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Chikungunya Viruses That Escape Monoclonal Antibody Therapy Are Clinically Attenuated, Stable, and Not Purified in Mosquitoes

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ABSTRACT

Chikungunya virus (CHIKV) is a reemerging mosquito-transmitted alphavirus that causes epidemics of debilitating polyarthritis in humans. A prior study identified two anti-CHIKV monoclonal antibodies ([MAbs] CHK-152 and CHK-166) against the E2 and E1 structural proteins, which had therapeutic efficacy in immunocompetent and immunocompromised mice. Combination MAb therapy was required as administration of a single MAb resulted in the rapid selection of neutralization escape variants and treatment failure in mice. Here, we initially evaluated the efficacy of combination MAb therapy in a nonhuman primate model of CHIKV infection. Treatment of rhesus macaques with CHK-152 and CHK-166 reduced viral spread and infection in distant tissue sites and also neutralized reservoirs of infectious virus. Escape viruses were not detected in the residual viral RNA present in tissues and organs of rhesus macaques. To evaluate the possible significance of MAb resistance, we engineered neutralization escape variant viruses (E1-K61T, E2-D59N, and the double mutant E1-K61T E2-D59N) that conferred resistance to CHK-152 and CHK-166 and tested them for fitness in mosquito cells, mammalian cells, mice, and *Aedes albopictus* mosquitoes. In both cell culture and mosquitoes, the mutant viruses grew equivalently and did not revert to wild-type (WT) sequence. All escape variants showed evidence of mild clinical attenuation, with decreased musculoskeletal disease at early times after infection in WT mice and a prolonged survival time in immunocompromised *Ifnar1*$^{-/-}$ mice. Unexpectedly, this was not associated with decreased infectivity, and consensus sequencing from tissues revealed no evidence of reversion or compensatory mutations. Competition studies with CHIKV WT also revealed no fitness compromise of the double mutant (E1-K61T E2-D59N) neutralization escape variant in WT mice. Collectively, our study suggests that neutralization escape viruses selected during combination MAb therapy with CHK-152 plus CHK-166 retain fitness, cause less severe clinical disease, and likely would not be purified during the enzootic cycle.

IMPORTANCE

Chikungunya virus (CHIKV) causes explosive epidemics of acute and chronic arthritis in humans in Africa, the Indian subcontinent, and Southeast Asia and recently has spread to the New World. As there are no approved vaccines or therapies for human use, the possibility of CHIKV-induced debilitating disease is high in many parts of the world. To this end, our laboratory recently generated a combination monoclonal antibody therapy that aborted lethal and arthritogenic disease in wild-type and immunocompromised mice when administered as a single dose several days after infection. In this study, we show the efficacy of the antibody combination in nonhuman primates and also evaluate the significance of possible neutralization escape mutations in mosquito and mammalian cells, mice, and *Aedes albopictus* vector mosquitoes. Our experiments show that escape viruses from combination antibody therapy cause less severe CHIKV clinical disease, retain fitness, and likely would not be purified by mosquito vectors.

Although chikungunya virus (CHIKV) was first isolated from a febrile patient with severe joint pain in Tanzania in 1953 (1), it is believed that the virus has caused disease in Africa and Southeast Asia since the late 1700s (2). Historically, CHIKV infection caused periodic, contained outbreaks across Africa and Asia (2). Between 2005 and 2007, however, an explosive epidemic of CHIKV infection of unprecedented magnitude occurred; it initiated on the coast of Kenya in 2004, from which it dispersed to the French island of La Reunion, other Indian Ocean islands, and many nations in Africa and Asia (2–4). The recent CHIKV epidemic has affected over 5 million people, including one-third of the population (~300,000 people) of La Reunion Island (4, 5). Although travelers returning from countries of endemicity to Canada, Europe, and the United States have acquired CHIKV infection and disease, local epidemics in the developed world did not occur until 2007, with the onset of the first European outbreak,

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which caused 229 cases and one fatality in Northern Italy (6, 7). In 2010, cases of CHIKV infection also were reported in France (8). Most recently, in 2013 and 2014, local epidemics of CHIKV infection were reported in the Americas in several countries in the Caribbean, providing the first evidence of autochthonous transmission in the New World (http://www.cdc.gov/chikungunya/geo/americas.html).

CHIKV is transmitted by the *Aedes* species mosquitoes and is maintained in a sylvatic cycle in Africa, where nonhuman primates and rodents are reservoirs and where forest-dwelling mosquitoes (chiefly the *Aedes* species *furcier*, *taylori*, *luteocephalus*, and *africanus*) are vectors for transmission (9, 10). During epidemics, humans serve as the principal reservoirs. *Aedes aegypti*, an urban mosquito that maintains close association with humans, is the primary vector in Asia. *Aedes albopictus* was the vector primarily responsible for the La Reunion epidemic in 2005 to 2007 due to a single amino acid mutation that enhanced vector infectivity and epidemic potential (11). *A. albopictus* mosquitoes have spread to include all continents, tropical and temperate (12), so CHIKV epidemics could occur anywhere (13). The changing epidemiology of CHIKV and ubiquity of its mosquito vectors highlight the likelihood of its continued global spread.

Acute CHIKV infection manifests 3 to 7 days after inoculation by an *Aedes* mosquito bite. Symptoms include an abrupt onset of a high fever, rash, polyarthralgia, and myalgia (14, 15). Polyarthralgia is mainly symmetric and tends to occur in previously injured or distal joints (16). Acute symptoms persist for about 14 days, but chronic arthralgia can linger and cause morbidity for weeks or even years. Tenosynovitis is observed commonly in the chronic, recurring form of CHIKV disease and often affects the wrists, fingers, and ankles (17). Joint pain can be debilitating; a recent study showed that severe arthralgia persisted for at least 36 months in ~60% of a cohort of CHIKV-infected patients (18).

The CHIKV genome is an 11.8-kb single-stranded, positive-sense RNA with two open reading frames (ORFs). It is one of 29 alphaviruses and belongs to the *Togaviridae* family of enveloped viruses. There are three genotypes of CHIKV: East/Central/South African (ECSA), Asian, and West African, which are between 95.2 and 99.8% identical at the amino acid level (19, 20). The CHIKV genome is flanked by untranslated regions with a 5′ *N*-methyl guanosine cap and poly(A) tail, between which the two ORFs reside. The 5′ two-thirds of the genome encodes four nonstructural proteins (nsP1, -2, -3, and -4). The second ORF, which is downstream of a separate 26S subgenomic promoter (21), encodes the structural proteins: C (nucleocapsid protein), E3, E2, 6K, TF, and E1. The mature virion is comprised of the nucleocapsid protein C and two glycoproteins, E1 and E2, with E2 functioning in attachment to cells and E1 participating in virus fusion. Each 700-Å CHIKV virion contains 240 copies of the envelope and capsid proteins, which are arranged in T=4 quasi-icosahedral symmetry. E1-E2 heterodimers assemble into 80 trimeric spikes on the virus surface (22). X-ray crystallographic structures (23–26) have elucidated the architecture of the glycoprotein shell. The E1 ectodomain consists of three domains. Domain I (DI) lies between DII and DIII, the latter of which adopts an immunoglobulin-like fold. The fusion peptide is located at the distal end of DII. E1 monomers sit at the base of the surface spikes and form a trimer around each of the icosahedral axes. E2 localizes to a long, thin, leaf-like structure on the top of the spike and contains three domains with immunoglobulin-like folds: the N-terminal domain A, located at the center; domain B at the tip; and the C-terminal domain C, located proximal to the viral membrane.

CHIKV infection *in vivo* has been studied extensively in mice (27–35). Newborn outbred mice exhibit gait instability, lethargy, and weight loss after CHIKV infection (27). Neonatal inbred C57BL/6 mice are vulnerable to fatal CHIKV infection; viral replication in these animals is observed in muscle, joint, skin, and brain (28). Adult mice with defective type I interferon (IFN) signaling (*Ifnar1*$^{-/-}$ mice) develop lethal infection, with muscle, joint, and skin serving as primary sites of replication (28, 34). CHIKV infection in 2- to 6-week-old wild-type (WT) inbred C57BL/6 and outbred CD-1 mice results in rheumatologic and musculoskeletal disease (29, 30, 36) that mimics human illness in some respects and can progress to persistent infection (31). Infected mice develop metatarsal foot swelling with histological evidence of arthritis, tenosynovitis, and myositis. In cynomolgus macaques, CHIKV persists in joints, muscles, lymphoid organs, and liver for at least 3 months (37), providing a possible explanation for the long-lasting CHIKV symptoms observed in humans. While adult rhesus macaques effectively control CHIKV infection, aged animals developed persistent infection in the spleen (38).

The importance of the humoral response in preventing or controlling CHIKV infection has been established (39). Immune gamma globulin from donors in the convalescent phase of CHIKV infection displays strong neutralizing activity *in vitro* and protective efficacy in *Ifnar1*$^{-/-}$ and neonatal WT mice (28). E1- or E2-specific monoclonal antibodies (MAbs) or immune sera exhibit protective efficacy *in vivo* against other alphaviruses (40–48). Protective human and mouse MAbs against CHIKV E2 protein that bind domain B, domain A, or the fusion loop groove neutralize CHIKV infection and prevent or mitigate disease *in vivo* (49–55). Anti-E1 MAbs against CHIKV with protective activity in mice also have been described (52).

