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Molecular Characterization of a Novel, Highly Protective Combination Monoclonal Antibody Therapy against Chikungunya Virus

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Therapy against Chikungunya Virus

By

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ABSTRACT OF THE DISSERTATION

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Doctor of Philosophy in Biology and Biomedical Sciences

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Professor Michael Diamond, Chair

Chikungunya virus (CHIKV) is an *Aedes* mosquito-transmitted alphavirus that causes epidemics of a debilitating, often chronic polyarthritis in humans. Over five million people in Africa and Asia have been infected since 2005, and an outbreak occurred recently in Italy demonstrating the potential for a global epidemic. A strong antibody response is elicited during infection and the aim of this thesis was to develop a better understanding of how the humoral immune response can control CHIKV infection. We identified 230 new anti-CHIKV monoclonal antibodies (MAbs) and tested their ability to inhibit infection of strains representing all three CHIKV genotypes (East/Central/South African, West African and Asian). We identified 36 of these MAbs that inhibit Chikungunya infection; almost half of them are potently neutralizing and have EC50 values of less than 15 ng/mL (0.1 nM) against CHIKV strains representing the three genotypes. Many of these MAbs exhibit cross-reactivity with a number of related alphaviruses including O'nyong'nyong, Ross River, Semliki Forest, Mayaro, Una, Getah, Bebaru,

Middleburg, Barmah Forest, Sindbis and Venezuelan equine encephalitis viruses. Four of these neutralizing MAbs provided complete protection as prophylaxis in highly susceptible immunocompromised mice and mapped to distinct antigenic epitopes on the E1 and E2 structural proteins. To define functional epitopes, we selected for escape mutants in vitro for these four MAbs. We identified most of these escape mutants in the brains and leg muscle of mice dying despite lower dose prophylaxis or monotherapy. The most protective MAb was humanized, shown to block viral fusion, and require Fc effector function for optimal activity in vivo. In post-exposure therapeutic trials, administration of a single dose of a combination of two neutralizing MAbs targeting different domains of the E2 surface glycoprotein or targeting both the E1 and E2 glycoproteins limited the development of resistance and protected immunocompromised mice against disease when given even 24 to 36 hours before CHIKV-induced death. These studies provide some insight into the location of neutralizing epitopes of CHIKV and how selected pairs of highly neutralizing MAbs may be a promising treatment option for CHIKV infection in humans.

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Chapter I

Introduction

Virology and Structure of Chikungunya Virus

Chikungunya virus (CHIKV) is encoded by an 11.8 kb single-stranded, positive sense RNA with two open reading frames (ORF). It is one of 29 alphaviruses and belongs to the *Togaviridae* family of enveloped viruses. The Alphavirus genus is split into the New World alphaviruses, which include Eastern, Western, and Venezuelan equine encephalitis viruses and the Old World alphaviruses, which are primarily arthitogenic and include CHIKV, Sindbis, Semliki Forest, Ross River and O'nyong'nyong viruses [1,2]. There are three genotypes of CHIKV: East/Central/South African (ECSA), Asian and West African. Based on E1 amino acid sequence analysis, the three genotypes are between 95.2 and 99.8% identical [3]. The CHIKV genome is flanked by untranslated regions with a 5' N-methyl guanosine cap and polyA tail, between which two ORFs reside. The 5' two-thirds of the genome encodes the four non-structural proteins (nsP 1, 2, 3, 4) [4,5]. As shown for other alphaviruses, it is predicted that two different non-structural polyproteins are translated. The predominant population is nsP123 and a minor amount of nsP1234 is produced when there is complete read-through of this ORF. As nsP4 is the RNA polymerase, limiting the production of nsP4 can help control RNA replication. These polyproteins are subsequently processed into the individual proteins by the nsP2 protease. The second ORF, which is downstream of a separate 26S subgenomic promoter [6], encodes the structural proteins: C (the nucleocapsid protein), E3, E2, 6K, and E1. [4,7]

