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Infection of Myofibers Contributes to Increased Pathogenicity during Infection with an Epidemic Strain of Chikungunya Virus

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ABSTRACT

Chikungunya virus (CHIKV) is an alphavirus transmitted by mosquitoes that is known to cause severe arthritis and myositis in affected patients. The ongoing epidemic began in eastern Africa in 2004 and then spread to islands of the Indian Ocean, India, and Southeast Asia, ultimately afflicting millions. During this outbreak, more severe disease manifestations, including fatalities, have been documented. The reasons for this change in pathogenesis are multifactorial but likely include mutations that have arisen in the viral genome which could alter disease pathogenesis. To test this hypothesis, we used a murine model of CHIKV to compare the disease pathogeneses of two recombinant strains of CHIKV, the first derived from the La Reunion outbreak in 2006 (LR2006 OPY1) and the second isolated from Senegal in 1983 (37997). While the two strains exhibited similar growth in mammalian cells *in vitro*, we observed more severe clinical disease and pathology in mice infected with the LR2006 OPY1 strain of CHIKV, which included prolonged viremia and elevated viral titers and persistence in the muscle, resulting in devastating myonecrosis. Both CHIKV strains infected connective tissue fibroblasts of the muscle, but only the LR2006 OPY1 strain replicated within myofibers *in vivo*, despite similar growth of the two strains in these cell types *in vitro*. However, when the 37997 strain was administered directly into muscle, myofiber infection was comparable to that in LR2006 OPY1-infected mice. These results indicate that differences in the ability of the strain of CHIKV to establish infection in myofibers may contribute to the increased disease severity.

IMPORTANCE

CHIKV is an emerging pathogen that causes significant morbidity. Little is known about the pathogenesis of the disease, and this study suggests that the ability of a recent epidemic strain to infect myofibers results in increased disease severity. Better understanding of how CHIKV causes disease contributes to the ultimate goal of creating therapeutics to alleviate the impact of this debilitating virus.

Chikungunya virus (CHIKV) is a mosquito-borne alphavirus in the *Togaviridae* family. It was first identified in 1952 in Tanzania and was named in the Makonde language for the painful arthritis it causes (1). Since then, periodic outbreaks of chikungunya fever have been reported intermittently in Africa and Asia. CHIKV was brought to attention most recently when an outbreak began in 2004 in Kenya. In short order, the virus spread to surrounding countries and islands off the coast. In 2006, nearly 40% of the population of La Reunion island was infected, and between 3 to 6.5 million cases are estimated to have occurred in India from 2006 to 2008 (2). Unlike previous outbreaks, which have been of limited duration, this epidemic has continued into 2013, with spread throughout India and into Malaysia and Thailand.

In previous outbreaks, CHIKV had behaved like the "Old World" alphaviruses, such as Sindbis virus or Ross River virus (RRV), with the disease characterized by abrupt high fever, incapacitating polyarthritis, and skin manifestations (3, 4). While quite debilitating, the infection is generally self-limited and is cleared within 2 weeks by most patients. However, a subset of patients can develop polyarthritis and tenosynovitis that can last for months or even years following the initial infection (5–7). Epidemiological studies from this ongoing outbreak have also revealed more severe disease manifestations. Mortality due to CHIKV infection was noted for the first time, with over 250 deaths attributable to CHIKV infection occurring during the La Reunion epidemic (8, 9). This increased mortality was observed predominantly in elderly patients with other comorbidities. Increased susceptibility of neonates to severe infection with subsequent long-term sequelae has also been noted (8, 10). Furthermore, around one-half of the mothers with ongoing CHIKV infection at the time of delivery transmitted CHIKV to their offspring (11). Neonatal infection consisted of fever, poor feeding, pain, and skin manifestations, with severe illness presenting as encephalopathy, often resulting in long-term sequelae (11).

The reasons for the increased severity during this outbreak are likely multifactorial and include the larger scale of the outbreak, more thorough documentation and follow-up by the medical community, and novel mutations that have accrued in the current circulating strain of CHIKV. The importance of these novel mu-

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tations has already been demonstrated by a recent study in which an alanine-to-valine mutation at residue 226 in the E1 protein of La Reunion strains of CHIKV was found to result in increased infectivity of a new mosquito vector, *Aedes albopictus*, the Asian tiger mosquito (12). The new vector is present in temperate regions and may have contributed to autochthonous outbreaks in France and Italy, and it has potential to spread the disease in North America as well (13–15). It is therefore also possible that some of the novel mutations could contribute to the increased disease pathology observed during this ongoing outbreak.

To test for differences in disease pathogenesis, we used the neonatal model of CHIKV infection. The infection of neonatal mice recapitulates many of the clinical and histological findings of human CHIKV infection. Infected neonatal mice exhibit significant viral dissemination to multiple tissues, develop neurological signs of disease, and succumb to infection (16), similar to what has been observed in patients, especially in highly susceptible neonates (11). Biopsy samples from patients that have been stained for CHIKV antigen localize the staining to the muscle connective tissue fibroblasts and satellite cells (17). An evaluation of CHIKV localization following the infection of 9-day-old mice also localized CHIKV to the muscle fibroblasts, which support muscle health, as well as myoblasts (16). Finally, while the pathogenesis of alphavirus-induced disease is incompletely understood, the inflammatory response clearly contributes to disease. The expression of proinflammatory cytokines, including interleukin-6 (IL-6) and IL-1β, correlates with the severity of CHIKV-induced disease in patients (18). In the neonatal mouse model, we observe the induction of these proinflammatory cytokines following infection. Therefore, the infection of neonatal mice recapitulates many of the clinical and histological findings of human CHIKV infection and provides us with a small-animal model to evaluate potential differences in disease pathogenesis between the CHIKV recombinant clones.

To evaluate whether an epidemic strain of CHIKV differs in disease characteristics from a past strain from a previous outbreak, we utilized a recombinant clone of CHIKV isolated from the La Reunion outbreak in 2006 (LR2006 OPY1) and compared this to an earlier recombinant clone isolated from Senegal in 1983 (37997). The 37997 strain belongs to the West African clade of CHIKV, while LR2006 belongs to the East Central South African clade, which diverged many years ago (19, 20); the LR2006 OPY1 strain has sequence changes similar to those of other clinical isolates from the Indian Ocean outbreak (21). An earlier analysis of these two recombinant clones found that the LR2006 OPY1 strain showed increased infectivity of and transmission by *A. albopictus*, though the two strains grow similarly in C6/36 cells derived from *A. albopictus* (12, 22). We observed that despite similar *in vitro* growth in various mammalian cell types, the LR2006 OPY1 strain displayed a more severe disease phenotype, resulting in hind limb weakness and high muscle titers in neonatal mice. While both strains were able to infect muscle connective tissue fibroblasts early in infection, only LR2006 OPY1 was able to infect myofibers. However, this was not due to a difference in tropism, as both strains were able to replicate in myoblasts and myofibers *in vitro*. When 37997 was injected directly into the muscle, myofiber infection comparable to that of LR2006 OPY1 was observed. This suggests that the ability of a viral strain to establish infection in myofibers can impact the severity of infection.

MATERIALS AND METHODS

Mice. Mice were maintained at Washington University School of Medicine in accordance with all federal and university guidelines. For mouse studies, the principles of good laboratory animal care were adhered to in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the NIH (23). The protocols were approved by the Animal Studies Committee at Washington University. All efforts were made to minimize suffering. C57BL/6 and alpha/beta interferon (IFN-α/β) receptor 1 ($IFNAR^{-/-}$) mice on the C57BL/6 background were bred and maintained in our mouse colony. For neonatal experiments, mice were infected at 6 days of age. Litters were weight matched at the initiation of the experiments.

Virus production. The construction of recombinant plasmids for the LR2006 OPY1, 37997, LR2006-green fluorescent protein (GFP), and 37997-GFP strains of CHIVK has been previously described (19, 20). Recombinant viral stocks were generated from viral cDNA clones by *in vitro* transcription of linearized cDNA templates followed by RNA transfection with Lipofectamine 2000 (Invitrogen) into baby hamster kidney 21 cells (BHK cells) as previously described (24). After 48 h, the supernatant was collected, centrifuged, aliquoted, and stored at −80°C. Titers of viral stocks were determined by plaque assay as previously described (25). All work with live virus was performed in a biosafety level 3 facility and followed strict guidelines established by the Environmental Health and Safety committee at Washington University School of Medicine.