Recently, we identified several neutralizing anti-CHIKV MAbs that provided complete protection against lethality as prophylaxis in highly susceptible *Ifnar1*$^{-/-}$ mice (52). Our two most protective MAbs (CHK-152 and CHK-166) mapped to distinct epitopes on the E2 and E1 structural proteins. In postexposure therapeutic trials, a single dose of a combination of the two MAbs (CHK-152 plus CHK-166) limited the development of resistance and protected *Ifnar1*$^{-/-}$ mice against disease when it was administered 24 to 36 h before CHIKV-induced death. Here, we evaluated the efficacy of combination MAb therapy against CHIKV infection in rhesus macaques and the significance of possible resistance in mosquito and mammalian cells, immunocompetent and immunocompromised mice, and *A. albopictus* mosquitoes.

MATERIALS AND METHODS

Ethics statement. All rhesus macaques were handled in accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies. Infection studies with rhesus macaques were approved by the Oregon National Primate Research Center (ONPRC) Institutional Animal Care and Use Committee, which is accredited by the Assessment and Accreditation of Laboratory Animal Care International. For blood collection, monkeys were anesthetized with ketamine by intramuscular injection. Euthanasia was conducted under anesthesia with ketamine, followed by an overdose of sodium pentobarbital, as recommended by the American Veterinary Medical Association. Experiments with *Ifnar1*$^{-/-}$ mice were performed according to the guidelines and with the approval of the Washington University Institutional Animal Care and Use Committee. Experiments with WT C57BL/6 mice were performed in

accordance and with approval of the University of Colorado School of Medicine Institutional Animal Care and Use Committee guidelines. Footpad injections were performed under anesthesia that was induced and maintained with ketamine hydrochloride and xylazine or isoflurane, and all efforts were made to minimize suffering.

Cells and viruses. Vero and BHK21 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% and 10% fetal bovine serum (FBS; Omega Scientific), respectively, 10 mM HEPES, nonessential amino acids (Cellgro), and antibiotics (penicillin and streptomycin) at 37°C in a 5% CO_2 incubator. C6/36 *A. albopictus* cells were cultivated in Leibovitz-15 medium (Invitrogen) supplemented with 10% FBS, 10 mM HEPES, and antibiotics (penicillin and streptomycin) at 27°C. The infectious clone of CHIKV LR 2006 OPY-1 (CHIKV-LR) has been described previously (56). The CHIKV pDonor221 plasmid containing the WT or mutated structural genes was published previously (52) and was provided by K. Dowd and T. Pierson (Bethesda, MD). Single point mutations (E1-K61T or E2-D59N) were introduced into the infectious clone after QuikChange site-directed mutagenesis (Agilent Technologies) (primers are listed in Table S1 in the supplemental material) by ligating an SgrA1 and SfiI (New England BioLabs) doubly digested pDonor221 fragment. The double mutant E1-K61T E2-D59N was created by performing sequential mutagenesis reactions. All WT and mutant infectious clones were sequenced in their entirety.

To produce recombinant virus from infectious cDNA clones, plasmids were linearized with the restriction endonuclease NotI (New England BioLabs), and N7-methyl-guanosine-capped RNA was produced *in vitro* using an SP6 DNA-dependent RNA polymerase transcription kit according to the manufacturer's instructions (mMessage kit; Ambion). CHIKV RNA was electroporated with three pulses at 850 V, with 25 μF of capacitance and infinite resistance in a 2-mm cuvette into BHK21 cells. The supernatant was harvested 28 h later, aliquoted, and stored frozen at −80°C to generate the passage 0 (P0) stock. C6/36 cells were infected with the P0 CHIKV-LR at a multiplicity of infection (MOI) of 0.01, in the presence of 10 μg/ml of either CHK-166 (for CHIKV E1-K61T), CHK-152 (for CHIKV E2-D59N) and 10 μg/ml of CHK-166 and CHK-152 (CHIKV E1-K61T E2-D59N). Supernatant was harvested 68 to 72 h later. Virus titers were determined on Vero cells by focus-forming unit (FFU) assay. Virus from insect or mammalian cell stocks was sequenced to confirm retention of the mutated amino acids.

Virus titration assays. Vero cells (seeded overnight at 3×10^4 cells/well, in 96-well plates) in DMEM with 5% FBS were infected at 37°C for 90 min with serial dilutions of virus stocks and then overlaid with 1% (wt/vol) methylcellulose in modified Eagle medium (MEM) supplemented with 4% FBS. Plates were harvested 18 h later and fixed with 1% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS). The plates were incubated sequentially with 500 ng/ml of a chimeric mouse-human anti-CHIKV MAb (ch-CHK-9) as described previously (52) and a 0.3 μg/ml solution of horseradish peroxidase (HRP)-conjugated goat anti-human IgG secondary antibody (Sigma) in PBS supplemented with 0.1% saponin and 0.1% bovine serum albumin (BSA). CHIKV-infected foci were visualized using TrueBlue peroxidase substrate (KPL) and quantitated on an ImmunoSpot, version 5.0.37, macroanalyzer (Cellular Technologies, Ltd.).

Viral growth curves. Vero cells were seeded overnight at 6×10^4 cells/well in 12-well plates in 1 ml of DMEM containing 5% FBS. C6/36 cell-derived CHIKV (WT or mutant) was incubated with 10 μg/ml of CHK-166, CHK-152, both CHK-166 and CHK-152, or no MAb for 1 h at 37°C. Medium was removed from Vero cells, and virus-MAb complexes in 500 μl were added for 1 h at 37°C. Subsequently, the inoculum was removed, and cells were rinsed with PBS. Medium containing the respective MAb or no MAb was added back to the cells. Cells were harvested at time points 1, 12, 24, and 36 h postinfection. Analogously, growth curves were performed on C6/36 cells with P0 BHK-derived CHIKV (WT and mutant), and cells were harvested at time points 1, 24, 48, and 72 h postinfection. Virus titers were determined by focus-forming assay as described above.

Mouse experiments. *Ifnar1*$^{-/-}$ mice were obtained from J. Sprent (Scripps Institute, San Diego, CA) and backcrossed 10 times onto the C57BL/6 background. *Rag1*$^{-/-}$ mice were purchased commercially (Jackson Laboratories). *Ifnar1*$^{-/-}$ and *Rag1*$^{-/-}$ mice were bred in the pathogen-free animal facilities of Washington University School of Medicine. Some of the 6- to 8-week-old *Ifnar*$^{-/-}$ mice were treated at day −1 with 50 μg of CHK-152 and CHK-166 by intraperitoneal (i.p.) injection. Other *Ifnar1*$^{-/-}$ or *Rag1*$^{-/-}$ mice received the WT and mutant viruses in the absence of MAb pretreatment. *Ifnar1*$^{-/-}$ or *Rag1*$^{-/-}$ mice were inoculated subcutaneously in the footpad with 10 FFU or 10^3 FFU, respectively, of C6/36 cell-derived CHIKV-LR (WT, E1-K61T, E2-D59N, or E1-K61T E2-D59N) in 50 μl of Hanks balanced salt solution (HBSS) supplemented with 1% heat-inactivated FBS.

C57BL/6 WT mice were obtained from the Jackson Laboratory and bred in specific-pathogen-free facilities at the University of Colorado. Three-week-old mice were used for all of these studies. Mice were inoculated in the left rear footpad with 10^3 FFU of CHIKV (WT, mutant viruses, or a defined mixture of WT and mutant viruses) in PBS supplemented with 1% FBS in a volume of 10 μl. Mice were monitored for disease signs and weighed at 24-h intervals. On the termination day of each experiment, mice were sedated with isoflurane and euthanized by thoracotomy and exsanguination. Mice were perfused by intracardiac injection with PBS, and tissues were removed by dissection and homogenized in TRIzol reagent (Life Technologies) for RNA isolation.

Nonhuman primate experiments. Adult male and female rhesus macaques (ranging from 6 to 13 years old) were infected subcutaneously in both arms with 10^7 PFU of the CHIKV-LR strain in 1 ml of PBS. At days 1 and 3 postinfection, animals were injected intravenously ($n = 6$ per group in two independent experiments) with a total of 15 mg/kg of MAbs against CHIKV E1 and E2 (mixture of MAb CHK-152 and CHK-166) or West Nile virus (WNV) E (WNV E16, negative control) while under sedation. MAbs were diluted in approximately 15 to 20 ml of saline solution for a total volume of 25 ml. Blood samples were obtained following ketamine sedation (10 mg/kg) on the day of infection (day 0) and at days 1, 2, 3, 4, 5, and 7 postinfection. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by centrifugation over Histopaque gradient (Sigma-Aldrich). Animals were euthanized at 7 days postinfection (dpi). At the time of harvest, organs, lymphoid tissues, joints, and muscles were collected and processed for nucleic acid detection and plaque assays to quantify levels of CHIKV.

qRT-PCR analysis for CHIKV. (i) Rhesus macaques. Viral RNA load in the tissues at necropsy of the rhesus macaques was quantified using real-time reverse transcription-PCR (RT-PCR) (38). Primers and probes included the following: CHIKV-9482F, 5′-GGAACGAGCAGCAACCTTTG-3′; CHIKV-9931R, 5′-ATGGTAAGAGTCTCAGACAGTTGCA-3′; and the TaqMan probe CHIKV-9870F, 5′-GGAATAAGGGCTTGT-3′. Total RNA was prepared from tissue specimens using TRIzol according to the manufacturer's instructions (Life Technologies). The isolated RNA was quantified using a Nanodrop spectrophotometer. RNA was treated with RNase-free DNase, and then single-stranded cDNA was generated using random hexamers and Superscript III RT (Invitrogen, Carlsbad, CA). Gene amplicons served as quantification standards (sensitivity, 10 to 100 copies). Quantitative RT-PCR (qRT-PCR) was performed and analyzed using ABI StepOne Plus real-time PCR system (Applied Biosystems) as described previously (31).

