α/β (3, 50, 62). For instance, based on human and mouse studies, low levels of IFN-β can efficiently inhibit CHIKV infection (50). Mice deficient in the IFN-α/β receptor (IFN-α/β R⁻/⁻) are highly susceptible to CHIKV infection, with infection resulting in death within 3 days (8). Similarly, RRV infection resulted in substantial mortality in IFN-α receptor deficient mice (IFN-α R⁻/⁻), while wild-type mice were able to resist infection (J. Podger and S. Mahalingam, unpublished data). In terms of induction and sensitivity to IFN, our results show that RRV_PERS induces less IFN-β and exhibits resistance to its antiviral effects when compared to the parent virus.

Mortality and disease severity was greatly enhanced in mice infected with RRV_PERS compared to those infected with RRV-T48. The reduction in IFN-β production observed in vitro following infection with RRV_PERS was also observed in vivo, with a 50% reduction in IFN-β.
levels in the lymph nodes of mice infected with RRV\textsubscript{PERS} compared to RRV-T48. It is likely that the virus-mediated inhibition of type I IFN response contributes to disease exacerbation following infection with RRV\textsubscript{PERS}. Our results are reminiscent of findings with VEEV, in which infection of mice with an IFN resistant strain resulted in enhanced clinical disease (51). Similar results were obtained with an IFN-\textsubscript{α}/\textsubscript{β}-resistant EEE virus strain; infection of mice with this strain resulted in enhanced encephalitis, an effect that mapped to both structural and nonstructural genes (1).