Cells. Murine embryonic fibroblasts (MEFs) were generated from C57BL/6 or $IFNAR^{-/-}$ mice and were grown in Dulbecco modified Eagle medium (DMEM) (Mediatech) supplemented with 10% fetal bovine serum (FBS) (Sigma), 1% penicillin-streptomycin (Mediatech), 1% L-glutamine (Mediatech), and 1% HEPES (Mediatech). MEFs were used prior to passage number 5 for these studies. Primary muscle fibroblasts were isolated from newborn mice as previously described (26). Briefly, skeletal muscle obtained from newborn mice was dissected and digested with collagenase (Worthington). Cells were cultured in F12 medium (Invitrogen) with 10% FBS and 1% penicillin-streptomycin for 2 days before replating the cells for growth curve analysis. C2C12 cells were obtained from ATCC (ATCC CRL1772) and cultured as recommended. To obtain myofibers, C2C12 cells were plated at 5×10^4 cells/well in 24-well plates, differentiated into myofibers by the substitution of 2% horse serum for FBS, and then cultured for 10 days prior to infection as described below.

Viral growth curves. MEFs, primary connective tissue fibroblasts, and C2C12 cells were grown and/or differentiated as described above. For viral growth curves, MEFs were plated at 1×10^5 cells/well and all other cell types were plated at 5×10^4 cells/well in 24-well tissue culture-treated plates and allowed 24 h to adhere. For interferon sensitivity assays, cells were then either pretreated with IFN-β (PBL InterferonSource) at the indicated dose or mock treated with medium only for 24 h. Cells were infected at the indicated multiplicity of infection (MOI). Cells were rinsed with DMEM with 1% FBS, after which diluted virus was added and allowed to adhere for 1 h. The virus was then aspirated and replaced with fresh medium. At each time point, a plate was frozen at −80°C and underwent three freeze-thaw cycles before titers were determined on BHK cells by standard plaque assay.

Viral studies. Six- to nine-day-old pups were infected with between 5×10^4 and 1×10^5 PFU of the indicated recombinant CHIKV clone (37997 or LR2006 OPY1) diluted in 15 μl of phosphate-buffered saline (PBS) and injected intradermally (i.d.) to mimic a mosquito bite, using a Hamilton syringe and 30-gauge needle, into the left upper pectoral area. For survival experiments, pups were monitored daily for clinical signs of disease (including ruffling of fur, altered gait, and hind limb paralysis) and lethality for 21 days postinfection (dpi). For viral titers, the mice were infected as described above and the various tissues (injection site, serum, muscle, and brain) were harvested into PBS and stored at −80°C. Injection site refers to the skin and underlying connective tissue and fat at the site of infection. Serum samples were pooled when collected, with each sample containing sera from two mice from a single experiment. For

muscle titers, the hind limb ipsilateral to the site of infection was skinned and the hamstring was collected. For the analysis of viral loads, the samples were homogenized with 100 μl of 1.0-mm-diameter zirconia-silica beads using a MagnaLyzer (Roche) by alternating two pulses of 7,000 rpm for 30 s each, with chilling on ice for 30 s. The homogenates were serially diluted, and titers were determined by plaque assay on BHK cells. For serum collection, pups were bled at the designated time postinfection into BD Serum Separator tubes. These were spun for 9 min at 12,000 × *g* and then frozen at −80°C until their titers were determined.

Histopathology. Six-day-old pups were infected i.d. with 5×10^4 PFU of either CHIKV recombinant clone or were mock infected i.d. with PBS in the left upper pectoral area. Mice were sacrificed at the indicated day postinfection, and the hind limb and brain were fixed with 10% formalin for 48 h at room temperature and then rinsed with PBS for 15 min and stored in 70% ethanol at 4°C until being submitted for embedding in paraffin blocks and sectioning. The sections were stained with hematoxylin and eosin to examine for histopathology. Embedding, sectioning, and staining were performed by the Anatomic and Molecular Pathology Histology Core at Washington University in St. Louis. All slides were evaluated and scored blindly by one of the authors (J.C.C.) for the presence of histopathological changes consistent with mengoencephalitis in the brain samples and for histological changes within the muscle. Changes in the muscle were scored as follows: 0, normal; 1, predominantly normal tissue, only minimal chronic inflammatory infiltrates and some regenerative fibers; 2, focal, mild chronic inflammatory infiltrates and some degeneration/regeneration; 3, mild myonecrosis with chronic inflammatory infiltrates and degeneration/regeneration; 4, moderate myonecrosis with chronic inflammatory infiltrates and degeneration/regeneration; and 5, severe myonecrosis with chronic inflammatory infiltrates and degeneration/regeneration.

Cytokine/chemokine analysis. Sera were harvested from infected mice at the indicated time points and stored at −80°C for analysis. Serum cytokine levels were measured using Luminex technology with the Bioplex Pro mouse cytokine 23-plex assay (Bio-Rad).

Immunofluorescence. For immunofluorescence experiments, the mice were infected as described above, but we utilized recombinant clones for the 37997 and LR2006 OPY1 strains expressing green fluorescent protein (GFP) (19). For intradermal infections, 1×10^5 PFU of either GFP strain was injected into the skin of the upper left chest of 6-day-old pups. For intramuscular (i.m.) injections, 2.5×10^4 PFU of either the GFP-expressing, or parental recombinant strains was injected directly into the left hamstrings of 6-day-old pups. Tissue was harvested from the mice at the indicated times and fixed in 4% paraformaldehyde for 24 h at 4°C. It was then rinsed with PBS for 24 h and allowed to equilibrate in 30% sucrose for 2 h before embedding in OCT. Sections were cut using a cryostat and blocked with 5% normal goat serum (Sigma). Sections were stained with an anti-collagen IV antibody (Abcam) and with DAPI (4′,6′-diamidino-2-phenylindole) (Vector Laboratories). The slides were developed with a donkey anti-rabbit 546 secondary antibody using Alexa Fluor (Invitrogen).

Quantification of histology. Photographs were taken using a Nikon Eclipse 80i microscope and processed with Metamorph software (Molecular Devices). To quantify, 6 high-power (×40 or ×20) fields (hpf) were taken per section, with a single section per mouse. For i.m. injections, GFP-positive (GFP^+) and GFP-negative fibers were counted in each hpf, and the percent positive cells was calculated. For i.d. injections, only GFP^+ cells per hpf were counted. The mean and standard error of the mean (SEM) of all high-power fields per sections were used to calculate significance.

RT-PCR. Mice were infected with 1×10^5 PFU i.d. Tissues were excised and flash frozen in liquid nitrogen. Tissues were homogenized using the MagnaLyzer (Roche), and RNA was extracted using the RNeasy fibrous tissue kit (Qiagen). RNA was used to run real-time PCR (RT-PCR) on the infected tissues, normalizing to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (IDT). Monocyte chemoattractant protein 1 (MCP-1), RANTES, and interferon-stimulated gene 15 (ISG15) gene expression assays were purchased from Applied Biosystems. IFN-β primers and probes were provided courtesy of Michael Diamond (Washington University, St. Louis, MO) and are forward primer 5′-GGC TTC CAT CAT GAA CAA CAG-3′, reverse primer 5′-GTT GAT GGA GAG GGC TGT G-3′, and probe 5′-5/6FAM/CTG CGT TCC TGC TGT GCT TCT C/36-TAMSp/-3′. Pan-IFN-α primers and probes were used as previously reported (27).

ELISA. Mice were infected with 1×10^5 PFU i.d., and serum was harvested at the indicated time points and stored at −80°C. An IFN-α enzyme-linked immunosorbent assay (ELISA) kit from PBL InterferonSource was used according to the manufacturer's instructions.

Chimeric viruses. Chimeric viruses were constructed using a method described previously (28). Briefly, the E2 region of each strain was amplified using the forward primer 5′-AATGTCTATAAAGCCACAAGAC-3′ and the reverse primer 5′-ACGTTGACAGACTCTGAGAA-3′. The segments were gel extracted and used as primers for the next PCR with the opposite strain with the QuikChange II XL mutagenesis kit (Agilent). The reaction mix then underwent an initial incubation for 1 min at 98°C, followed by 18 cycles of 98°C for 30s, 60°C for 30s, and 72°C for 8 min and a final extension of 72°C for 10 min. Clones were sequenced using the forward and reverse primers. Viruses were then generated as described in "Virus production" above.