Of the five structural proteins, the glycosylated envelope proteins E1 and E2 form heterodimers in a trimeric array. Eighty such trimers compose the icosahedral lattice which is embedded into a host plasma-membrane derived lipid bilayer by a single transmembrane

helix for each of E1 and E2. This constitutes the envelope and outermost layer of CHIKV. Two-hundred and forty copies of the capsid protein also arrange into an icosahedral structure to form the nucleocapsid layer (with T=4 symmetry), within which the ssRNA genome is located. [7]

The E2 precursor protein, p62 is cleaved by the cellular enzyme furin en-route to the plasma membrane to yield the mature E2 and E3 proteins. The small protein E3 is composed of the 64 N-terminal amino acids of p62 and it remains attached to the virion on some alphaviruses [8-10]. Glycoprotein E1 is composed of the N-terminal domain I and domains II and III; the fusion loop is located at the tip of domain II. E2 is composed of domains A, B and C. Domains III and C are oriented closest to the viral membrane. Recently, the crystal structures of CHIKV p62-E1 and mature E3-E2-E1 were solved [8]. E2 covers much of E1 in a twisted plate morphology on the viral surface and the fusion loop of E1 lies between the groove formed by domains A and B of E2. These studies also indicate that the immature and mature forms of the heterodimer are similar, except for the tether region connecting E3 to p62, which is disordered in the mature post-furin cleavage form [8].

Epidemiology

Although Chikungunya virus was first isolated from a febrile patient during an epidemic of fever and severe joint pain in Tanzania in 1953 [11-14], based on retrospective case-study and phylogenetic analysis, it is estimated that CHIKV has caused disease in Africa and Southeast Asia since the late 1700's. CHIKV has epidemic potential, as reflected by the initial description in which it spread from village to village, infecting between 80-90% of the

3

inhabitants [15]. Over the last few decades, CHIKV has re-emerged and periodically caused outbreaks across Africa and Asia [1,16]. Significant morbidity has been observed, although historically, infection did not cause much mortality. Between 2005 and 2007, however, there was an explosive CHIKV epidemic of unprecedented magnitude; it initiated on the coast of Kenya in 2004 and spread to the French island of La Reunion (LR), from which it quickly dispersed to other Indian Ocean islands, India and many nations in Africa [1,17,18]. The ECSA genotype of CHIKV gave rise to this epidemic strain of circulating CHIKV which affected over five million people including approximately one third of the population of La Reunion island (~300,000 people) [18,19]. Although travelers returning from endemic countries to Canada, Europe and the United States have developed Chikungunya disease, local epidemics did not occur in these temperate climates. This changed in 2007 with the onset of the first European autochthonous epidemic, which caused 229 cases and one fatality in Northern Italy [20-22]. Three years later, in 2010, autochthonous cases of Chikungunya were reported in France [22,23]. This highlights the possibility of CHIKV spread across the world and the importance of developing a vaccine or specific antiviral agents.

Vertical transmission of CHIKV also has been observed and results in peripartum disease (4 days prior to or 2 days post delivery), likely due to intrapartum contamination of fetal blood with CHKV, before the mother makes protective antibodies [24,25]. Studies estimate that vertical transmission rates are 40 to 50% for viremic mothers [25-27]. These infants require intensive care and have a high propensity for developing neurological symptoms. [24,25]

Clinical Disease of CHIKV infection

The word "chikungunya" comes from the Makonde language spoken in Tanzania and it means "that which bends up"; this describes the contorted posture of patients afflicted with this disease. Acute infection with CHIKV manifests three to seven days post transmission by an *Aedes* mosquito bite. Symptoms include abrupt onset of a high fever, pruritic, maculopapular rash (occurring in 40 to 50% of patients) which extends over the trunk and limbs and sometimes the face, polyarthralgias (occurring in >95% of patients) and myalgias (occurring in 90% of patients) [4,24,28-31]. Tenosynovitis is another common sign observed in the chronic, recurring form of CHIK disease and most often affects the wrists, fingers and ankles [32,33]. Polyarthralgias are mainly symmetric and tend to occur in previously injured joints or distal joints [24]. Acute symptoms persist for about 14 days but chronic arthralgias can linger and are a significant cause of morbidity for weeks to even years. Pain can fluctuate in intensity, but does not usually change anatomical location. Joint pain is often debilitating; a recent prospective study showed that arthralgias persisted for at least 36 months in over 60% of a cohort of patients [34]. Rheumatological manifestations, consisting of a febrile arthritis predominantly affecting the extremities, were detected 15 months post-infection in 57% of another cohort of CHIKV patients, although there was no bone or cartilage erosion [24,35,36]. Prior osteoarthritis, hypertension or age > 45 were identified as risk factors for developing chronic joint manifestations [36].