(ii) Mice. RNA was isolated using a PureLink RNA minikit (Life Technologies), and the amount of CHIKV positive-strand RNA present in tissues was quantified by qRT-PCR as previously described (31). Briefly, the first-strand cDNA reaction was primed with 250 ng of random primers (Life Technologies). A CHIKV sequence-specific forward primer (CHIKV-2411F, 5′-AGAGACCAGTCGACGTGTTGTAC-3′) and reverse primer (CHIKV-2676R, 5′-GTGCGCATTTTGCCTTCGTA-3′) were used in conjunction with a TaqMan probe (CHIKV-2579F, 5′-ATC

TGCACCCAAGTGTACCA-3′). For absolute quantification of CHIKV RNA, a standard curve was generated: 10-fold dilutions from 10^8 to 10^0 copies of CHIKV positive-strand genomic RNA, synthesized *in vitro*, were spiked into RNA from BHK-21 cells, and reverse transcription and quantitative PCR (qPCR) were performed in an identical manner. No template controls were run in parallel.

Quantitative analysis for infectious CHIKV. Infectious viral load from tissues was determined by limiting-dilution plaque or focus-forming assay. Tissues were homogenized using a bead beater (Precellys 24 homogenizer), and cellular debris was pelleted by centrifugation (5,000 × *g* for 2 min). The titer of a 100-μl sample of the clarified tissue supernatant or blood plasma was determined on Vero or BHK21 cells. The genome-to-FFU ratios of stock WT and mutated viruses were determined by measuring the number of viral RNA copies per FFU using a standard curve and quantitative RT-PCR.

Mosquito infection experiments. *A. albopictus* mosquitoes (La Reunion strain) were reared according to standard procedures. Adult females were fed a mixture of defibrinated sheep blood and P0 stocks of WT or CHIKV mutants (E1-K61T, E2-D59N, and E1-K61T E2-D59N) that were generated in BHK21 cells in a 1:1 blood/culture supernatant ratio. The titers of the WT, E1 K61T mutant, E2 D59N mutant, and E1 K61T E2 D59N double mutant were, respectively, 7.56, 7.36, 7.59, and 7.49 $\log_{10}$ tissue culture infectious dose 50% endpoint titers ($TCID_{50}$)/ml. After feeding, mosquitoes were anesthetized, and engorged females were separated into cartons and held for a 14-day extrinsic incubation period at 28°C, 70 to 80% relative humidity, and a 16:8 photoperiod. In separate experiments, mosquitoes were fed a blood meal using the same virus stocks and harvested within 1 h of feeding. Mosquitoes were collected at 7, 10, and 14 days postinfection and titrated to determine viral titer of abdomens containing the midguts with surrounding tissues or secondary tissues, including heads, wings, and legs for dissemination, or used for RNA extraction and sequencing. Viral titers from mosquito samples were determined using Vero cells and expressed as $\log_{10}$ $TCID_{50}$/ml as previously described (11).

Viral RNA sequencing from cells and tissues. (i) Cell culture. Vero or C6/36 cells were infected with WT or CHIKV mutants as described above. Cell supernatants were collected at 36 or 72 h after infection, respectively, and RNA was isolated using a QIAamp viral RNA minikit (Qiagen). cDNA was produced using a Superscript III First Strand System (Life Technologies). Phusion DNA polymerase (New England BioLabs) was used to produce a PCR amplicon spanning the E2-E1 structural genes. Amplicons were purified by agarose gel electrophoresis, sequenced using overlapping primers (see Table S1 in the supplemental material), and analyzed using Geneious software.

(ii) Mouse tissues. Tissues were perfused extensively with PBS and then removed by dissection and homogenized in TRIzol reagent (Life Technologies). Total RNA was isolated from the right ankle using a PureLink RNA kit (Life Technologies). The first-strand cDNA reaction was primed with 250 ng of random primers. PCRs were performed with a set of overlapping primers that amplified the complete E2 and E1 coding regions (see Table S1 in the supplemental material). Amplicons were purified by agarose gel electrophoresis and sequenced directly on a fluorescent capillary automated sequencer at the University of Colorado DNA sequencing and analysis core. Sequence alignments were performed using Geneious Pro software.

(iii) Mosquitoes. Whole mosquito bodies were collected and homogenized in RLT lysis buffer, and RNA was extracted using RNeasy kits (Qiagen). Samples were screened for CHIKV infection using a OneStep RT-PCR kit (Qiagen), and primers that amplified the complete E2-E1 genes were used for sequencing (see Table S1).

(iv) Nonhuman primates. Rhesus macaque joint and organ tissues were homogenized in 1 ml of TRIzol reagent plus approximately 250 μl of SiLiBeads, type S (1.7 to 2.1 mm; VWR), using a Precellys 24 homogenizer. Total RNA was isolated according to the manufacturer's specifications, and first-strand cDNA was synthesized using a 10 μM oligonucleotide mix consisting of random primers and oligo(dT). PCRs were performed with AmpliTaq Gold 360 (Invitrogen) and primers adjacent to E1 and E2 (see Table S1 in the supplemental material). Amplicons were cloned into the pGEM Teasy vector (Promega), transformed in bacteria, and expanded overnight in LB medium. Plasmids were purified using a Genelute Plasmid Mini Prep kit (Sigma), and sequencing was performed by the ONPRC Sequencing Core using a fluorescent capillary automated sequencer.

Cytokine bioplex assay. WT mice were inoculated with 10^3 FFU of CHIKV WT or CHIKV E1-K61T E2-D59N virus; at day 3 after infection blood was collected, and serum was isolated. A BioPlex Pro assay was performed according to the manufacturer's protocol (Bio-Rad). The cytokine screen included interleukin 1α (IL-1α), IL-1β, IL-2, IL-3 IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 p40, IL-12 p70, IL-13, IL-17, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), gamma interferon (IFN-γ), monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α), MIP-1β, RANTES, and tumor necrosis factor alpha (TNF-α).

Statistical analysis. All data were analyzed using Prism software (GraphPad software). For survival analysis, Kaplan-Meier survival curves were analyzed by log rank test. For growth kinetics and neutralization, an unpaired *t* test or analysis of variance (ANOVA) was used to determine statistical significance. A Mann-Whitney test was used to analyze cytokine and chemokine levels in serum.

RESULTS

Anti-CHIKV MAb therapy reduces viral spread in nonhuman primates. A prior study established the therapeutic efficacy of a combination of two MAbs (CHK-152 and CHK-166) against CHIKV infection in mice (52). As a next step toward evaluating the possible utility of this approach in humans, we assessed the efficacy of combination MAb therapy in nonhuman primates, using a recently developed rhesus macaque model of CHIKV infection (38). We infected adult rhesus macaques subcutaneously in both arms with 10^7 PFU of the epidemic CHIKV-LR strain. On days 1 and 3 postinfection, the infected animals were administered mouse MAbs (15 mg/kg, intravenously) directed against CHIKV E2 and E1 (CHK-152 and CHK-166) or, as a negative control, against WNV E protein (WNV E16). Blood was collected at day 0 immediately prior to infection, on days 1, 2, 3, 4, and 5 after infection, and at necropsy on day 7. Plasma mouse MAb concentrations were determined by enzyme-linked immunosorbent assay (ELISA) (Fig. 1A); similar peak levels were observed for both treatment groups, with the highest levels following the second injection. Combination MAb therapy completely neutralized infectious CHIKV in blood compared to animals treated with the control anti-WNV MAb (Fig. 1B). CHIKV RNA loads in the arm joints (finger, wrist, and elbow) and muscles (biceps, triceps, and brachial radius) were similar for both treatments, suggesting that by 1 day after infection the virus established infection in these tissues. However, combination anti-CHIKV MAb treatment reduced viral burden in the joints and muscles of the legs, indicating that therapy reduced viral dissemination, as follows: knee, 270-fold ($P < 0.003$); ankle, 114-fold ($P < 0.002$); toes, 62-fold ($P < 0.0006$); soleus, 78-fold ($P < 0.05$); and hamstring (semitendinosus and semimembranosus), 3-fold ($P < 0.05$) muscles (Fig. 2A and B). Analogously, viral burdens in the axillary lymph nodes were similar after anti-CHIKV or control MAb treatment (Fig. 2C) but were lower in the anti-CHIKV MAb-treated animals in the mesenteric (27-fold lower; $P < 0.009$) and inguinal (287-fold lower; $P < 0.003$) lymph nodes, which drain the gut and legs, respectively. While trends were present, anti-CHIKV therapy did