Statistical analysis. All data were analyzed using the Prism software (GraphPad Software, San Diego, CA). Survival data were analyzed using Gehan-Breslow-Wilcoxon test, with death as the primary variable. Acute-titer data and cytokine data were analyzed using the Mann-Whitney test. Errors bars in figures represent the standard error of the mean.

RESULTS

CHIKV strains 37997 and LR2006 OPY1 display similar growth and sensitivity to an IFN-β-induced antiviral state in primary MEFs. A previous comparison of the sequences of the 37997 and the LR2006 OPY1 strains of CHIKV revealed 162 amino acid differences between these two strains (22). Since these differences could impact the ability of these viruses to replicate, we first wanted to evaluate their growth in mammalian cells prior to evaluating potential differences in pathogenesis. Both BHK cells and MEFs were infected with the two strains of CHIKV at a low MOI, and viral growth over time was analyzed. Both the LR2006 OPY1 and the 37997 strains of virus grew with similar kinetics in BHK cells (data not shown). We also observed no difference in the viral growth rate or in the peak viral titers achieved in MEFs infected with either strain at any of the time points analyzed (Fig. 1A).

Previous studies with other alphaviruses have shown that mutations controlling the sensitivity of a virus to type I interferons could impact its *in vivo* pathogenesis (29, 30). Mutations mapped to the 5′ nontranslated region and the nsP1 and nsP2 genes in Sindbis virus have been found to affect interferon sensitivity (29, 30). There are residue differences in these genes between the LR2006 OPY1 and 37997 strains (22). Therefore, we next tested whether the two strains of CHIKV displayed similar *in vitro* sensitivities to type I IFNs. C57BL/6 MEFs were either mock treated or treated for 24 h prior to infection with IFN-β at a dose that would induce an antiviral response and partially inhibit viral replication. The cells were then infected, and viral growth was monitored at several time points postinfection. As seen in Fig. 1B (dashed lines), IFN pretreatment was able to equivalently inhibit both the LR2006 OPY1 and the 37997 strains of CHIKV. We also observed a similar increase in replication of the two CHIKV strains following the infection of $IFNAR^{-/-}$ MEFs, with the virus growing to titers that were nearly 100-fold higher than in B6 MEFs

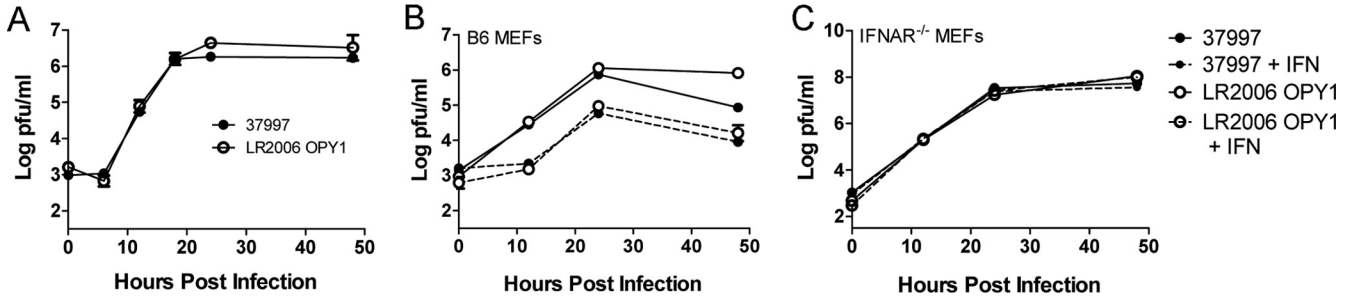

FIG 1 Recombinant CHIKV strains LR2006 OPY1 and 37997 display similar growth characteristics *in vitro*. (A) WT MEFs were infected at an MOI of 0.05 for 1 h and analyzed for titer by plaque assay at several time points postinfection. (B and C) WT MEFs (B) or IFNAR$^{-/-}$ MEFs (C) were pretreated with 8 U/ml of IFN-β for 24 h before infection and then infected at an MOI of 0.05 for 1 h, and titers were determined by plaque assay as for panel A. Growth curves were determined in two independent experiments, each in duplicate.

(Fig. 1C). As expected, pretreatment of these cells had no impact on CHIKV replication. Together, these results indicate that the LR2006 OPY1 and the 37997 recombinant clones of CHIKV displayed similar growth kinetics in mammalian cells and similar sensitivities to IFN *in vitro*.

The LR2006 OPY1 strain of CHIKV induces more severe disease than the 37997 strain of CHIKV. Next, we evaluated the pathogeneses of these two clones using a neonatal murine model of CHIKV infection. In this model, the infection of wild-type (WT) mice less than 10 days of age results in the development of a disseminated infection with significant lethality (16, 31). We infected 6-day-old C57BL/6 pups i.d. in the upper left chest with 5×10^4 PFU of either the LR2006 OPY1 or the 37997 strain of CHIKV. Infection with either strain of CHIKV resulted in significant lethality with a similar median onset of lethality of 10 days for both viruses, with 54% of the LR2006 OPY1-infected pups and 68% of the 37997-infected pups succumbing to infection (Fig. 2A). However, despite the similarity in lethality induced by both strains of CHIKV, we observed more severe clinical signs of disease in the LR2006 OPY1-infected pups than in the 37997-infected mice. While mice infected with either strain of CHIKV displayed ruffled fur, sluggishness, and altered gait, the LR2006 OPY1-infected mice often developed hind limb weakness and paralysis, which were rarely seen in the 37997-infected mice (data not shown). This severe weakness was first observed at 7 days postinfection (dpi) and lasted throughout the course of infection in surviving mice.

To further investigate the differences in the clinical courses induced by the two strains, we next evaluated viral dissemination and replication of the two clones in six-day-old mice at different times postinfection. An analysis of the injection site, which included the skin and underlying connective tissue, revealed similar viral loads at all of the time points analyzed, with peak titers obtained at 1 dpi and a steady decrease in viral replication until 9 dpi, when replicating virus was no longer detected (Fig. 2B). It has been previously reported that in the neonatal model of CHIKV infection, mice develop viremia, resulting in viral dissemination to multiple tissues. Mice infected with either strain of CHIKV developed viremia, with similar viral levels detected in the LR2006 OPY1- and the 37997-infected mice at both 1 and 3 dpi (Fig. 2C). However, mice infected with the 37997 strain cleared the virus from the serum by 4 dpi. In contrast, mice infected with the LR2006 OPY1 strain maintained persistent viremia until 7 dpi, when serum viremia was no longer detected.

The hind limb weakness and ataxic gait that we observed in the LR2006 OPY1-infected mice could be due to CHIKV infection impacting either the central nervous systems or the muscles, or both, of these mice. Therefore, we evaluated viral loads in both the brains and the muscles of mice infected with either the LR2006 OPY1 or the 37997 strain. While we did observe an increase in viral titers in the brains of the LR2006 OPY1-infected mice at 3 and 4 dpi, by 5 dpi similar titers were observed in mice infected with either CHIKV strain, and both strains were cleared by 12 dpi in surviving mice (Fig. 2D). Furthermore, histological evaluation of the brains from these mice at the various times postinfection revealed no significant pathology induced by either strain of CHIKV (data not shown). In contrast to the case for the brain, an analysis of viral titers in the muscle revealed a striking difference between the two strains. We found that infection with either strain of CHIKV resulted in detectable viral titers in the muscle as early as 1 dpi (Fig. 2E). Consistent with our clinical observations, however, the LR2006 OPY1-infected mice had significantly greater viral burdens in their muscles. By 3 dpi the mice infected with the LR2006 OPY1 strain of CHIKV displayed greater than 100-fold more virus in their muscles than those infected with the 37997 strain, even though serum viral loads were similar for the two strains at this time point. The increased viral burden observed in the muscles of the LR2006-infected mice persisted until at least 9 dpi, with the LR2006 OPY1-infected mice having between 3- and 4-log-higher viral titers in their muscle (Fig. 2E). Mice infected with the 37997 strain had peak replication at 5 dpi with a mean titer of 3.0×10^4 PFU/ml, while the LR2006 OPY1 strain peaked at 6 dpi with a mean titer of 6.17×10^7 PFU/ml (Fig. 2E). While the 37997 strain was largely cleared from the muscle by 9 dpi, mice infected with the LR2006 OPY1 strain continued to have greater than 10,000-fold more virus, even at 9 dpi. Even increasing the infecting dose of 37997 to 20 times the dose (2×10^6 PFU i.d.) did not result in an increase in viral loads detected in the muscles of the infected mice (data not shown). To determine if the adaptive immune response played a role in clearing the viral infection from the muscle, we also infected Rag$^{-/-}$ mice with either strain of CHIKV and observed no differences in viral loads compared to those in WT mice (data not shown). Therefore, consistent with the hind limb weakness observed in the mice infected with the LR2006 OPY1 strain of CHIKV, we observed increased peak viral loads and prolonged replication in the muscles of mice infected with the LR2006 OPY1 strain.