Prior to the Indian Ocean outbreak, CHIKV infection was not associated with mortality but in 2005, this and other severe disease manifestations surfaced. During the epidemic, ~250 deaths were attributed to CHIKV infection on the island of La Reunion, which corresponds

to one death per 1,000 infections [37-39]. Patients with atypical presentations, however, had a death rate of 10% [26,27]. Some atypical manifestations included neurological (encephalitis, meningoencephalitis, seizures, and Guillain-Barre syndrome), cardiac (myocarditis, pericarditis, and heart failure) and renal (nephritis and acute renal failure) [1,24,26,28,40] signs and symptoms.

Enzootic cycle

CHIKV is transmitted by the *Aedes* species mosquitoes and is maintained in a sylvatic cycle in Africa, where non-human primates and rodents are the reservoirs and forest-dwelling mosquitoes (chiefly the *Aedes* species *furcier, taylori*, *luteocephalus and africanus*) and are the vectors for transmission. [4,41-43] During epidemics, humans serve as the reservoirs. In Asia, there is only mosquito-human-mosquito transmission, as a sylvatic cycle has not been documented [24,42]. *Aedes aegypti*, an urban mosquito that maintains close association with humans, is the primary vector in Asia. In comparison, *Aedes albopictus* was the vector primarily responsible for the La Reunion epidemic in 2005-2007 [1,4]. The global distribution of *Aedes albopictus* mosquitoes has spread over the last few decades to include all continents, either tropical or temperate, so CHIKV epidemics could theoretically occur anywhere. [37]

An A226V mutation on the E1 glycoprotein served as a gain-of-function adaptive change that resulted in enhanced infectivity of *Aedes albopictus* mosquitoes (via enhanced dissemination of the virus from the midgut to secondary organs), which ultimately caused an

increase in transmission of CHIKV to humans [4,24,44]. At the beginning of the outbreak, all viral isolates possessed A226 on the E1 glycoprotein, whereas by the end of the epidemic over 90% of CHIKV isolated from La Reunion had acquired the valine point mutation [44,45]. The autochthonous cases of Chikungunya reported in Italy were caused by an A226V isolate of CHIKV, but the autochthonous cases in Italy originated from the Asian genotype and lacked this mutation [22]. Notably, the ratio of clinical apparent to inapparent cases is high for CHIKV; only 3.2% of people tested in one study conducted on La Reunion were seropositive for CHIKV yet failed to develop acute symptoms consistent with infection [28].

Replication Cycle and Virus Assembly

Although the protein NRAMP was recently discovered as a necessary cell surface factor for binding and entry of Sindbis virus, a related alphavirus, a bona fide entry receptor for CHIKV has not been defined [46]. The E2 glycoprotein binds to the cell surface and this is followed by internalization of CHIKV into endosomes, likely in a clathrin-dependent fashion [47]. Upon acidification of the late endosome, the E1-E2 heterodimer dissociates and the E1 glycoprotein (which is a class II fusion protein) undergoes conformational changes exposing the fusion loop [41,48]. E1 rearranges into homotrimers which induce fusion between the viral and endosomal membranes, thus releasing the nucleocapsid into the cytoplasm [8,10,48]. The CHIKV genome is an infectious RNA and can be translated immediately in the cytoplasm [7] without modification. Following translation of the non-structural proteins (nsP1, nsP2: the helicase, protease and protein also involved in host transcriptional shut-off,

nsP3 and nsP4, the RNA polymerase) and replication of the genome to yield negative sense RNA, the 3' 26S subgenomic RNA is produced and translated into a polyprotein, which is autoproteolytically cleaved into the five structural proteins that form the virion [4]. After the N-terminally located capsid protein is released from this polyprotein, pE2 and E1 are inserted into the endoplasmic reticulum, form dimers and passaged through the Golgi apparatus. En route to the plasma membrane, furin, a cellular calcium-dependent serine protease processes pE2 into E2 and E3. These envelope proteins insert into the plasma membrane [5,38].