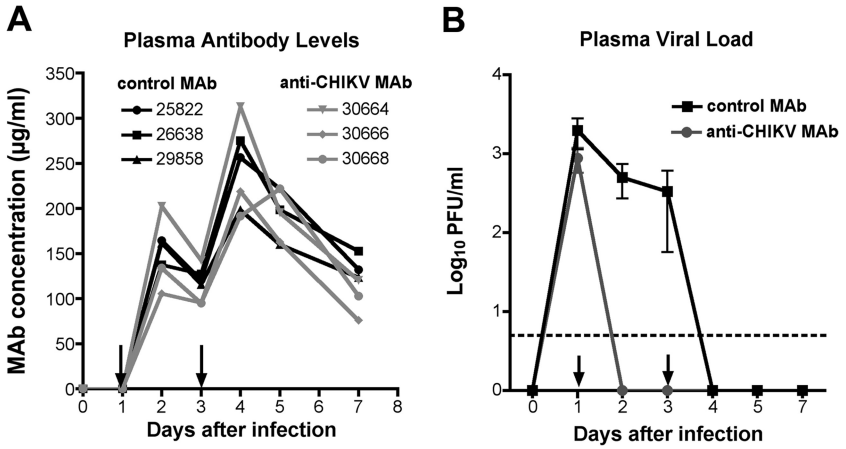

FIG 1 Anti-CHIKV therapy reduces viremia in rhesus macaques. Rhesus macaques were infected with 10^7 PFU of CHIKV-LR and injected intravenously at 1 and 3 dpi with anti-CHIKV antibodies or a control WNV MAb (15 mg/kg). At 0, 1, 2, 3, 4, 5, and 7 dpi, peripheral blood samples were collected and processed for plasma. (A) Plasma anti-CHIKV mouse MAb titers were measured by ELISA ($n = 3$/group). The numbers under each treatment refer to the individual rhesus macaques used in this study. (B) Levels of CHIKV in plasma were measured by limiting-dilution plaque assay. Antibodies directed against CHIKV but not WNV lowered viremia to undetectable levels at 2 dpi, and this effect was maintained until the study endpoint (7 dpi) ($n = 3$ per group; $P < 0.01$ at 2 dpi, two-way ANOVA).

not reduce the viral load in the lung or kidney in a statistically significant manner. Although infectious CHIKV was isolated from tissues from all of the control MAb-treated animals, we failed to detect any infectious virus in harvested tissues from rhesus macaques treated with anti-CHIKV MAbs (data not shown), despite the presence of viral RNA. Consensus sequencing of the remaining viral RNA in tissues at day 7 failed to reveal any amino acid changes in the E1 or E2 gene (see Fig. S1 in the supplemental material). These findings indicate that within 24 h of inoculation in rhesus macaques, CHIKV has established itself within tissues near the site of infection and the adjacent draining lymph nodes. Combination therapy with anti-CHIKV MAbs reduced viral load at distant sites of infection, neutralized the existing reservoir of infectious virus, and limited the development of resistance.

Stability of neutralization escape variants in mosquito and mammalian cells. As part of prior epitope mapping studies, we identified neutralization escape variants in cell culture and *in vivo* against CHK-152 and CHK-166 (52). E2-D59N was the dominant amino acid mutant selected under immune pressure from CHK-152 both *in vivo* and in cell culture. Analogously, an E1-K61T mutation consistently was selected in the presence of CHK-166 in cell culture. To understand the significance of these mutations in the context of combination MAb therapy, we engineered E2-D59N, E1-K61T, and E2-D59N E1-K61T mutations into the in-

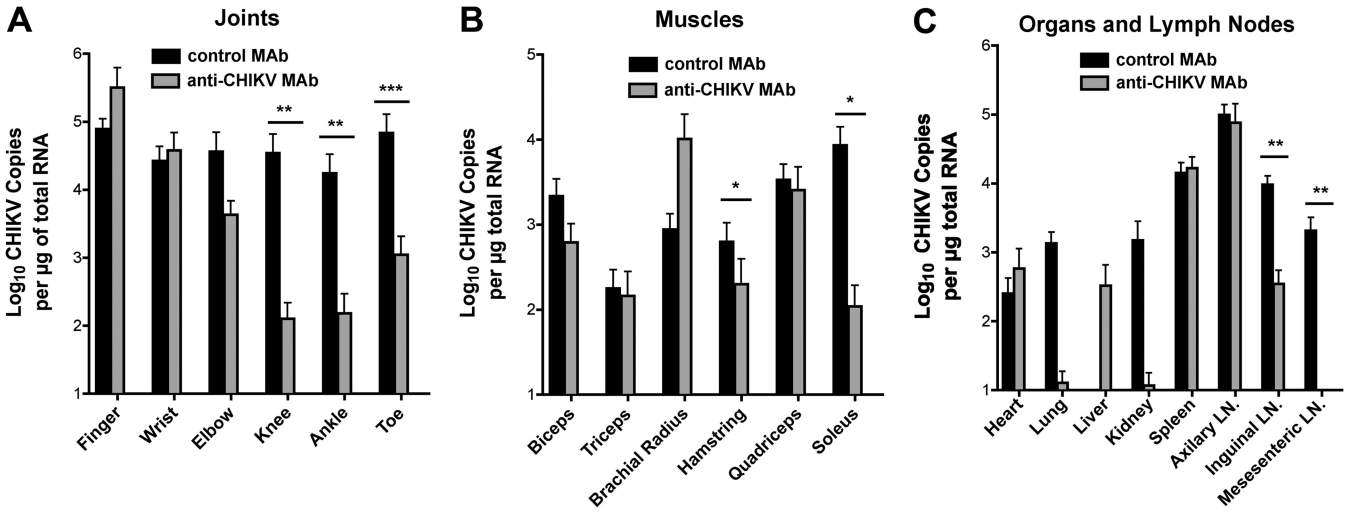

FIG 2 Anti-CHIKV therapy reduces CHIKV dissemination in rhesus macaques. Rhesus macaques were treated at 1 and 3 dpi with anti-CHIKV antibodies or control anti-WNV antibody (15 mg/kg). Necropsy occurred at 7 dpi, and monkey tissue samples were processed for total RNA by the TRIzol method ($n = 6$ per group). Quantitative RT-PCR was used to detect CHIKV loads in joints, muscle, and other organs and lymph nodes. Virus dissemination to peripheral muscles and joints (leg), organs (lung and kidney), and lymph nodes ([LN] inguinal and mesenteric) was greatly reduced by anti-CHIKV treatment. Viral loads in the arm muscles and joints (site of infection) as well as spleen and draining lymph nodes (axillary) were not affected by the antibody treatment. Asterisks indicate statistically significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) as judged by a Mann-Whitney test.