LR2006 and 37997 elicit similar serum type I IFN and proinflammatory cytokine and chemokine responses during the

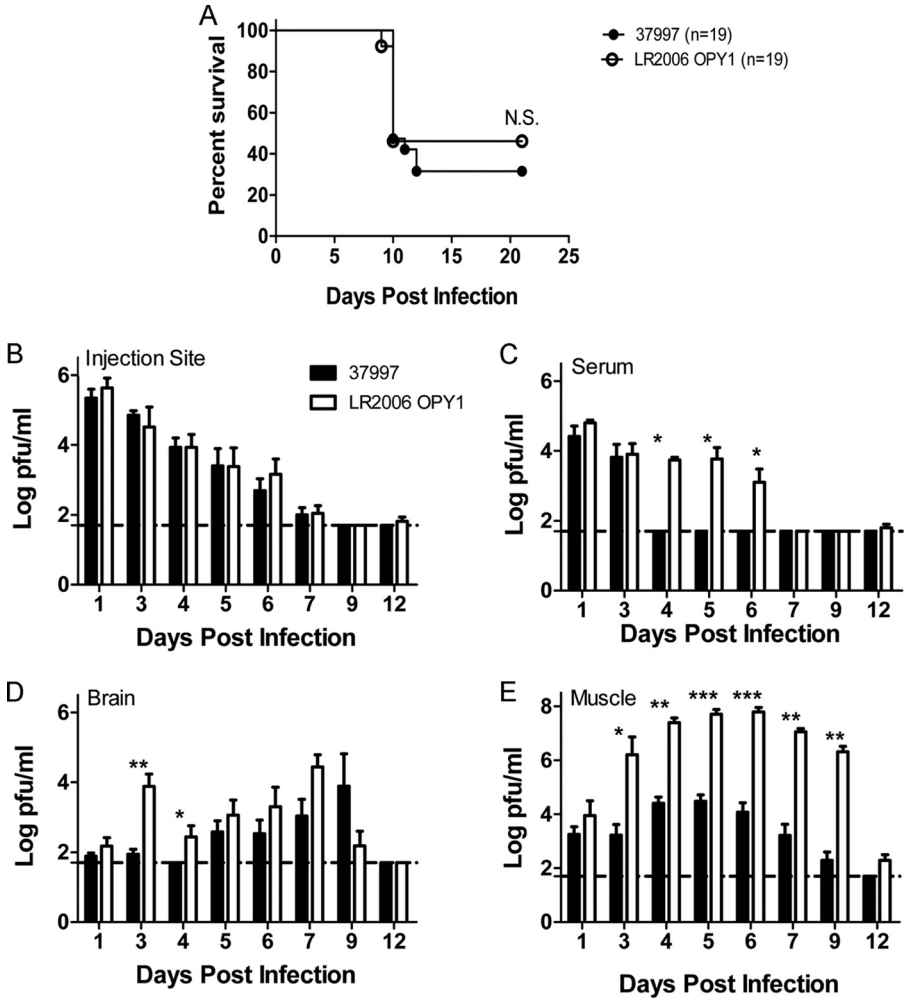

FIG 2 LR2006 OPY1 achieves higher muscle titers and persistence in the serum. Pups were infected i.d. at 6 days of age with 5×10^4 PFU of either the LR2006 OPY1 or 37997 CHIKV strain. (A) Infected mice were monitored for clinical signs of disease and death each day until 21 days postinfection. (B to E) Infected mice were harvested at the indicated time points; injection site (B), serum (C), brain (D), and distal hind limb muscle (E) were collected, and titers were determined by plaque assay ($n = 5$ to 8 pups per group per time point, except at 12 dpi, where $n = 4$ pups per time point). The dashed lines indicate the limit of detection for the assay. Significance for survival was evaluated by both the log rank and the Gehan-Breslow-Wilcoxon tests. Statistical significance for panels B to E was evaluated with the Mann-Whitney test. *, $P = 0.01$ to 0.05; **, $P = 0.001$ to 0.01; ***, $P < 0.001$; N.S., not significant.

course of infection. One explanation for the dramatic difference in viral titers could be altered production of type I IFNs by the two viruses or perhaps differential antagonism of this response. To begin to assess this possibility we first evaluated whether there was a difference in the systemic induction of type I IFNs by the LR2006 OPY1 and the 37997 strains of CHIV. Mice were infected with either the LR2006 OPY1 or the 37997 strain of CHIKV, and serum IFN-α levels were measured at various time postinfection (Fig. 3A). We detected very little IFN at 8 h postinfection. Levels peaked at 16 h postinfection and slowly diminished during the next 48 h. However, we observed no differences in the levels of systemic IFN-α induced by either viral strain. Since we observed the largest differences in viral loads in the muscles of infected mice, we also evaluated the type I IFN response in the muscles of mice infected with either strain of CHIKV. RT-PCR was used to measure the levels of IFN-β, IFN-α, or ISG15 in the hind limb muscle at various times postinfection (Fig. 3B). While the expression of all three was increased compared to levels in mock-infected mice, there was no difference in the induction of IFN-α, IFN-β, or ISG15 between the different CHIVK strains. Based upon this analysis, it does not appear that the differences in disease pathogenesis seen in the LR2006 OPY1 and the 37997 strains of CHIKV are due to differences in the induction of type I interferon.

We have previously seen that neonatal mice infected with CHIKV develop a systemic inflammatory response (25). Furthermore, in a neonatal model of Sindbis virus infection, this systemic inflammatory cytokine response likely contributes to the lethality (32). We therefore next determined if there was a difference in the cytokines induced by either the 37997 or LR2006 OPY1 strain of CHIKV that may account for the differences in pathogenesis that we observed. We analyzed 23 cytokines and chemokines in the sera of infected mice at 1, 3, 5, and 7 dpi. As previously observed, several cytokines were induced by CHIKV infection, including IL-1α, IL-1β, IL-6, IL-10, and granulocyte colony-stimulating factor (G-CSF) (Fig. 3C). Both CHIKV strains induced elevated levels of IL-1β, IL-6, and RANTES, cytokines found to be associ-