To counteract type I IFN responses, many viruses encode proteins that disrupt type I IFN signaling and downstream responses. These evasion strategies have been linked to viral pathogenesis and the emergence of virus in new host populations (24, 51). Suppression of type I IFN responses has been observed for influenza virus (4, 14), dengue virus (20, 39), West Nile virus (32) and other RNA viruses (reviewed in 15, 35). Many viral proteins have been reported to block transcription factors that control production of type I IFN (reviewed in references 14, 15, 35). In this study we have identified RRV as an additional member capable of developing mechanisms to suppress IFN responses. Alphavirus infection results in rapid shut down of host cell protein synthesis in favor of viral protein synthesis, and the conventional view has been that this mechanism underlies the observed suppression of IFN-\textsubscript{α}/\textsubscript{β} production (44). For example, the \textit{nsP2} proteins of sindbis virus and Semliki Forest virus (SFV) can inhibit type I IFN responses via host protein shut down (5, 12), while the capsid proteins of Eastern equine encephalitis virus (EEEV) and Venezuelan equine encephalitis virus (VEEV) perform a similar IFN-targeting function (1, 2, 51). Mutating the nuclear localisation sequence of \textit{nsP2} of SFV resulted in a virus that induced more IFN-\textsubscript{α}/\textsubscript{β}, suggesting that \textit{nsP2} inhibits IFN-\textsubscript{α}/\textsubscript{β} induction (5). However, the authors admit that an alternative explanation is that re-localisation of \textit{nsP2} may simply result in
more effective induction of IFN-α/β (5). Alphaviruses might also exert much more specific
effects on the IFN-α/β system independent of host protein shutoff. Our earlier studies with RRV
demonstrated that Fc receptor bearing cells (DCs and macrophages) can be infected via a
mechanism involving antibody dependent enhancement (ADE) of infection, and that this results
in IL-10-dependent inhibition of cellular IFN-α/β production (34). We have also demonstrated
that the presence of high mannose glycans on virus derived from mosquito cells interferes with
type I IFN induction in myeloid DCs (46). Recently, Yin and colleagues reported that the
phosphorylation of STAT-1 and STAT-2 was partially blocked by VEEV and Sindbis virus and
the effect was dependent on the expression of viral nsP (61). In another study, Simmons et al
showed that VEEV can antagonize STAT-1 activation following type I IFN treatment and that
the inhibition of type I IFN signaling occurred via distinct mechanisms independent of host
protein shutoff (48). Cruz et al have identified a mutation in the nsP1/nsP2 cleavage domains of
Sindbis virus and RRV was associated with a specific enhancement of IFN production
independent of virus-induced host shutoff (9). More recently, Fros et al showed that
chikungunya virus infection blocked IFN-induced STAT-1 phosphorylation and that this
inhibition was mediated by nsP2 and was independent of host shut-off (13). Several lines of
evidence also suggest that shut down of host protein synthesis is not responsible for the
RRV<sub>PERS</sub>-mediated IFN-α/β signaling inhibition. First, the reduced levels of phosphorylated
STAT-1 was not associated with reduced total STAT-1 levels, which suggests that the inhibition
was not due to decreased synthesis of total STAT-1. Second, phosphorylated STAT-2, total
STAT-2 and house keeping gene α-actin levels were not decreased by RRV<sub>PERS</sub> infection, which
indicates that the small plaque variant specifically targets STAT-1. Third, the STAT-1 and
STAT-2 levels in uninfected control treated with IFN were comparable to levels in cells
infected with RRV-T48 treated with IFN suggesting that RRV-T48 does not inhibit phosphorylation of STAT-1 or 2 and that the effects are specific to RRV<sub>PERS</sub>. The ability of viruses to develop resistance to host antiviral activity by genetic sequence variation has a major effect on virus virulence and persistence in the host. Mutational studies have shown that alphaviruses appear to attain these effects mainly through the action of nsPs (1, 2, 5, 9, 13, 51). We identified a number of mutations in nsP<sub>1-4</sub> and a single mutation in E2 in RRV<sub>PERS</sub>. Using site-directed mutagenesis, we introduced the RRV<sub>PERS</sub> E2 mutation into the parent RRV-T48 cDNA infectious clone (adenine to uracil (GAG ▹ GUG) at position E2-347). Interestingly, the mutation in E2 did not affect IFN induction and resistance of RRV<sub>PERS</sub> (data not shown). Future studies will investigate whether these mutations in the nsP regions are involved in the perturbation of IFN signaling pathways that we observed. Our preliminary studies (using chimeric virus and pcDNA plasmid expressing non-structural genes) show that RRV<sub>PERS</sub> nsP<sub>1</sub> and nsP<sub>2</sub> are able to inhibit IFN-α/β signaling (data not shown) and we are currently attempting to identify the mechanisms of action. Future studies will introduce the mutations that we identified in the RRV<sub>PERS</sub> nsPs into the wild-type RRV infectious clone (pRR64), followed by testing the IFN resistance phenotype and pathogenicity of the recombinant virus. If the IFN resistance phenotype of RRV<sub>PERS</sub> is recovered, examination of the expression of downstream IFN-induced proteins will be investigated. These studies will allow us to identify the specific nsP mutation(s) that lead to the IFN suppression phenotype induced by enhanced macrophage inflammatory activity.

In conclusion, we describe in vitro and in vivo studies characterising the genetic and phenotypic properties of a novel small plaque, persistent strain of RRV. The enhanced suppression of IFN signaling mediated by RRV<sub>PERS</sub> is likely to play a key role in the enhanced pathogenicity exhibited by this virus. This study demonstrates that selective pressure exerted by
host antiviral activity can promote the evolution of an IFN-resistant, persistent, and highly pathogenic alphavirus strain. It is possible that a similar evolutionary mechanism may operate in vivo, and sequencing of clinical isolates from persistent alphavirus infections will provide new insights into viral pathogenesis in human disease.

Acknowledgments

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Figure legends

Figure 1. Differential plaque morphology and growth kinetics of RRV_{PERS} compared to RRV-T48. A. Plaque morphology on Vero cell monolayers for parent Ross River virus strain T48 (RRV-T48) and RRV_{PERS}. Both RRV-T48 and RRV_{PERS} plaques were purified from the supernatants of RRV-T48-infected RAW 264.7 cultures at day 14 post-infection, and new viral stocks re-grown in fresh Vero cell cultures, after which plaque morphology was confirmed by plaque assay. B-D. Growth kinetics of RRV-T48 and RRV_{PERS} in Vero (B), HEp-2 (C) or RAW 264.7 (D) cells infected with 0.1 MOI virus. (E) RAW 264.7 cells were pretreated with 10^4 unit/ml of anti-murine IFN-\(\alpha\) and anti-murine IFN-\(\beta\) antibodies (R&D Systems) for 1 h at 37°C. The cells were then infected with 0.1 MOI RRV-T48 or RRV_{PERS} for 1 h at 37°C. The virus inoculum was discarded and cells rinsed with media. Fresh media containing 10^4 unit/ml of anti-murine IFN-\(\alpha\) and anti-murine IFN-\(\beta\) antibodies was added to the monolayer for an additional 48 h at 37°C. Culture supernatants were collected at various time points post RRV-infection and viral growth assessed by plaque assay on Vero cell monolayers. Assay limit of detection is 2.0 Log_{10} PFU/mL. Significant differences in virus titers are marked with an asterisk, * \(P < 0.05\).