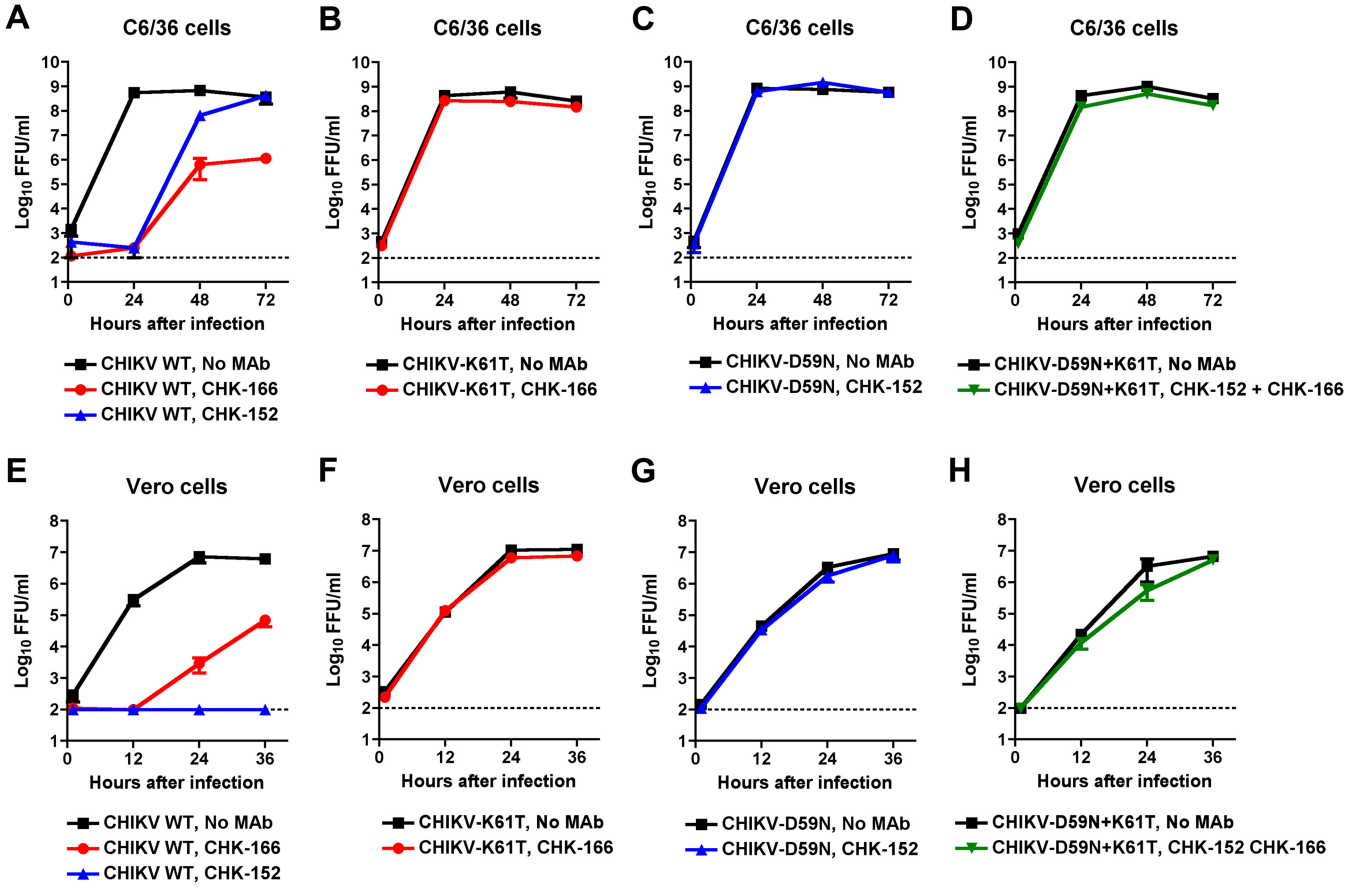

FIG 3 Growth kinetics of WT and mutant CHIKV in insect and mammalian cells. (A to D) C6/36 *A. albopictus* cells were infected with P0 BHK cell-derived CHIKV WT (A), CHIKV E1-K61T (B), CHIKV E2-D59N (C), or CHIKV E1-K61T E2-D59N (D) virus. For some experiments, CHIKVs (WT or mutants) were preincubated with 10 μg/ml of CHK-166, CHK-152, or both CHK-166 and CHK-152 for 1 h at 37°C, as indicated. Virus or virus-MAb complexes were added to C6/36 cells for 1 h at 37°C. After samples were washed to remove free virus and antibody, supernatants were harvested at 1, 24, 48, and 72 h postinfection for titration by FFU assay. The results are the average of three independent experiments performed in triplicate. Statistically significant differences are indicated (*, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$). For the mutant viruses, none of the differences with and without MAb treatment were significantly different. (E to H) African green monkey Vero cells were infected with P0 C6/36 cell-derived CHIKV WT (E), CHIKV E1-K61T (F), CHIKV E2-D59N (G), or CHIKV E1-K61T E2-D59N (H) virus. For some experiments, CHIKVs (WT or mutants) were preincubated with 10 μg/ml of CHK-166, CHK-152, or both CHK-166 and CHK-152 for 1 h at 37°C, as indicated. Virus or virus-MAb complexes were added to Vero cells for 1 h at 37°C. After samples were washed to remove free virus and antibody, supernatants were harvested at 1, 12, 24, and 36 h postinfection for titration by FFU assay. The results are the average of three independent experiments performed in triplicate. Statistically significant differences are indicated (*, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$). For the mutant viruses, none of the differences with and without MAb treatment were significantly different.

fectious cDNA clone of CHIKV (strain LR 2006 OPY-1), generated the recombinant viruses, and tested them for growth in insect and mammalian cells in the presence or absence of individual or combinations of neutralizing MAbs. We hypothesized that individual or combinations of mutations might be attenuating and, thus, revert to WT sequences in the absence of antibody pressure. Initial studies were performed in C6/36 *A. albopictus* cells. As expected, CHIKV WT grew efficiently in C6/36 cells, reaching titers in the supernatant of ~10^8 to 10^9 FFU/ml by 72 h. Infectious levels of CHIKV WT were reduced markedly in the supernatants of C6/36 cells that were pretreated with 10 μg/ml of CHK-152 or CHK-166 (Fig. 3A). CHIKV E1-K61T, CHIKV E2-D59N, and CHIKV E1-K61T E2-D59N viruses all grew similarly in C6/36 cells, regardless of whether the corresponding neutralizing MAb (CHK-166, CHK-152, or both) was included (Fig. 3B to D). No growth defect of any of the mutants was observed compared to the parent CHIKV WT virus in the absence of MAbs (e.g., titers in FFU/ml at 72 h: WT, 3.3×10^8; E1-K61T, 2.7×10^8; E2-D59N, 3.3×10^8; E1-K61T E2-D59N, 3.4×10^8). This suggested that the individual or combination mutants did not have a fitness defect in C6/36 cells. To confirm this, we sequenced the E1 and E2 genes of the virus harvested at the end of the growth curves (72-h time point) of CHIKV WT, E1-K61T, E2-D59N, and E1-K61T E2-D59N viruses performed in the absence of MAb treatment. At the level of consensus sequencing, all mutations were maintained, and no reversion to WT virus was apparent (see Fig. S2 in the supplemental material).

We repeated these studies in primate Vero cells, with the idea that individual or combinations of mutations could impact entry, assembly, or replication in a different host species. As anticipated, CHIKV WT grew efficiently in Vero cells, reaching titers in the supernatant of 10^7 FFU/ml by 36 h. In comparison, markedly reduced or no infectious WT virus was observed in Vero cell supernatants that contained 10 μg/ml of CHK-166 or CHK-152,

TABLE 1 Infection rates of CHIKVs in *A. albopictus* at days 7, 10, and 14 after infection

Viremic blood meal	Infection rate (no. of infected mosquitoes/total no. in group [%]) at:[a]			Titer at day 14 p.i. (log_{10} $TCID_{50}$/ml [*n*])[b]
	Day 7 p.i.	Day 10 p.i.	Day 14 p.i.	
Mock (defibrinated sheep blood)	0/15 (0)	0/13 (0)	0/26 (0)	ND
WT	15/16 (94)	22/22 (100)	30/35 (86)	4.99 ± 0.50 (11)
E1-K61T	36/41 (88)	14/16 (88)	36/44 (82)	4.65 ± 0.91 (12)
E2-D59N	30/33 (91)	14/15 (93)	40/43 (93)	5.44 ± 0.64 (11)
E1-K61T E2-D59N	34/42 (81)	12/13 (92)	33/44 (75)	5.04 ± 0.90 (11)

[a] *A. albopictus* mosquitoes were fed a blood meal of equivalent amounts of WT and the indicated mutant CHIKVs. Mosquitoes were collected at different days postinfection (p.i.), dissected, and analyzed for viral antigen expression in the abdomen using indirect immunofluorescence microscopy (expressed as rate of infection). None of the values for mutant viruses were significantly different ($P > 0.05$) from the WT level at any of the days tested, as judged by a Fisher's exact test.

[b] Whole mosquitoes were collected at day 14 postinfection and homogenized, and titers of infectious virus were determined on Vero cells. The data are expressed as log_{10} $TCID_{50}$/ml ± standard deviation, and the number of individual mosquitoes tested per group is indicated in parentheses. ND, not determined.

respectively (Fig. 3E). The CHIKV E1-K61T and E2-D59N viruses grew equivalently in the presence or absence of CHK-166 and CHK-152 (Fig. 3F and G); this suggested that these mutant viruses did not have a fitness defect in Vero cells. Similar growth kinetics were observed with the double mutant (E1-K61T E2-D59N) virus in the presence or absence of the combination of MAbs (Fig. 3H). Consistent with the absence of attenuation in Vero cells, sequencing studies of viruses propagated in the absence of MAb treatment confirmed that all mutations were maintained at the 36-h time point as no reversion to WT virus was present (see Fig. S3 in the supplemental material). These studies could not be extended further in time due to the cytopathic effect of CHIKV in mammalian cells.

Phenotype of neutralization escape variants in mosquitoes. Although a growth defect of the neutralization escape mutants was not observed in C6/36 insect cells, infectivity and dissemination in the *A. albopictus* vector might differ because of additional cellular and immune barriers. To evaluate this, live mosquitoes were fed blood meals of CHIKV WT, CHIKV E1-K61T, CHIKV E2-D59N, or CHIKV E1-K61T E2-D59N virus. In these studies, we used a relatively high virus dose (10^7 $TCID_{50}$ per blood meal) to ensure optimal infection rates of the mosquitoes. At days 7, 10, and 14 the abdomens and secondary tissues were harvested to determine the rates of infection and dissemination and the quantity of infectious viruses. In general, infectivity levels of the WT and mutant viruses were similar in the abdomens of *A. albopictus* mosquitoes over the first 2 weeks (Table 1). Dissemination also was equivalent as 100% of the secondary tissues were infected with CHIKV WT, CHIKV E1-K61T, CHIKV E2-D59N, or CHIKV E1-K61T E2-D59N at days 7, 10, and 14, with the exception of one nondisseminated infection with the E2-D59N mutant at day 7 (Table 2). No differences were observed among the four CHIKV strains in the mean infectious titers of whole-mosquito homogenates at day 14 postinfection (Table 1). Sequencing studies of the E2-E1 genes harvested from whole-mosquito homogenates at day 14 after infection with the mutant CHIKV revealed no evidence of reversion to WT virus despite multiple rounds of replication in the mosquito tissues (see Fig. S4 in the supplemental material). Thus, and similar to the results seen in C6/36 cells in culture, the neutralization escape variants showed no evidence of restricted tropism or altered virulence *in vivo* in the vector host.

Phenotype of neutralization escape variants during acute infection of immunocompromised mice. Although no growth defects of the mutant viruses were observed in cell culture and mosquitoes, these replication dynamics might not reliably predict phenotypes in mammals due to differences in tropism and immune responses. Initially, we confirmed that preadministration of a combination (50 μg each) of CHK-152 and CHK-166 prevented CHIKV WT-induced lethality in highly vulnerable 6- to 8-week-old *Ifnar1*$^{-/-}$ mice. In the absence of MAbs, all mice succumbed rapidly to infection with 10 FFU of CHIKV WT, with a mean survival time of 3.8 ± 0.1 days, similar to that reported previously (28, 52, 57). In the presence of CHK-152 and CHK-166, as expected, the *Ifnar1*$^{-/-}$ mice failed to develop disease and survived infection (Fig. 4A and Table 3). Moreover, no viral RNA was recovered at day 3 from muscle of CHIKV WT-infected *Ifnar1*$^{-/-}$ mice pretreated with CHK-152 and CHK-166, which suggests that sterilizing immunity was achieved (data not shown).