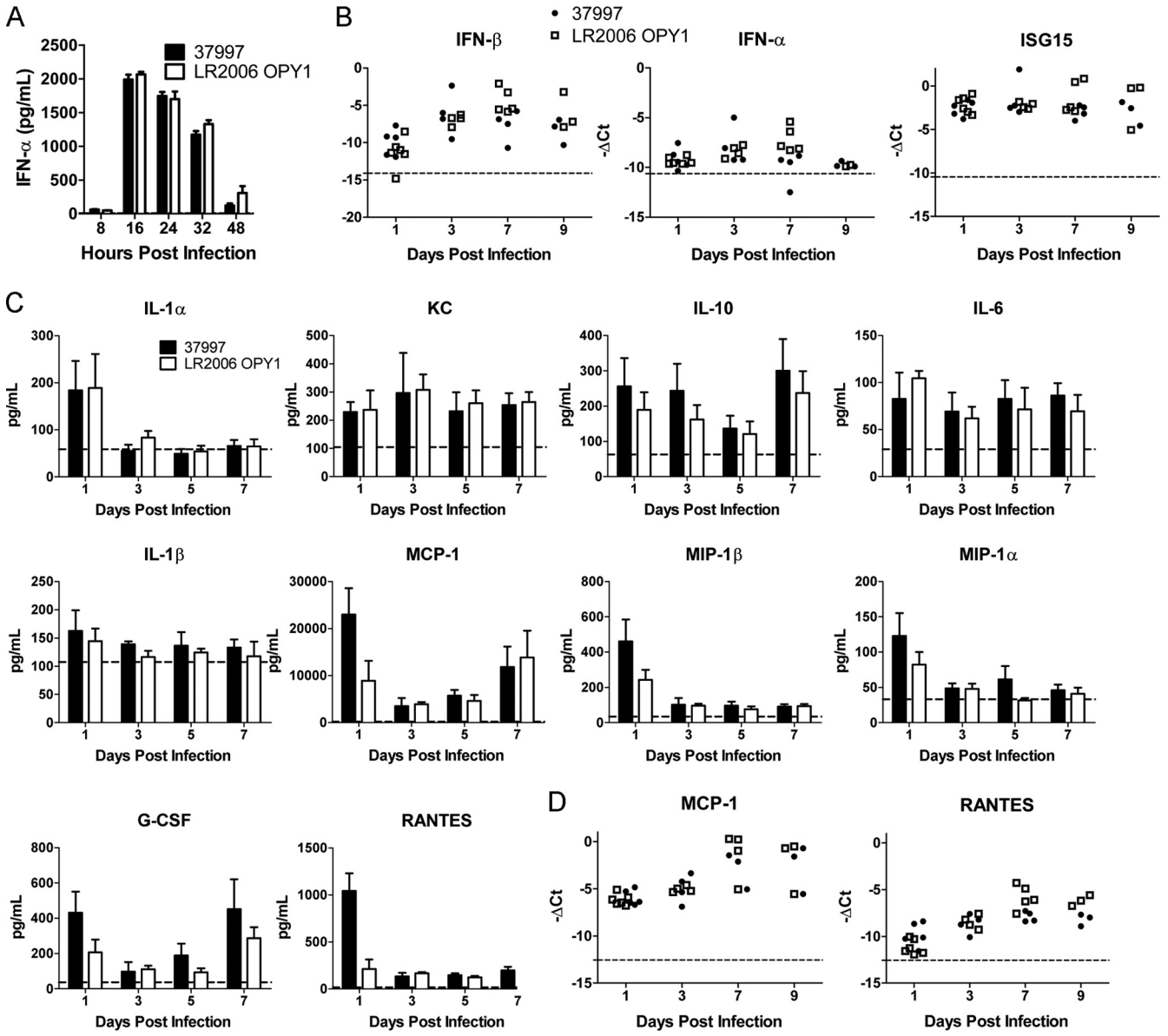

FIG 3 Similar type I interferon and proinflammatory cytokine and chemokine profiles are induced by both strains of CHIKV. Six-day-old pups were infected with 5×10^4 PFU of either CHIKV strain or mock treated with PBS i.d. (A) Sera were collected from infected mice at several hours postinfection, and IFN-α levels were measured by ELISA. The IFN-α level in mock-infected mice was 8.37 pg/ml. (B and D) Mice were infected with 1×10^5 PFU of either CHIKV strain. RNA was extracted from hind limb muscle, and RT-PCR was performed. Samples were normalized to GAPDH. The dashed lines indicate RNA levels in mock-infected mice. (C) Sera were collected at the indicated times and analyzed using a Bioplex Pro mouse 23-plex array. The dashed lines indicate the level of cytokine or chemokine in mock-infected samples. $n = 4$ per time point. The results did not reach statistical significance at any time points analyzed.

ated with severe disease in human patients afflicted with CHIKV (18). Surprisingly, despite the increased viral loads detected in the LR2006 OPY1-infected mice during the course of viral infection, we observed no significant difference in the cytokine levels between the strains at any time point we analyzed. We did observe a trend toward an increase in several chemokines, including KC, MCP-1, MIP-1α, MIP-1β, and RANTES, at 1 dpi in mice infected with the 37997 strain of CHIKV (Fig. 3C), although these differences did not reach statistical significance. To determine whether elevations in these chemokines were also present in the muscle, where we observed the differences in viral titers, we analyzed the hind limb muscle for expression of MCP-1 and RANTES RNAs by RT-PCR (Fig. 3D). RNA levels of both chemokines were elevated over levels in mock-infected mice as early as 1 dpi. However, we observed no differences in the induction of either MCP-1 or RANTES RNA between the two strains of CHIKV. At 7 dpi, the RNA levels of both chemokines were further elevated, with mice infected with LR2006 expressing levels of MCP-1 and RANTES RNAs similar to those in mice infected with the 37997 strain, despite the increased viral burden present in these mice at this time (Fig. 3D). Together, these results indicate that both the 37997 and the LR2006 OPY1 strains of CHIKV induce a systemic cytokine and chemokine response in this neonatal model; however, no significant differences in the responses induced by the two viral strains were noted.

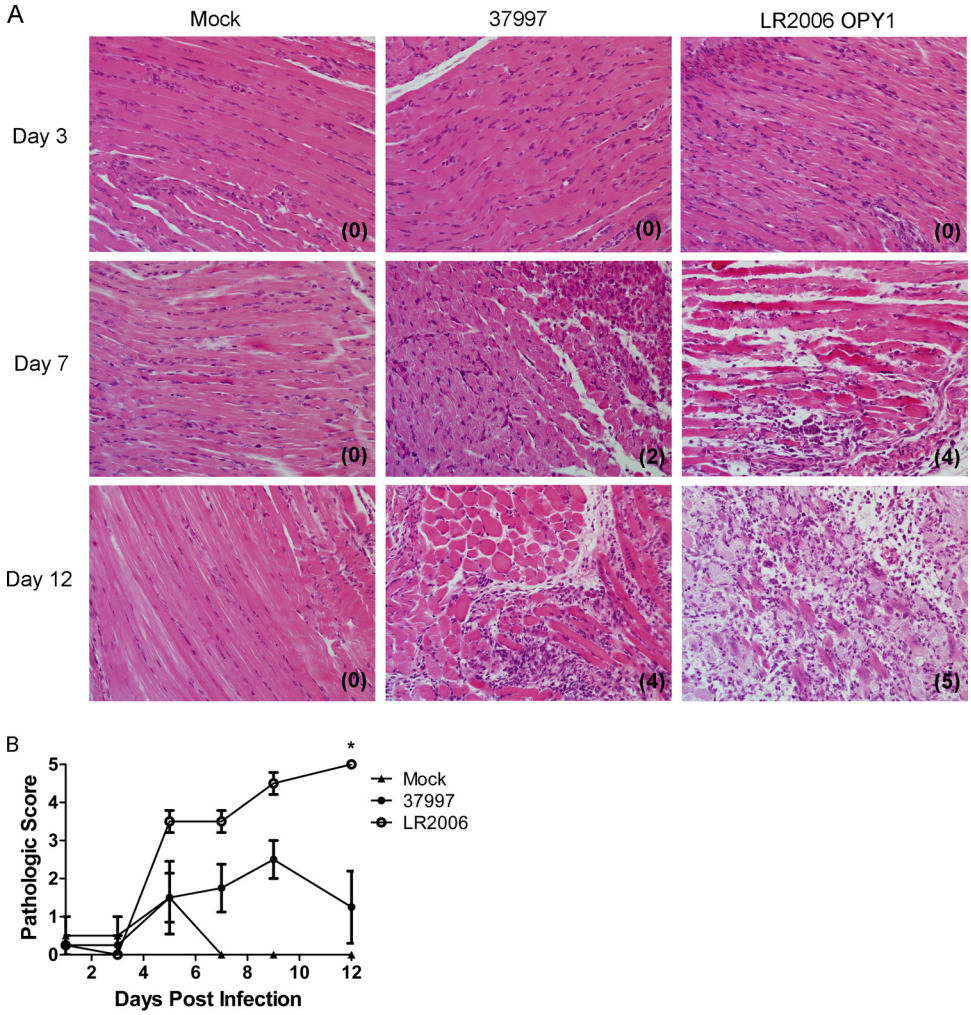

FIG 4 The LR2006 OPY1 strain induces dramatic myositis and necrosis. Six-day-old pups were infected with 5×10^4 PFU of CHIKV i.d. or were mock treated with PBS. The hind limb was isolated on the indicated days postinfection and fixed in 10% formalin for 48 h. Sections were submitted for embedding, sectioning, and staining. (A) The pathology of each sample was scored by a pathologist on a scale of 0 to 5 as described in Materials and Methods. Representative H&E-stained sections at several days postinfection at a magnification of ×40 are shown, with the pathological score indicated in parentheses. (B) Graphical representation of the scores of all of the histological sections. *, $P = 0.01$ to 0.05.