Figure 2. RRV_{PERS} infection of RAW 264.7 cells triggers lower IFN-\(\beta\) expression and production. (A) Quantitative real time PCR analysis of transcript for IFN-\(\beta\) mRNA in RAW 264.7 cells 12-hours after infection with 0.1 MOI of RRV-T48 or RRV_{PERS}. Data represent the mean fold change in mRNA levels, compared to uninfected controls. GAPDH was used as a reference gene. Statistical analysis of relative expression results was performed using the REST software. Significant differences in expression are marked with an asterisk, * \(P < 0.05\). (B, C)
Semi-quantitative RT-PCR analysis of transcript for RRV E2 mRNA in RAW 264.7 cells 12-hours after infection with 0.1 MOI of RRV-T48 or RRV<sub>PERS</sub>. HPRT was used as a reference gene. Relative transcript levels were quantified in arbitrary units using ImageJ (Image Processing and Analysis in Java; http://rsb.info.nih.gov/ij/). (D) Luciferase reporter gene activity at 12 hours after infection of RAW 264.7 cells with 0.1 MOI of RRV-T48 or RRV<sub>PERS</sub>. RAW264.7 cells were transiently transfected with pIFN-β (-125/+72) Lucter reporter plasmid. Twenty-four hours later, cells were inoculated with 0.1 MOI RRV-T48 or RRV<sub>PERS</sub>, followed by cell lysis at 12 hours post-infection. Luciferase activity was normalised to β-Galactosidase reporter expression. Significant differences in expression are marked with an asterisk, *<i>P</i> < 0.05. (E) ELISA analysis of IFN-β protein production in RAW 264.7 cells 12-hours after infection with 0.1 MOI of RRV-T48 or RRV<sub>PERS</sub>. Significant differences in protein levels are marked with an asterisk, *<i>P</i> < 0.05. (F) Percentage of infected cells at 6, 12 and 24 hrs post-infection with 0.1 MOI RRV-T48 or RRV<sub>PERS</sub>. Infected cells were detected using mouse anti-RRV hyperimmune ascitic fluid and fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG antibody. The number of infected cells was counted with a Leica fluorescent microscope. Significant differences in cell numbers are indicated with an asterisk, *<i>P</i> < 0.05.

**Figure 3.** RRV<sub>PERS</sub> exhibits enhanced resistance to antiviral stimuli. Growth of RRV-T48 and RRV<sub>PERS</sub> in RAW 264.7 cultures stimulated with 10IU/ml (A), 50IU/ml (B) or 100 IU/ml (C) IFN-β 24-hours prior to RRV infection (MOI = 0.1). Culture supernatants were collected at 12, 24 and 48 hours post RRV-infection and viral growth assessed by plaque assay on Vero cell monolayers. Assay limit of Detection is 2.0 Log<sub>10</sub> PFU/mL. Significant differences in virus titers are marked with an asterisk, *<i>P</i> < 0.05. (D) Quantitation of RRV genome by real time PCR.
RAW 264.7 cells stimulated with 50 IU/ml IFN-β 24 hours prior to RRV infection (MOI = 0.1).

RNA extracted at 24 hours post infection for the measurement of RRV genome copy numbers.

Significant differences in viral genome copy numbers are indicated with an asterisk, * P < 0.05. (E) Total protein was obtained from RAW 264.7 cells stimulated with 50 IU/ml IFN-β 24-hours prior to RRV infection (MOI = 0.1). Cell lysates were collected at 24 hours post infection for Western blot analysis using a polyclonal antibody against RRV E2 glycoprotein. Detection of host cell protein expression by anti-α-actin was used to demonstrate equal loading of protein.