Parallel infection experiments using analogously passaged (P0) stocks of CHIKV E1-K61T, CHIKV E2-D59N, or CHIKV E1-K61T E2-D59N virus revealed a slightly protracted mean survival time compared to infection with CHIKV WT (WT, 3.8 days; E1-K61T, 4.9 days; E2-D59N, 4.9 days; and E1-K61T E2-D59N, 5.4 days; $P < 0.0001$ for all mutants compared to the WT), suggesting that *in vivo* the engineered neutralization escape viruses were slightly attenuated (Fig. 4B to D and Table 3) yet still pathogenic. As it remained possible that the delay in lethality in *Ifnar1*$^{-/-}$ mice was associated with reversion in the absence of immune pressure, infection experiments were repeated in the presence of the respective neutralizing MAb(s). In each case, the mean survival time in the presence of neutralizing MAb was longer (CHIKV E1-K61T, 5.8 days; CHIKV E2-D59N, 5.4 days; CHIKV E1-K61T E2-D59N, 6.4 days) than that with CHIKV WT in the absence of MAb. To

TABLE 2 Dissemination rates of WT and variant CHIKVs in *A. albopictus* at days 7, 10, and 14 after infection

Viremic blood meal	Dissemination rate (no. of positive mosquitoes/total no. of mosquitoes [%]) at:[a]		
	Day 7 p.i.	Day 10 p.i.	Day 14 p.i.
WT	10/10 (100)	12/12 (100)	21/21 (100)
E1-K61T	25/25 (100)	11/11 (100)	26/26 (100)
E2-D59N	17/18 (94)	10/10 (100)	29/29 (100)
E1-K61T E2-D59N	23/23 (100)	8/8 (100)	25/25 (100)

[a] *A. albopictus* mosquitoes were fed a blood meal of equivalent amounts of WT and indicated mutant CHIKV. Mosquitoes were collected at different days postinfection (p.i.), dissected, and analyzed for viral antigen expression in the secondary tissues using indirect immunofluorescence microscopy.

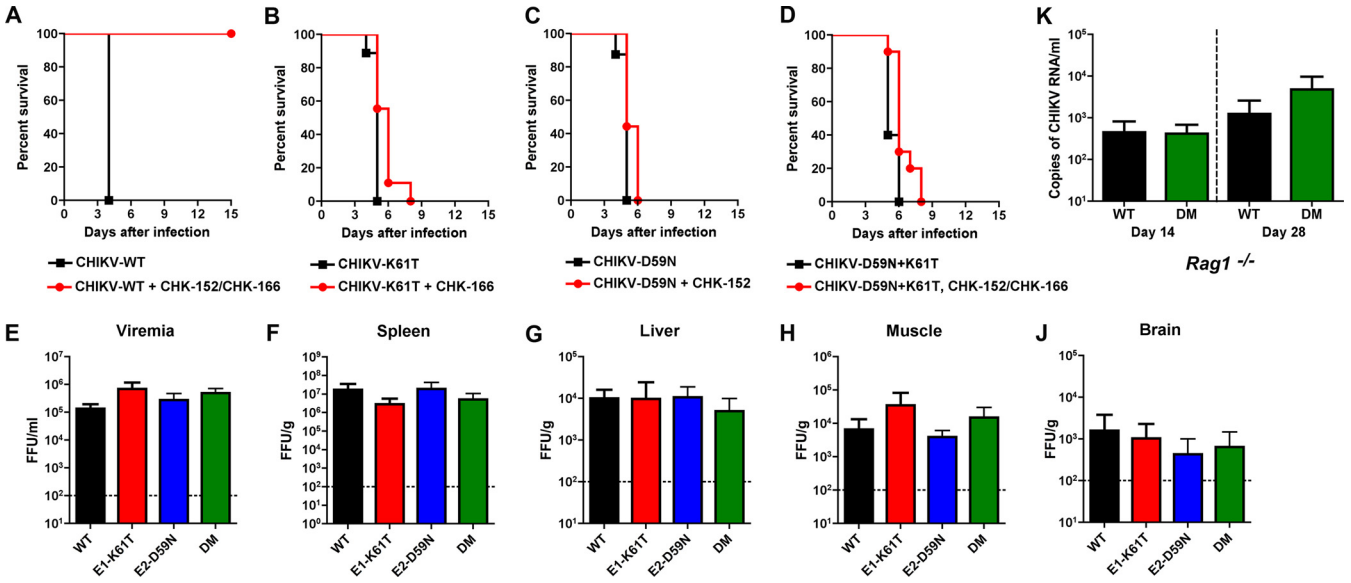

FIG 4 Virulence of WT and mutant CHIKV strains in immunocompromised *Ifnar1*$^{-/-}$ or *Rag1*$^{-/-}$ mice. (A to D) Six- to eight-week-old *Ifnar1*$^{-/-}$ mice were passively transferred saline or 50 μg of each antibody, CHK-166 and CHK-152, via an intraperitoneal injection 1 day before infection with 10 FFU of CHIKV WT (A), CHIKV E1-K61T (B), CHIKV E2-D59N (C), or CHIKV E1-K61T E2-D59N (D) virus via a subcutaneous route. The survival curves were constructed from data of at least two independent experiments with between 7 and 10 mice per group. (E to J) Six week-old *Ifnar1*$^{-/-}$ mice were infected with 10 FFU of CHIKV WT, CHIKV E1-K61T, CHIKV E2-D59N, or CHIKV E1-K61T E2-D59N (DM) virus via a subcutaneous route. At day 2 after infection, serum, spleen, liver, muscle (right leg), and brain were harvested from individual mice for virus titration by focus-forming assay. The data are the average ± standard deviation from 4 to 6 mice per group. The differences in viral burden did not attain statistical significance ($P > 0.1$, Mann-Whitney test). (K) *Rag1*$^{-/-}$ mice were infected with 10^3 FFU of CHIKV WT or CHIKV E1-K61T E2-D59N (DM) via a subcutaneous route. Blood samples were obtained from individual animals at days 14 and 28 after infection, and CHIKV RNA was measured by qRT-PCR. No statistically significant differences were observed.

evaluate this further, we recovered virus from the muscle of three independent moribund mice infected with CHIKV WT, CHIKV E1-K61T, CHIKV E2-D59N, and CHIKV E1-K61T E2-D59N viruses in the absence or presence of neutralizing antibody treatment and sequenced the E1 and E2 genes directly. We predicted that highly attenuating mutations *in vivo* might revert to WT sequences in the absence of immune pressure. Despite the slight differences in virulence reflected by prolongation of the survival time, the engineered mutations (E1-K61T, E2-D59N, and E1-K61T E2-D59N) were retained in muscle tissue in the absence or presence of the respective neutralizing MAb(s), as judged by consensus sequencing (see Fig. S5 in the supplemental material). We also observed no statistically significant difference in viral burdens at day 2 after infection for any of the mutant viruses relative to the WT virus in the serum, spleen, liver, muscle, or brain (Fig. 4E to J). Thus, the escape mutant viruses were stable and pathogenic in *Ifnar1*$^{-/-}$ mice although there was a slight delay in the kinetics of lethal infection that did not reflect major differences in tissue viral burden.

TABLE 3 Virulence of WT and mutant CHIKVs in the presence or absence of MAbs

Virus	Treatment[a]	Survival rate (%)	Mean survival time (days)	No. of mice
CHIKV WT	PBS	0	3.8 ± 0.1	10
CHIKV WT	CHK-152 + CHK-166	100	>21	7
CHIKV E1-K61T	PBS	0	4.9 ± 0.1	9
CHIKV E1-K61T	CHK-166	0	5.8 ± 0.3	9
CHIKV E2-D59N	PBS	0	4.9 ± 0.1	8
CHIKV E2-D59N	CHK-152	0	5.4 + 0.2	9
CHIKV E1-K61T E2-D59N	PBS	0	5.4 ± 0.2	10
CHIKV E1-K61T E2-D59N	CHK-152 + CHK-166	0	6.4 ± 0.3	10

[a] Six to 8 week-old *Ifnar1*$^{-/-}$ mice were passively transferred PBS or 50 μg of CHK-166, CHK-152, or CHK-166 plus CHK-152 via an intraperitoneal injection 1 day before infection with 10 FFU of CHIKV WT, CHIKV E1-K61T, CHIKV E2-D59N, or CHIKV E1-K61T E2-D59N virus via a subcutaneous route.

As it was unclear why the mutant viruses were slightly clinically attenuated yet stable and replicating to nearly WT virus levels, we speculated that there might be an adaptive immune pressure (e.g., IgM response) during the early host response which favored retention of the mutant strains. To assess this, we infected *Rag1*$^{-/-}$ C57BL/6 mice, which lack functional B and T cells and do not produce antibody, with WT or CHIKV E1-K61T E2-D59N virus. Prior studies had established that *Rag1*$^{-/-}$ mice develop persistent CHIKV infection (31), so these animals also allowed us to follow the stability of the mutant virus in an immunocompromised mammalian host over several weeks. At days 14 and 28 after infection, viremia reached equivalent levels in *Rag1*$^{-/-}$ mice infected with CHIKV WT and CHIKV E1-K61T E2-D59N (Fig. 4K). Consensus sequencing of the E2-E1 genes from tissues harvested from *Rag1*$^{-/-}$ mice at day 28 revealed no changes to the WT virus and no evidence of reversion of CHIKV E1-K61T E2-D59N virus despite the many rounds of replication that occurred during the 4-week interval (see Fig. S6 in the supplemental material). Thus, in the absence of adaptive immune pressure, the mutant CHIKV E1-K61T E2-D59N replicated to normal levels and was remarkably stable.