The nucleocapsid is composed of 80 copies of the capsid protein. It forms independently in the cytoplasm and encloses a single copy of viral genomic RNA. CHIKV particles bud from the plasma membrane as the nucleocapsid associates with the type I integral membrane protein E2. Each budding particle contains 80 surface spikes, each composed of a trimer of E1-E2 heterodimers arranged in T=4 quasi icosahedral symmetry [5,8].

Pathogenesis of CHIKV

Upon delivery of CHIKV into the skin, the virus spreads into the subcutaneous capillaries and predominantly infects fibroblasts in the connective tissue [38,49]. It disseminates through the blood to lymph nodes and can infect cells in other target organs, such as the liver, spleen, muscle and joints [38,49]. CHIKV does not infect osteoclasts, osteoblasts, lymphocytes or dendritic cells and there are contradictory reports on its infectivity of monocytes [39,50]. CHIKV also selectively infects muscle satellite cells (but not myocytes), endothelial cells and monocyte-derived macrophages. Muscle satellite cell infection has been observed in patient biopsies and *in vitro* [51]. Muscle satellite cell infection is particularly

significant because these cells are progenitors for new muscle fibers and augmentation of pre-existing muscle fibers; satellite cells will undergo cell division and differentiation after exercise or muscle injury. If these muscle stem cells are infected, they may help maintain a persistent reservoir of CHIKV in the muscle tissue, which may contribute to recurrent episodes of arthralgias and myalgias [51,52].

CHIKV replicates efficiently in humans and titers can reach 10^{12} RNA copies/mL in the serum of infected patients [53]. Tissues targeted in mouse and primate models and in human patients include connective tissue, especially the muscle epimysium, the joint capsule, and deep dermis [49,52]. Viral dissemination throughout the body was evidenced in a macaque model of CHIKV infection, which recapitulates features of human disease. CHIKV RNA was detected in the liver, spleen, lymph nodes and joints of all animals tested in the acute phase and infectious CHIKV was still recovered from the spleen, muscle and liver 44 days post infection [54]. Persistence of CHIKV antigen was detected in splenic macrophages up to 44 days post-infection and has been hypothesized to serve as a reservoir for long-term persistence of CHIKV [54]. Identifying a potential chronic reservoir of CHIKV may be very important for understanding the pathophysiology underlying chronic disease. Macrophages may contribute to this reservoir; CHIKV was detected 18 months post-infection in the peri-vascular synovial macrophages of one patient [53]. Additionally, Binadarit (an inhibitor of MCP-1) was shown to ameliorate arthritis-like disease symptoms in mice [55]. MCP-1 (monocyte chemotactic protein 1) is a crucial recruitment factor for macrophages into inflamed sites and is unregulated in both the serum and tissue of CHIKV-LR infected mice and acutely infected patients [53,55].

In mouse models of CHIKV disease, lethality to CHIKV infection is both age and IFNAR status-dependent [49]. *IFNAR*^{-/-} C57BL/6 mice are highly vulnerable and will die three days post intradermal infection with 10 PFU CHIKV-LR. Similarly, 6 day old C57BL/6 wild-type neonates will uniformly die when administered 10^6 PFU of CHIKV-LR intradermally , but if they are allowed to age just three more days, 60% will survive and at 12 days of age these mice are no longer susceptible to death following CHIKV infection [49].

Although CHIKV is an Old World arthritogenic virus, it has been shown to cause CNS disease, (encephalitis and meningitis), especially in neonatal patients and in mouse models [26]. CHIKV infects the choroid plexus and ependymal cells and the meninges but not brain endothelial cells, neurons, or microglia [49].