Figure 4. Type I IFN signaling is inhibited in cells infected with RRV\textsubscript{PERS}. (A) These studies were performed with Vero cells, which do not produce IFN but are able to respond to IFN. Vero cells were transfected with pISRE (9-27) Lucer plasmid and then infected with RRV-T48 or RRV\textsubscript{PERS} (5 MOI). IFN-β (100 IU/ml) was added 12 h later, and luciferase expression was measured following incubation for 6 hours. Luciferase activity was normalised to β-Galactosidase reporter expression. Significant differences in expression are marked with an asterisk, * P < 0.05. (B, C) Western blot analysis of STAT-1 and STAT-2 expression and phosphorylation in HEp2 cells infected with RRV-T48 or RRV\textsubscript{PERS} (5 MOI) for 12 h followed by IFN-β (100 IU/ml) treatment for 30 mins or left untreated. The cell lysates were examined by Western blotting with antibodies to STAT-1, pY-STAT-1, STAT-2 or pY-STAT-2. Control included the detection of host cell protein expression by anti-α actin antibody.

Figure 5. RRV\textsubscript{PERS} infection in mice results in enhanced mortality associated with reduced IFN-β expression. (A-C). Survival of 14-day old Swiss outbred mice after intraperitoneal (i.p.)
infection by RRV-T48 (open circles) or RRV_{PERS} (closed circles). Virus was inoculated at doses ranging from $10^4$ (A), $10^5$ (B) or $10^6$ (C) PFU/mouse ($n = 10$). **Differences between survival curves were calculated using Log rank test.** $P < 0.05$ was considered to be significant. **D.** Titre of RRV-T48 and RRV_{PERS} (Log$_{10}$ PFU/mL) in the serum of 14-day old Swiss outbred mice infected subcutaneous (s.c) 24-hours previously with $10^4$ PFU/mouse. Plaque assay limit of detection is 2.0 Log$_{10}$ PFU/mL. Significant differences in virus titers are marked with an asterisk, $* P < 0.05$.

**E.** IFN-$\beta$ concentration in homogenised lymph nodes of 5 week old Swiss outbred mice 24-hours after s.c. infection by $10^4$ PFU RRV-T48 or RRV_{PERS} ($n=3$ mice per group). Significant differences in protein levels are marked with an asterisk, $* P < 0.05$.

**Figure 6.** **RRV_{PERS} induced severe RRVD in a mouse model of disease.** Eighteen-day old outbred mice were infected with $10^4$ PFU of RRV-T48 or RRV_{PERS} by injection in the left rear footpad. (A) Mice were scored for development of hind limb dysfunction and disease based on the following scale: 0, no disease; 1, ruffled fur; 2, very mild hind limb weakness; 3, mild hind limb weakness; 4, moderate hind limb weakness; 5, severe hind limb weakness/dragging; 6, complete loss of hind limb function; 7, moribund; 8 dead. ($n = 5$). **Data for disease scores were analyzed by the Mann-Whitney test.** Significant differences in disease score are indicated with an asterisk, $* P < 0.05$. (B) Eighteen day old outbred mice were infected with $10^4$ PFU of RRV by injection in the left rear footpad. At 5 days p.i., mice were perfused with 4% paraformaldehyde and 5 µm thick paraffin-embedded sections generated from quadriceps muscle were H & E stained; (a) Mock, (b) RRV-T48 infected mice, (c) RRV_{PERS}-infected mice. Images are representative of at least 3 mice per group. **Magnifications:** a, c, e (100x); b, d, f (200x).
TABLE 1. Primers used for the RT-PCR isolation and nucleotide sequence analysis of the RRV-E2 gene from a small plaque variant of RRV-T48.

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<td>10035</td>
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* Position of primer in complete RRV genome (refer to ref 10).

# Primers used for RT-PCR amplification of RRV-E2.

E2 gene is 1268 bp in length (position 8566-9834) and is situated between the E3 and 6K coding regions of the RRV genome (10).
TABLE 2. Primers used for nucleotide sequence analysis of the RRV-nsP1-4 genes from RRV PERS.