The LR2006 OPY1 strain of CHIKV infects myofibers and results in greater hind limb muscle damage than the 37997 strain of CHIKV. To further characterize the disease induced by these two CHIKV strains, we next performed a histological analysis of the muscles of infected mice. Muscle from the hind limb distal to the site of infection was collected at various times postinfection and stained with hematoxylin and eosin (H&E). These slides were evaluated by a pathologist in a blinded fashion. Muscle sections were given a severity score from 0 (normal) to 5 (most severe) as described in Materials and Methods. An analysis of the muscles from CHIKV-infected mice revealed significant pathological changes (Fig. 4A and B). In mice infected with the 37997 strain, we observed focal areas of myositis and necrosis, with the majority of the muscle being largely undamaged. The peak damage seen was at 9 dpi for 37997-infected mice (Fig. 4B). Consistent with the profound muscle weakness observed clinically and the increased viral titers, muscle from mice infected with the LR2006 OPY1 strain displayed widespread destruction beginning at 5 dpi and lasting until at least 12 dpi, with peak damage occurring at 12 dpi (Fig. 4A and B). In these samples there were dense inflammatory infiltrates accompanying massive myonecrosis (mean score = 5 at 12 dpi) (Fig. 4A). Therefore, these histological data support the clinical findings that the LR2006 strain of CHIKV induces more severe muscle disease in these mice.

To further evaluate the differences seen in the muscles of these mice, we next determined if there were differences in viral tropism between the two strains. Previous work has shown that CHIKV replicates in the connective tissue fibroblasts and myoblasts in both mice and humans (16). While other studies have noted myofiber necrosis and attributed this to infection of those fibers by CHIKV, previous studies have not detected infection of the myofibers (33). We utilized recombinant CHIKV strains in which GFP expression was driven by a subgenomic promoter in either the 37997 or LR2006 OPY1 backbone. When these viruses are utilized, only cells that harbor active CHIKV infection will express GFP. Mice were infected i.d. with either the 37997-GFP- or LR2006-GFP-expressing CHIKV strain, and at different times postinfection the hind limb muscle was evaluated by immunofluorescence.

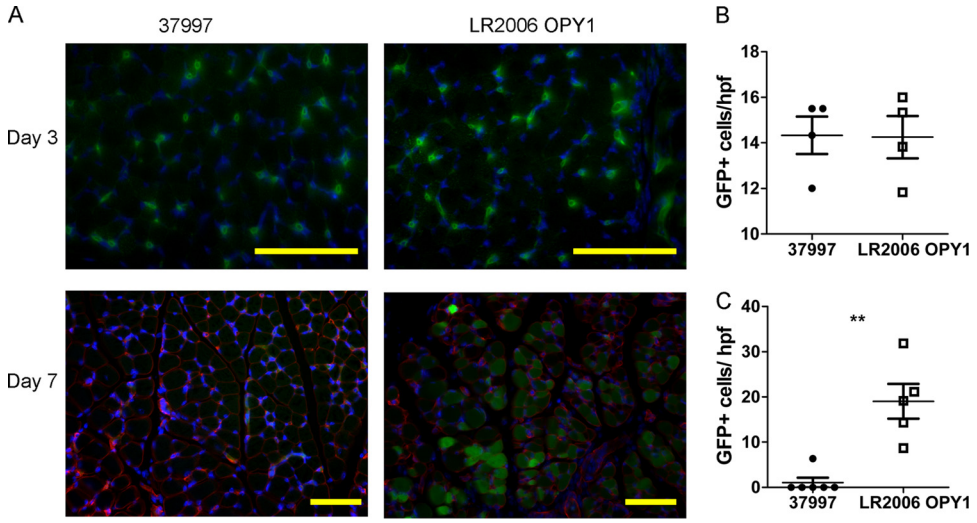

FIG 5 Intradermal infection results in infection of myofibers by LR2006 OPY1 but not by 37997. Mice were infected with either 37997-GFP or LR2006 OPY1-GFP i.d. at a dose of 5×10^4 PFU. Muscle from the hind limb was stained for collagen IV (red), GFP (green), or nuclei (blue). (A) Top panels, muscle harvested at 3 dpi (magnification, ×40); bottom panels, muscle harvested at 7 dpi (magnification, ×20). (B) Connective tissue fibroblasts were counted in ×40 high-power fields at 3 dpi. (C) Myofibers per ×20 high-power field at 7 dpi. Scale bars, 100 μm.

Similar to what has been previously reported, we detected GFP staining in the connective tissue fibroblasts surrounding the muscle fibers following infection with either the LR2006 or 37997 strain of CHIKV (Fig. 5A, top panels). Staining was detected as early as 3 days postinfection and increased throughout the course of infection until myofibers were destroyed. Quantification of GFP^+ fibroblasts revealed that similar quantities of cells were infected following infection with either strain of CHIKV (Fig. 5B). Surprisingly, in mice infected with LR2006 OPY1, we also detected GFP^+ staining of the myofibers, particularly at late time points after infection, although GFP^+ fibers could be seen as early as 3 dpi. In contrast, there was almost a complete absence of GFP^+ myofibers in the hind limbs of mice infected with 37997-GFP (Fig. 5A, bottom panels, and 5C). These data suggest that the ability of the LR2006 OPY1 strain to gain access to the myofibers and establish infection results in increased viral loads in the muscle and increased muscle damage and likely contributes to the increased pathogenicity of this CHIKV strain.

During i.m. injection, both strains are able to infect myofibers. The inability of the 37997 strain to infect the myofibers following an intradermal infection could be due to an inability of the virus to directly infect the myoblasts/myofibers, an inability to grow in the myoblasts, or difficulty in gaining access to this compartment. To test these possibilities, we first evaluated the abilities of the LR2006 OPY1 and 37997 strains to infect and replicate in primary connective tissue fibroblasts and in myoblasts and myofibers. Primary muscle connective tissue fibroblasts were isolated from neonatal mice and infected with either strain of CHIKV at an MOI of 0.05. Viral growth was assessed at various times postinfection. The two strains grew at similar rates and reached comparable peak titers after 48 h postinfection (Fig. 6A). This supported our *in vivo* observation that both strains infected and replicated within the connective tissue fibroblast compartment (Fig. 5A and B). We next evaluated the abilities of the LR2006 OPY1 and 37997 strains to infect and replicate within myoblasts and myofibers. We first used the C2C12 myoblast cell line to assess growth *in vitro*. Both CHIKV strains were able to infect the C2C12 cells, and an analysis of viral growth revealed that the two strains displayed similar growth kinetics (Fig. 6B). Next, myoblasts were differentiated into myofibers, forming a mixed culture of both myoblasts and myofibers. They were also infected with a low MOI, which produced comparable growth of the strains (Fig. 6C). These results suggest that both viral strains have the ability to infect connective tissue fibroblasts, myoblasts, and myofibers. Furthermore, it appears that the strains display similar growth characteristics *in vitro*. Therefore, it does not appear that the 37997 strain cannot infect and/or replicate in the myoblasts but rather appears that this viral strain may not be able to gain access to this compartment *in vivo*.

To determine whether the difference in viral replication was due to the inability of 37997 to infect myofibers *in vivo*, intramuscular injections were performed by injecting 2.5×10^4 PFU directly into the left hamstrings of six-day-old mice. Hind limb muscle from the site of injection was collected and stained by immunofluorescence. In contrast to the intradermal infection, following the direct intramuscular administration of virus, both 37997-GFP and LR2006-GFP were able to infect myofibers, as shown at 3 dpi (Fig. 6D), and the numbers of GFP^+ myofibers were similar for the two viral strains (Fig. 6E). At later time points, massive tissue destruction was seen in mice infected by either strain, and few GFP^+ myofibers remained (data not shown). This finding was further supported by an analysis of viral loads in the muscle. Mice were infected with the parental strains of LR2006 OPY1 and 37997, which lack GFP, and viral titers in the infected muscle were analyzed at different times postinfection. Following direct inoculation, the two strains reached similar viral titers, with only a 3.54-fold increase in titers in the LR2006 OPY1- versus the 37997-infected muscle, compared to the 1,000-fold difference seen during i.d. administration (Fig. 6F). Following direct inoculation, the 37997 strain reached peak titers of 6.77×10^5 PFU/ml, much higher than its peak viral load following administration by the i.d. route, despite using a lower inoculating dose of 2.5×10^4 PFU (Fig. 6F). The ability of the 37997 strain to replicate to high