Phenotype of neutralization escape variants in persistently infected immunocompetent mice. Models of acute and chronic

joint disease caused by persistent CHIKV infection in immunocompetent C57BL/6 mice recently have been established (30, 31, 34, 58). Because the time course of disease pathogenesis in *Ifnar1*$^{-/-}$ mice was short, we reasoned that the relative virulence and propensity for reversion of mutant strains might be revealed in a persistence infection model. Accordingly, we inoculated 3-week-old WT C57BL/6 mice with 10^3 FFU of BHK21 cell-derived CHIKV WT, CHIKV E1-K61T, CHIKV E2-D59N, or CHIKV E1-K61T E2-D59N virus in the left rear footpad. During the acute phase, we monitored swelling in the ipsilateral foot and ankle. At days 3 and 7 after inoculation, all three mutant viruses caused less swelling than the parent WT virus (~2-fold decrease in size, $P < 0.0001$) (Fig. 5A), suggesting a decrease in virulence during the acute phase. However, analysis of the levels of viral RNA or infectious virus in the ipsilateral (left) and contralateral (right) ankles or infectious virus in the serum failed to reveal a difference in replication levels of the mutant viruses (Fig. 5B and C), despite their attenuated clinical phenotype. As clinical attenuation and genetic stability of the CHIKV E1-K61T E2-D59N mutant virus also were observed at days 3 and 7 after infection with a C6/36 cell (P1)-passaged viral stock, it is unlikely that attenuated minority populations generated in the original BHK21 (P0) virus stock could explain the phenotype. Because prior studies with attenuated CHIKV strains containing mutations in the E2 gene (E2-E79K and E2-G82R) had observed less foot swelling (34) or greater weight loss (59) that was associated with differences in inflammatory mediators (36), we measured cytokine and chemokine responses at day 3 after infection in WT mice infected with CHIKV WT or CHIKV E1-K61T E2-D59N virus. Compared to infection with CHIKV WT, animals inoculated with CHIKV E1-K61T E2-D59N produced low serum levels of several proinflammatory cytokines and chemokines, including IL-5, IL-12 p40, MCP-1 (CCL2), and RANTES (CCL5), at day 3 (Fig. 5D to U).

These animals were followed into the persistence phase; at day 28 postinoculation, tissues were harvested, and qRT-PCR was performed. In this model, persistent CHIKV RNA and synovitis are present in joint-associated tissue although clinically apparent inflammation has receded (31). At day 28, viral RNA levels in the contralateral right ankle and left wrist, which reflect both viral spread and persistence, were similar between the WT and mutant viruses (Fig. 5V). Sequencing studies of the complete E2-E1 genes of viral RNA harvested from the right ankle of mice at day 28 after infection with the mutant CHIKV revealed no reversion at the site of the introduced mutation (see Fig. S7 in the supplemental material) nor emergence of a reproducibly selected second site mutation. One exception was a low-frequency G-to-A substitution at position 10513 in viral RNA from three mice (two of four CHIKV E2-D59N and one of four CHIKV E1-K61T mice), which results in a D-to-N coding change in the E1 gene at amino acid 174. The significance of this change remains unclear as it did not segregate with one of the two mutant viruses and also was not present in the animals infected with the double mutant (CHIKV E1-K61T E2-D59N) virus. Thus, although an attenuated clinical phenotype of the CHIKV mutant viruses was observed during the acute phase of infection and disease, in the absence of immune pressure reversion to the WT parental virus failed to occur, and the mutant viruses showed a similar ability to persist.

Competition studies reveal a fitness advantage of the mutant virus in mice. As a test of the relative stability and fitness of the double mutant (CHIKV E1-K61T E2-D59N) escape virus, we performed competition studies in WT C57BL/6 mice. CHIKV WT and CHIKV E1-K61T E2-D59N viruses were mixed in a 5:1, 1:1, or 1:5 FFU ratio and injected in the left rear footpad. As a control, we compared the genomic RNA-to-FFU ratios of the input viruses. CHIKV WT and CHIKV E1-K61T E2-D59N viruses had similar relative levels of genomic RNA per unit of infectious virus (1.04 ± 0.13 and 1.03 ± 0.20 copies of RNA per FFU for CHIKV WT and CHIKV E1-K61T + E2-D59N, respectively). At 28 days after initial infection, tissue from the right ankle was harvested, and consensus sequencing of the complete E2-E1 genes from viral RNA was performed. Unexpectedly, in tissue samples from four of four mice from each of the three cohorts, the mutant virus sequence (CHIKV E1-K61T E2-D59N) was detected regardless of the input ratio (see Fig. S8A and B in the supplemental material). In only one sample, did we observe sequence corresponding to CHIKV WT, and this was a minority population (Fig. S8C). Thus, the mutant virus not only was stable but also showed enhanced fitness as reflected by its dominance 4 weeks later in a tissue distant from the infection site.

DISCUSSION

Neutralizing MAbs are being explored as a platform for treatment of acute or chronic CHIKV infections (31, 49, 52, 54, 55). For CHIKV in particular, where humans are an integral part of the endemic and epidemic transmission cycles, the rapid emergence of resistance and transmission of such variants to mosquitoes could render therapeutic antibodies less effective. Previously, we identified a pair of neutralizing anti-CHIKV MAbs (CHK-152 and CHK-166) against the E2 and E1 structural proteins with therapeutic efficacy in immunocompetent and immunocompromised mice (31, 52). Combination MAb therapy was required as administration of a single MAb resulted in the selection of dominant single-mutation escape variants *in vitro* and *in vivo* and treatment failure. In the present study, we initially confirmed the efficacy of this combination MAb therapy in a nonhuman primate model of CHIKV infection. Combination CHK-152 and CHK-166 MAb therapy in rhesus macaques reduced viral infection and spread and neutralized reservoirs of infectious virus; for reasons that remain unclear, viral RNA persisted in the presence of MAb therapy although infectious virus was not recovered. At the level of consensus sequencing, escape viruses were not detected in the residual viral RNA present in tissues and organs of rhesus macaques. Although the significance of the persistent CHIKV RNA is uncertain, it could reflect populations of cells that actively propagate viral RNA. Why such cells are not targeted for elimination by cytolytic T cells or the effector mechanisms of CHK-152 and CHK-166 (e.g., antibody-dependent cellular cytotoxicity or phagocytosis), which should recognize the E2 and E1 proteins on the surface of infected cells, remains an area of future investigation.

To define the possible significance of neutralization escape, we engineered the mutations (E1-K61T, E2-D59N, and E1-K61T E2-D59N) into the infectious CHIKV cDNA clone and tested the infectivity of the recombinant viruses in mosquito cells and *A. albopictus* mosquitoes. In cell culture, the recombinant mutant viruses were stable without apparent attenuation as they grew equivalently in the presence or absence of antibody pressure. In *A. albopictus* mosquitoes, after a blood meal, the mutant viruses showed levels of midgut and salivary gland infection similar to those of the WT virus, again with no evidence of reversion at 14