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</tr>
<tr>
<td>P11</td>
<td>+</td>
<td>2939</td>
<td>GCTGTTGTGGAACACACTGTC</td>
<td>641</td>
</tr>
<tr>
<td>P12</td>
<td>-</td>
<td>3580</td>
<td>TGGTATCCCGGAAGCTTCTTC</td>
<td></td>
</tr>
<tr>
<td>P13</td>
<td>+</td>
<td>3361</td>
<td>GCGAACGCCTCGGACCTCTTC</td>
<td>776</td>
</tr>
<tr>
<td>P14</td>
<td>-</td>
<td>4137</td>
<td>CATTGCACCCCGCCCTGTTCC</td>
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</tr>
<tr>
<td>P15</td>
<td>+</td>
<td>4076</td>
<td>ACCCTCATACCGTGCTGGTAG</td>
<td>756</td>
</tr>
<tr>
<td>P16</td>
<td>-</td>
<td>4832</td>
<td>CGAAATCGCCGTTCCTGAACAG</td>
<td></td>
</tr>
<tr>
<td>P17</td>
<td>+</td>
<td>4795</td>
<td>GCATCAAGCACAATGCCCCGT</td>
<td>777</td>
</tr>
<tr>
<td>P18</td>
<td>-</td>
<td>5572</td>
<td>TCAACCGGCTCGCGCATTTC</td>
<td></td>
</tr>
<tr>
<td>P19</td>
<td>+</td>
<td>5514</td>
<td>CTGAAGATCTGGAGGTACTCAC</td>
<td>827</td>
</tr>
<tr>
<td>P20</td>
<td>-</td>
<td>6341</td>
<td>TAGAGTGGTAGCTCTCTCACT</td>
<td></td>
</tr>
<tr>
<td>P21</td>
<td>+</td>
<td>6242</td>
<td>CGCGTGCCATCACCATTTC</td>
<td>677</td>
</tr>
<tr>
<td>P22</td>
<td>-</td>
<td>6919</td>
<td>ATTCTCCGACAAGCTGCTTC</td>
<td></td>
</tr>
<tr>
<td>P23</td>
<td>+</td>
<td>6840</td>
<td>TTGACGGCAGTACTGCTGTTG</td>
<td>680</td>
</tr>
<tr>
<td>P24</td>
<td>-</td>
<td>7520</td>
<td>TTAGGACGGCCGTAGAGGGTG</td>
<td></td>
</tr>
<tr>
<td>P25</td>
<td>+</td>
<td>7311</td>
<td>CAAGATGAGATCTGAGGCGTG</td>
<td>439</td>
</tr>
<tr>
<td>P26</td>
<td>-</td>
<td>7750</td>
<td>CTGTTTGGTGGTGATGAGAG</td>
<td></td>
</tr>
</tbody>
</table>
TABLE 3. Mean RRV titers in RAW 264.7 murine macrophages over 21 days post-infection (p.i.). Cells were infected at an MOI of 0.1 on day 0 and cultured with 5 ng/mL LPS.

<table>
<thead>
<tr>
<th>Day post-infection</th>
<th>Mean RRV titer (Log_{10} PFU/mL) ± SEM (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.77 ± 0.02 (0 small plaques)</td>
</tr>
<tr>
<td>2</td>
<td>5.27 ± 0.16 (0 small plaques)</td>
</tr>
<tr>
<td>5</td>
<td>5.34 ± 0.11 (27%, 5.8%, 8.5%)*</td>
</tr>
<tr>
<td>14</td>
<td>5.93 ± 0.14 (89%, 93%, 91%)*</td>
</tr>
<tr>
<td>21</td>
<td>4.01 (n = 1) (80%)*</td>
</tr>
</tbody>
</table>

Virus used in this experiment was derived from the RRV-T48 genetic clone (RR64).

* Proportion of small RRV plaques observed on Vero cell monolayers after RAW 264.7 macrophage infection (n = 3) over a 21-day period. Parent virus (RRV-T48) presents as a “large” plaque on Vero cell monolayers (Fig. 1). At days 1 and 2 post RRV-T48 infection only large plaques were observed.
TABLE 4. Antibody-mediated inhibition of RRV plaque formation on Vero cell monolayers by anti-RRV polyclonal sera or RRV-E2 protein specific monoclonal antibodies.