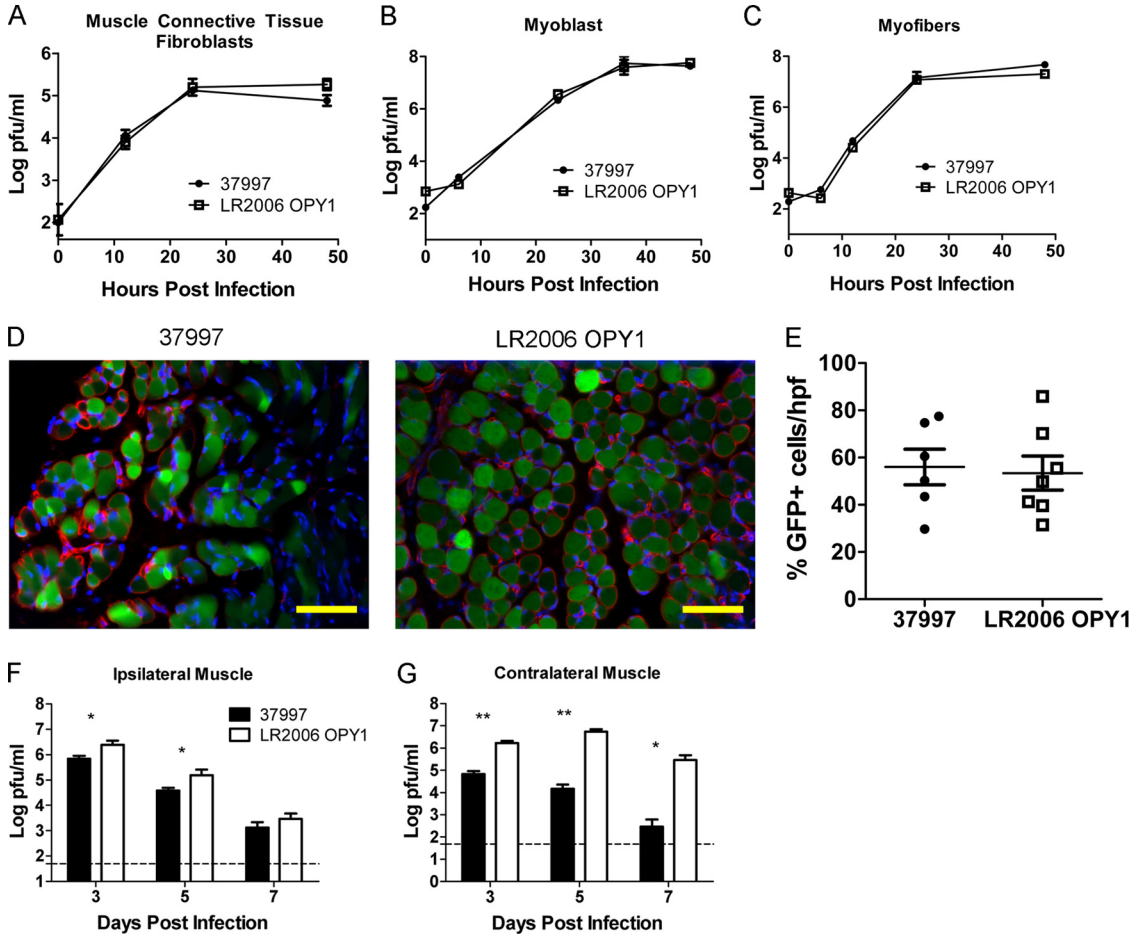

FIG 6 Both strains can infect muscle when given intramuscularly. (A to C) Primary muscle fibroblasts (A), C2C12 myoblasts (B), and differentiated C2C12 myofibers (C) were infected with either LR2006 OPY1 or 37997 at an MOI of 0.05, and titers were determined at various times postinfection by plaque assay. (D and E) Mice were infected in the hind limb with 2.5×10^4 PFU LR2006 OPY1-GFP or 37997-GFP and sacrificed at 3 days postinfection. (D) Frozen sections of hind limb muscle was stained for collagen IV (red), GFP (green), and nuclei (blue). Scale bar, 100 μm. (E) Quantification of GFP^+ myofibers per high-power field. (F and G) Mice were infected in the hind limb with the parental strains at 5×10^4 PFU; muscles ipsilateral (F) and contralateral (G) to the site of infection were harvested at the indicated days, and titers were determined by plaque assay. *, $P = 0.01$ to 0.05; ** $P = 0.001$ to 0.01; *** $P < 0.001$.

viral titers following direct i.m. inoculation indicates that the two viral strains display similar fitness once they gain access to the muscle fiber compartment. During the course of these direct intramuscular infections, we once again noted that the contralateral legs of the mice infected with the LR2006 OPY1 strain of CHIKV developed severe weakness, while in the mice infected with the 37997 strain of CHIKV, we did not detect contralateral leg weakness. When titers in the contralateral legs were determined, we found that similar to the case for the i.d. route of infection, the LR2006 OPY1-infected mice achieved significantly higher viral titers in their contralateral legs than the titers obtained in the 37997-infected mice, with viral titers reaching 5.5×10^6 PFU/ml in the LR2006 OPY1-infected mice at 5 dpi, compared to titers of only 1.5×10^4 PFU/ml in the 37997-infected mice (Fig. 6G). Thus, injecting virus at a different location, in this case directly into the muscle, does not alter its ability to spread throughout the host. Taken together, these results demonstrate that both the LR2006 OPY1 and the 37997 strains of CHIKV have the ability to replicate within the primary cells of the muscle, namely, the connective tissue fibroblasts and the myoblasts when access is provided. The differences in pathogenesis that we have seen in our model appear to reflect the ability of the LR2006 OPY1 strain to access the myoblast/myofiber niche, which it can do following either i.d. or i.m. inoculation. In contrast, the 37997 strain of CHIKV, while able to establish viremia and infect the connective tissue fibroblasts, fails to establish a robust infection of the myoblasts/myofibers unless it is provided direct access via i.m. inoculation. This results in decreased viral loads and diminished muscle damage.

DISCUSSION

The ongoing epidemic of CHIKV infection has highlighted the need for a better understanding of the pathogenesis of this reemerging pathogen. The dominant disease presentation seen during this outbreak consists of fever, rash, and arthritis, as in past outbreaks. However, new reports suggest increased disease severity, including neurological symptoms and mortality that had not previously been seen in CHIKV outbreaks. The reasons for altered pathogenesis are multifactorial and include the large scale of the outbreak, better reporting of CHIKV cases, and accrual of viral mutations.

In this study, we compared the pathogeneses of two different CHIKV strains, one isolated during the La Reunion outbreak in 2006 and another isolated from Senegal in 1983. Importantly, both viruses utilized in these studies were generated from recombinant clones to minimize viral heterogeneity. The clones were not mouse adapted and represent viruses that were actively circulating when isolated. Neonatal mice infected with the La Reunion recombinant strain (LR2006 OPY1) developed more severe disease, with progressive hind limb weakness and severe myonecrosis, than those infected with the Senegal strain (37997). 37997 and another strain from the 2006 outbreak (DHS-4263) were also compared in a macaque model, where slightly elevated viremia was seen with the 37997 strain (33). Mild muscle fiber necrosis was also seen in several animals, but muscle titers or pathology during acute infection was not determined (33). In the murine arthritis model, more severe joint swelling was seen in mice infected with the LR2006 OPY1 strain than in those infected with an Asian isolate from the 1960s (34). Additional studies that carefully evaluate viral pathogenesis and correlate this with myofiber infection will be important to perform to try to identify the viral determinants contributing to this phenotype.

The most striking difference in the disease induced by each strain was the very high muscle titer in the mice infected with LR2006 OPY1, which resulted in severe myonecrosis (Fig. 2E and 4). Both strains are able to spread from the site of infection and establish infection in the distal muscle by infecting connective tissue fibroblasts early in infection. CHIKV has been shown to infect this cell type in murine models and in human biopsy specimens (16). However, as early as 3 dpi, when viral loads in the serum were similar for the two strains, mice infected with the LR2006 OPY1 strain had viral titers within their hind limb muscles that were 1,000-fold greater than those observed in the 37997-infected mice. This increased viral load persisted until at least 9 dpi. Most strikingly, this increase in viral replication correlated with the ability of the LR2006 OPY1 strain, but not the 37997 strain, to infect the myofibers within the muscle.