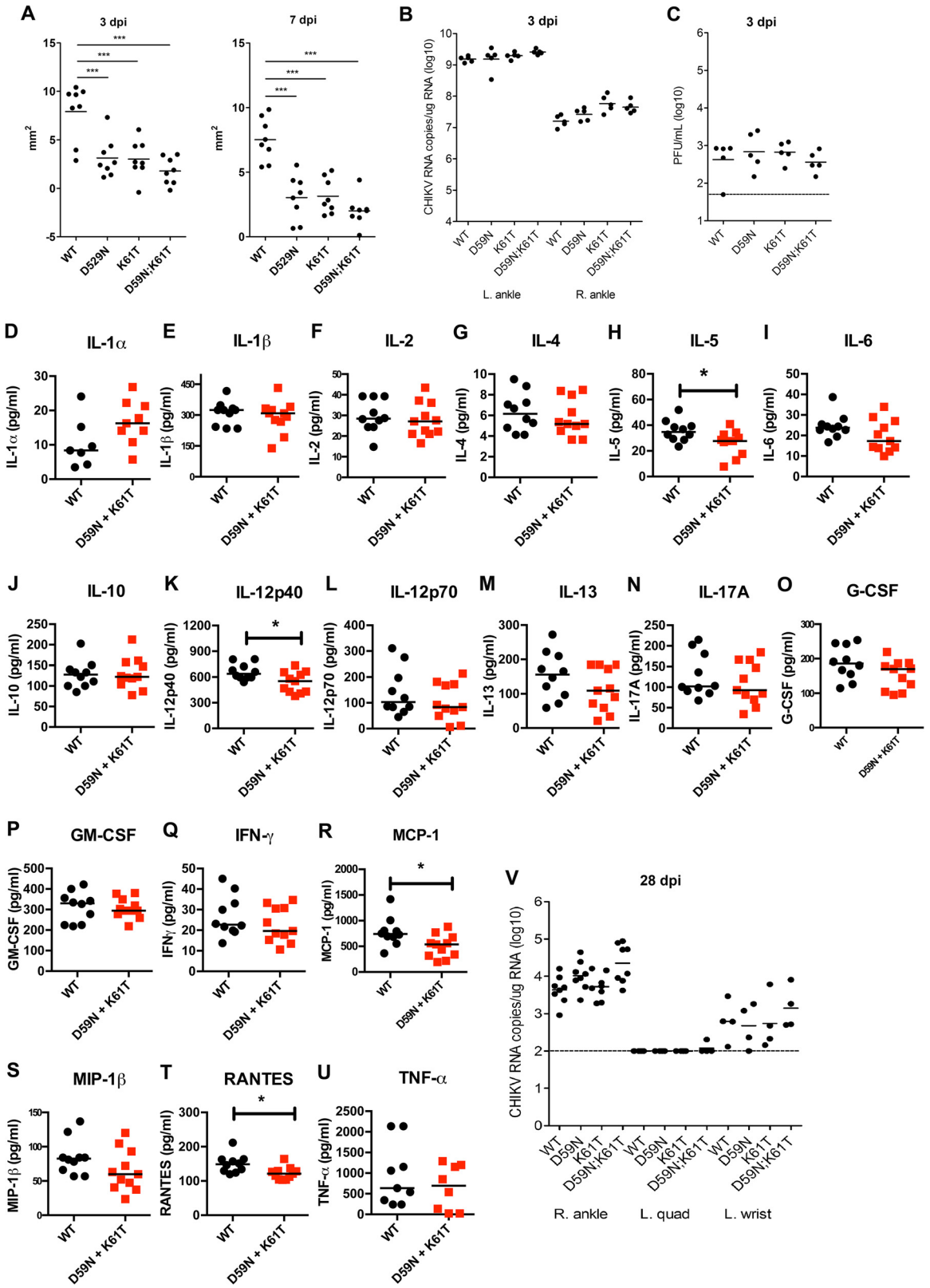

FIG 5 Virulence of WT and neutralization escape variant viruses in WT C57BL/6 mice. Three-week-old WT C57BL/6 mice were infected with 10^3 FFU of CHIKV WT, CHIKV E1-K61T, CHIKV E2-D59N, or CHIKV E1-K61T E2-D59N virus via a subcutaneous route. (A) At days 3 and 7 after infection, measurements of joint swelling were made. Statistically significant differences are indicated (***, $P < 0.001$). (B and C) At day 3, the ipsilateral (left) and contralateral (right) ankle to the site of injection were harvested, and virus was titrated by qRT-PCR (B) or plaque assay (C, for serum). (D to U) At day 3 after infection with CHIKV WT or CHIKV E1-K61T E2-D59N virus, serum was harvested and processed for the indicated cytokines and chemokines. The results are displayed as a scatter plot from three independent experiments from a total of nine mice. Statistically significant differences are indicated (*, $P < 0.05$). (V) At day 28, the indicated tissues were harvested, and yield was analyzed by qRT-PCR. None of the values obtained with the mutant viruses was statistically different from that of the WT virus. These results were pooled from two independent experiments with a total of 4 to 8 mice per group.

days, at least in the bulk population. Thus, in cell culture and mosquitoes, the mutant viruses maintained infection and dissemination potential and were not purified, nor did they acquire compensatory mutations in the structural genes. This finding in mosquitoes was unexpected because alignment of the E1-K61 and E2-D59 sequences with 140 different historical and circulating CHIKV strains had revealed complete conservation of the WT residues (52). One caveat to our study is that the infection conditions of *A. albopictus* in the laboratory differ from those in the field such that selection bias against certain mutant viruses could still occur. Natural populations vary widely with respect to vector competence, and as such there is no one "representative" wild population. Consequently, although our results apply to colonized mosquitoes infected under laboratory conditions, further validation with field strains may be required.

We performed analogous selection studies with the engineered mutant CHIKV strains in mammalian cells and immunocompromised (*Ifnar1*$^{-/-}$) and immunocompetent mice. In Vero cell culture, no difference in infections was observed between the WT and variant viruses in multistep growth curve analysis. In *Ifnar1*$^{-/-}$ mice, in the absence of MAb pressure, the escape viruses were stable at the level of consensus sequencing but exhibited mild clinical attenuation; this was reflected by a longer mean survival time and occurred regardless of whether MAb pressure was present. However, this was not associated with differences in tissue viral burden among the WT and mutant viruses, at least early during the course of infection. In immunocompetent C57BL/6 mice, the mutant viruses also showed evidence of clinical attenuation as decreased joint swelling was observed during the acute phase of infection compared to the parent WT virus; again, this was not associated with decreased replication in the joint tissues. Moreover, the mutant viruses were not cleared more rapidly, and persistent viral RNA was present at equivalent levels relative to the WT virus in several tissues at day 28. Consensus sequencing of the E1 and E2 genes during the persistence stage failed to reveal reversion. Unexpectedly, in direct competition studies in WT mice, the double mutant (CHIKV-E1-K61T E2-D59N) showed enhanced fitness as the mutant but not WT virus sequence was detected in tissue samples from all (12 of 12) mice 4 weeks after infection, regardless of the starting infection ratio. Although the mechanistic basis for the relative fitness of the double mutant (CHIKV-E1-K61T E2-D59N) *in vivo* remains uncertain, amino acid substitutions in a structural protein could impact steps that modulate viral tropism (attachment, entry, or fusion) or assembly. Overall, the dominant neutralization escape mutants to CHK-152 and CHK-166 show evidence of disease attenuation in mice although reversion and compromised fitness were not observed in the acute and persistent phases of infection. Additionally, the magnitude of the clinical phenotype during the acute phase did not predict the development of viral persistence in mice. In humans, while one study showed that chronic infection and symptoms were associated with higher viral loads during the acute phase (60), another, analogous to our findings, found that high viral loads and severity of acute disease failed to correlate with development of chronic disease (61). Clearly, the relationship between acute disease severity, acute viral loads, and chronic disease/infection warrants further study.

The retention of replication efficiency in Vero cells suggests that the mutations in the E1 and E2 proteins do not affect the fundamental processes of attachment, entry, fusion, or assembly. So why are the escape viruses clinically attenuated *in vivo* in both WT and *Ifnar1*$^{-/-}$ mice? Plausible explanations exist. (i) The cellular tropism *in vivo* (e.g., muscle cells or fibroblasts) is affected by the mutations in E1 and E2 (62). Mutations in the structural genes could impact infection of one cell type but not another. (ii) The mutant viruses are preferentially recognized by antibody or T cells due to slight changes in epitopes, which could impact pathogenesis (63). The latter hypothesis seems less likely, given our results showing sequence stability in *Rag1*$^{-/-}$ mice, which lack mature B and T cell responses. The significance of these results for antibody therapy in humans remains uncertain. While it is possible that these escape mutants (E2-D59N, E1-K61T, E2-D59N E1-K61T) against CHK-152 and CHK-166 will be attenuating, species differences in cellular tropism or immune recognition may not translate directly in humans.

Although the variant (E1-K61T and E2-D59N) CHIKVs replicated equivalently, they caused reduced disease with delayed kinetics compared to the parental virus. The absence of a link between viral burden in the joint and the clinical phenotype of joint swelling in the context of infection by CHIKV has been reported. *Ifnar1*$^{-/-}$ mice inoculated with a candidate vaccine strain of CHIKV (CHIKV-181/25) failed to cause arthritis despite attaining titers that were similar to those achieved with disease-causing WT strains (34, 59). A genetic analysis of CHIKV-181/25 identified two mutations in the E2 gene (E2-T12I and E2-G82R) that were associated with decreased footpad swelling and clinical virulence (59), and the E2-G82R mutation has been demonstrated to promote heparan sulfate binding (34, 64). Although it remains unclear how changes at E1-K61 and E2-D59 altered the clinical phenotype, these substitutions affected the early proinflammatory response. At day 3 after CHIKV-E1-K61T E2-D59N infection, when tissue swelling was lower, decreased levels of several cytokines and chemokines (IL-5, IL-12 p40, MCP-1, and RANTES) were detected in the serum. Notably, levels of IL-5, IL-12, and MCP-1 also were blunted in CD-1 mice infected with clinically attenuated CHIKV strains encoding mutations in the E2 gene that promoted heparan sulfate binding (36). Although immune system components may contribute to (e.g., IFN-γ [65] or macrophages [29], T and B cells [31, 63], and CD4 T cell-major histocompatibility complex [MHC] class II interactions [63, 65]) or limit (e.g., dendritic cell immunoreceptor, or DCIR [66]) joint swelling after CHIKV infection, the specific steps in pathogenesis remain to be determined. Detailed immunological and pathological studies in WT and gene-targeted mice with the E1-K61 and E2-D59 mutant viruses are planned to further define the mechanistic basis for their attenuated clinical phenotypes.

In both mosquito cell culture and *A. albopictus* mosquitoes, the escape variant viruses retained the ability to replicate and disseminate with kinetics that were essentially identical to those of the parent WT viruses. Quantitative viral yield analysis in the whole mosquito also failed to reveal growth defects of the variant viruses. Moreover, direct sequencing showed no reversion of the mutant viruses to the parent WT strain in mosquitoes. These data suggest that if resistant viruses were selected in the course of combination MAb therapy, the vector host might not purify them. This result, in theory, might make the combination of CHK-152 and CHK-166 a less attractive therapy. However, there are some considerations: (i) although single escape mutations readily were selected during monotherapy with CHK-152 or CHK-166, double mutations were never obtained during combination therapy despite

extensive passages *in vitro* or *in vivo* (52); (ii) it remains possible that mutant viruses will be more attenuated in humans than in mice. Although the CHK-152 and CHK-166 escape mutations (E2-D59 and E1-K61T) were not purified during passage through mosquitoes, this type of analysis will be useful with other combinations of neutralizing humanized or fully human anti-CHIKV MAbs to help define targets for clinical development.

In summary, our study confirmed the efficacy of combination MAb therapy against CHIKV infection in rhesus macaques and evaluated the significance of possible resistance in mosquito and mammalian cells, mice, and *Aedes albopictus* mosquitoes. Although purification and the acquisition of compensatory mutations were not observed, viruses resistant to CHK-152 and/or CHK-166 were clinically attenuated yet retained fitness in two different mouse models. Infectivity studies in nonhuman primates with mutant viruses, coupled with deep-sequencing approaches, may be needed to resolve the significance of the emergence of neutralization escape variants against CHK-152 and CHK-166. We suggest that similar studies should be performed with MAb-based therapeutics against arthropod-borne viruses that have epidemic cycles between humans and insect vectors.

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