<table>
<thead>
<tr>
<th>Anti-RRV antibody</th>
<th>Highest antibody dilution to achieve 50% RRV plaque inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RRV-T48</td>
</tr>
<tr>
<td>Polyclonal anti-RRV&lt;sup&gt;a&lt;/sup&gt;</td>
<td>$1.5 \times 10^{-5}$</td>
</tr>
<tr>
<td>Mab E7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>$7.5 \times 10^{-5}$</td>
</tr>
<tr>
<td>Mab 10C9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>$1.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>Mab 3C4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>$1.2 \times 10^{-6}$</td>
</tr>
</tbody>
</table>

a. Polyclonal antibody to RRV raised as an ascites to PEG-purified RRV-T48.

b. Specific for the E2 protein of RRV.

All antibodies titrated by 1/10 serial dilution from 1/50 – 1/5 x $10^6$ prior to mixing with 200 PFU of purified RRV.
TABLE 5: Predicted Amino Acid (AA) differences in the nsP1-4 and E2 protein of the persistent RRV\textsubscript{PERS} compared to the parent RRV-T48 based on the nucleotide sequence analysis of the nSP 1-4 region and E2, 6K and E1 (structural) regions of the RRV genome. (Silent mutations are not included).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Amino Acid</th>
<th>RRV-T48</th>
<th>RRV\textsubscript{PERS}</th>
<th>Conservative (+/-)</th>
<th>Nucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>nSP1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>143</td>
<td>R (AGA)</td>
<td>T (ACA)</td>
<td>-</td>
<td>450</td>
</tr>
<tr>
<td></td>
<td>147</td>
<td>A (GCT)</td>
<td>S (TCT)</td>
<td>-</td>
<td>461</td>
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<tr>
<td></td>
<td>154</td>
<td>X (NGG)</td>
<td>A (GCG)</td>
<td>+/-</td>
<td>483</td>
</tr>
<tr>
<td></td>
<td>303</td>
<td>R (GCC)</td>
<td>P (CCC)</td>
<td>-</td>
<td>929</td>
</tr>
<tr>
<td></td>
<td>499</td>
<td>K (AAG)</td>
<td>N (AAT)</td>
<td>-</td>
<td>1519</td>
</tr>
<tr>
<td>nSP2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>239</td>
<td>L (TTG)</td>
<td>F (TTT)</td>
<td>+</td>
<td>2341</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>N (AAC)</td>
<td>D (GAC)</td>
<td>-</td>
<td>2342</td>
</tr>
<tr>
<td></td>
<td>306</td>
<td>Y (TAC)</td>
<td>H (CAC)</td>
<td>-</td>
<td>2540</td>
</tr>
<tr>
<td></td>
<td>634</td>
<td>H (CAT)</td>
<td>Q (CAA)</td>
<td>-</td>
<td>3526</td>
</tr>
<tr>
<td>nSP3</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>337</td>
<td>S (AGC)</td>
<td>I (ATC)</td>
<td>-</td>
<td>5028</td>
</tr>
<tr>
<td>nSP4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>563</td>
<td>K (AAG)</td>
<td>N (AAC)</td>
<td>-</td>
<td>7321</td>
</tr>
<tr>
<td>E2</td>
<td>116</td>
<td>E (GAG)</td>
<td>V (GTG)</td>
<td>-</td>
<td>8913</td>
</tr>
</tbody>
</table>

AA residues are numbered from the N-terminal of each nsP and E2 protein. The single letter amino acid code is used. Conservative AA differences are indicated by (+). Amino acids that could not be deduced due to nucleotide ambiguity (N) are shown as a X. Nucleotides are numbered from the 5´ terminus of RRV-Sp sequence and are indicated by letters in brackets. Genebank accession number for RRV-E2 cDNA is M20162.
RRV<sub>PERS</sub> infected  RRV-T48 infected  Mock infected

A

Clinical score

Days post-infection

B

Mock infected

RRV-T48

RRV-PERS