There are several hypotheses that could explain the difference in behavior between the 37997 and LR2006 OPY1 strains. These include differences in viral fitness, viral tropism, and access to myofibers. A recent study of Ross River virus identified a single amino acid change within the E2 protein (Y18H) that resulted in a dramatic reduction in muscle titers and pathology. This switch to histidine resulted in a decrease in the fitness of this virus in mammalian cells, resulting in reduced replication in both murine and human cells but an increased ability to replicate in mosquito cells (35). An alignment of the E2 proteins from the LR2006 OPY1 and 37997 strains revealed that both have a histidine at this position. Furthermore, while the LR2006 strain does display an increase in viral replication within the muscle and induces more damage, we have not observed differences in viral fitness. Similar replication of the two strains was observed in several mammalian cell types, including BHK cells, MEFs, and connective tissue fibroblasts (Fig. 1 and 6 and data not shown), suggesting that the fitness of the virus does not explain our *in vivo* phenotypes.

Previous to this study, direct infection of myofibers by CHIKV had not been shown, though evidence of myofiber fragmentation and necrosis has been reported (33). Since we observed the infection of myofibers following i.d. administration of the LR2006 OPY1 strain but not the 37997 strain, differences in cellular tropism could also contribute to the altered pathogenesis. Mutations in the LR strain could result in increased receptor binding and/or replication within myoblasts or myofibers. However, our *in vitro* studies demonstrated that both strains were able to infect connective tissue fibroblasts, myoblasts, and myofibers, and the viruses grew with similar kinetics and to similar titers (Fig. 6). Furthermore, the administration of the 37997 strain directly into the muscle resulted in myofiber infection and dramatically reduced the differences in viral titers seen between the two strains (Fig. 6). Interestingly, while direct i.m. administration of the two strains equalized the viral loads within the injected muscle and allowed for 37997 to infect the myofibers, spread of the virus to the contralateral muscle was more efficient for the LR2006 strain, once again resulting in viral loads that were greater than 100-fold higher than those seen with the 37997 strain (Fig. 6). These data suggest that the LR2006 OPY1 strain may have an increased ability to access myofibers.

Previous studies evaluating the ability of gene therapy viral vectors, such as herpes simplex virus 1 (HSV-1) and adenoviruses, to infect skeletal muscle have shown that the basal lamina can function as a physical barrier to limit myofiber infection in adult muscle. Intact skeletal muscle was not permissive to HSV-1 infection of myofibers, but isolated myofibers cultured *in vitro* could be infected (36). The disruption of the basal lamina through the utilization of genetically defective mice (*dy/dy*) which have abnormal basal lamina synthesis allowed for myofiber infection *in vivo* (37). One potential explanation for the differential infection of myofibers with our viruses is that during the course of the LR2006 OPY1 infection, there is an alteration in the production of cytokines or proteases that can damage the basal lamina surrounding the muscle fibers, allowing the LR2006 OPY1 strain to establish infection. Proinflammatory cytokines and chemokines are induced in animal models of CHIKV, and in human studies elevated levels of IL-6, IL-1β, and RANTES have been associated with more severe disease manifestations (18). Infection with either the LR2006 OPY1 or the 37997 strain of CHIKV did induce a proinflammatory cytokine and chemokine response; however, despite the prolonged serum viremia following LR2006 OPY1 infection, no significant differences were observed between the two strains during the course of infection (Fig. 3). We also did not observe a difference in the induction of type I IFNs either systemically or within the muscle itself. While the levels of IFN did not differ, it is still possible that there is differential induction of ISGs by these two strains that contributes to differential control of viral replication within the muscle fiber. A limited analysis of ISGs has not revealed any differential expression within the muscle; however, a more complete analysis is required to fully rule out this possibility. Alternatively, within the context of the intact muscle architecture, differential induction in the expression of receptors for CHIKV on myoblasts or myofibers could account for differences in infectivity. Until the CHIKV receptor is identified, the latter hypothesis cannot be formally tested. Whether the prolonged serum viremia we observed during the LR2006 OPY1 infection also contributes to the myofiber infection by allowing the LR2006 OPY1 virus to reach higher concentrations along the basal lamina is unknown. However, the prolonged viremia cannot account for the entire muscle phenotype, as LR2006 OPY1-infected mice already have high titers at 3 dpi, when both strains cause similar serum viremia (Fig. 2).

Little is known about the viral determinants that regulate CHIKV-induced disease, in particular its ability to target the mus-

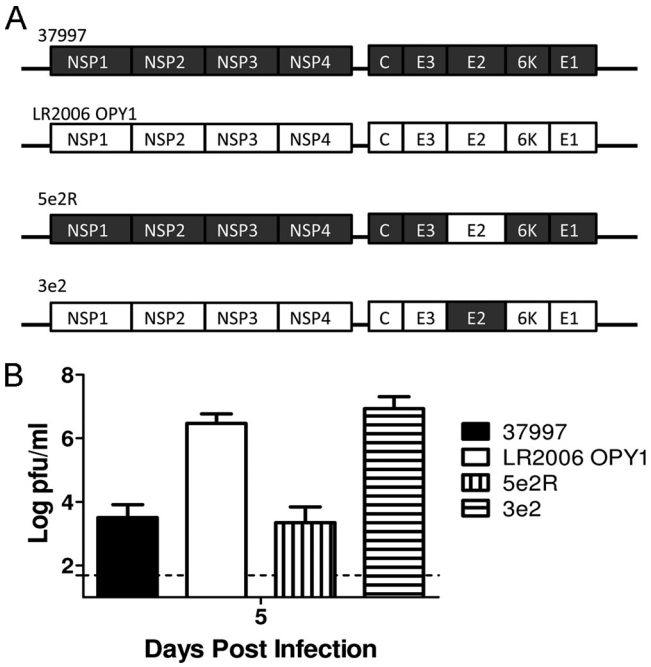

FIG 7 The E2 protein of LR2006 OPY1 strain of CHIKV is not responsible for the increased replication in the muscle fibers. (A) Chimeric viruses were constructed by swapping the E2 from one strain into the backbone of the other as shown in the schematic. Mice were infected by each strain at a dose of 5×10^4 PFU intradermally. (B) Hind limbs were harvested at 5 dpi, and titers were determined by plaque assay ($n = 3$ or 4).

culoskeletal system. Several studies have identified mutations within the E2 protein in different alphaviruses to have a significant impact upon virulence. The vaccine strain CHIKV 181/clone 25 was found to be attenuated in mice due to mutations in the E2 protein that arose during serial passaging in cell culture (38). Additionally, a residue found to be responsible for controlling RRV virulence in the muscle was localized to the E2 protein (35). To critically evaluate the role that the E2 protein may play in the ability to infect and replicate in the myofibers, we generated chimeric viruses in which the E2 proteins were exchanged, replacing the E2 of the LR2006 strain with the 37997 E2 or making the reciprocal virus (Fig. 7A). Following i.d. infection with the recombinant strains, we analyzed viral titers in the distal muscle, but in both cases we found that the substitution of the E2 protein did not alter the viral phenotype of the background virus, indicating that the E2 protein, in isolation, was not responsible for the increased replication within the muscle (Fig. 7B).

The prolonged serum viremia observed following LR2006 OPY1 infection could have many impacts upon CHIKV pathogenesis. An increase in the length of time that a patient exhibits high serum viremia would prolong the period of time in which a patient can transmit the infection to a new mosquito host and subsequently to another patient. It has previously been shown that an alanine-to-valine mutation within the LR2006 OPY1 strain resulted in increased infectivity of the Asian tiger mosquito, *A. albopictus*. Therefore, a prolonged period of serum viremia coupled with the ability of this strain to replicate to high titers in *A. albopictus* could contribute to the ease with which CHIKV has spread to infect many on La Reunion and ultimately millions in Southeast Asia. A prolonged viremic phase could also contribute to more severe disease manifestations, especially in the elderly and neonates with already-diminished immune systems. A prospective cohort study of patients on La Reunion found that those patients with chronic disease had higher viral loads than their counterparts who recovered fully, suggesting a deficit in viral clearance (39). Future studies evaluating whether the ability of CHIKV to access and infect myofibers contributes to more severe disease manifestations, such as the development of arthritis or the maintenance of chronic infection, will be important to further our understanding of CHIKV pathogenesis and in the identification of therapeutic interventions.

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