Antibody-mediated Protection against CHIKV

Polyclonal antibody response to CHIKV

Although the innate immune response plays an essential early role in protection against CHIKV infection, the antibody response against this virus is rapid and also important. IgG can be detected 3-8 days post symptom onset, even as early as 2 days post symptoms in some patients [31,56,57]. Many patients in a study of travelers returning back to Europe from the Indian Ocean region were positive for IgM antibody at the onset of symptoms (day 1) and all patients positive for CHIKV infection had both IgG and IgM by day 5 post symptom onset [57]. The rapid kinetics of antibody production may, in part, be attributed to the high CHIKV viremia, which results in a significant antigen load prior to symptom onset,[57]. Although IgM titers usually last for 1-3 months post-infection [31]; the persistence of IgM as late as 18

months after acute CHIKV infection in some patients suggests possible viral persistence [35,58].

The importance of the antibody response in clearing CHIKV infection was demonstrated in μMT mice that lack mature B cells. These mice sustain significantly higher CHIKV titers that start earlier in the course of disease, and they experience more joint swelling than wild type mice. Chronic infection occurred in μMT mice; viremia persisted for 79 days and has lasted as long as 402 days in surviving mice [59].

Passive transfer of polyclonal immunoglobulins from convalescent CHIKV infected patients protect against death of CHIKV-infected immunocompromised or neonatal mice. This demonstrates the neutralizing and protective potential of immune sera [60]. In a separate study, non-human primates were vaccinated with virus-like-particles (VLPs) containing CHIKV structural proteins; two milligrams of purified IgG from these animals was delivered passively to IFNAR$^{-/-}$ mice; this protected against death and viremia [61]. This further suggests that antibodies in immune sera alone can protect against CHIKV infection.

Monoclonal antibody response to CHIKV

Only a few studies involving CHIKV MAbs have been reported. Mouse MAbs against CHIKV that cross-react to O'nyong'nyong virus, some of which inhibit haemagglutination of both CHIKV and O'nyong'nyong virus, were identified in 1995 [62]. Years later, another study identified three antibodies that were shown to react with the E2 glycoprotein of CHIKV; neutralization potential was not determined [63].

Two neutralizing human MAbs (8B10 and 5F10) were identified from immortalized peripheral blood mononuclear cells (PBMCs) harvested from a patient previously infected

with CHIKV [64]. Escape mutants that were less sensitive to neutralization by these MAbs were isolated under antibody passage, but they still partially neutralized at higher concentrations of MAb [65]. The potential of one of these mutations (E2-R82G) to enhance potential cell-cell transmission of CHIKV was considered [65], however, further study is warranted. Although a high dose (250 µg) of 8B10 was protective against death in AGR129 mice (lacking the interferon α/β/γ receptor) when administered six hours before CHIKV infection, neither 8B10, 5F10 or the combination of these MAbs was able to prevent death therapeutically even when delivered just eight hours post infection [66].

Studies investigating the human antibody response to CHIKV have suggested that the dominant, neutralizing antibodies produced are mostly directed against the E2 glycoprotein and the major isotype represented is IgG3 [59,67-69]. One study found that neutralizing human antibodies primarily target a linear peptide consisting of 18 amino acids located at the N-terminus of E2 which they denoted E2EP3 [67]. In a different cohort consisting of nine patients, antibodies targeting nsP3 and E2 were identified 2-3 months post-infection, but only antibodies against E2 persisted 21 months post infection [69].

Patient studies also have correlated a more severe, acute disease with early, high viremia but this group also developed an early IgG3 response and completely cleared CHIKV infection without persistent arthralgia [68]. Early IgG3 responders were defined as patients producing IgG3 by 7- 10 days after the onset of infection and while no one in this group maintained chronic CHIKV symptoms, 30% of late IgG responders developed persistent arthrlagias [68].

Rationale

This work was initiated towards the end of one of the largest recorded CHIKV epidemics in history, when little was known about the immune response to CHIKV. We sought to generate a novel panel of anti-CHIKV MAbs because we were interested in developing a better understanding of how the humoral immune system restricts CHIKV infection. To encourage the diversity of epitopes targeted by MAbs, we immunized mice with CHIKV-LR and boosted four mice with either VLPs [61], E2 glycoprotein or CHIKV-LR. We aimed to identify highly neutralizing MAbs, determine what viral epitopes they targeted, identify mechanisms of neutralization and determine if these MAbs are protective in animal models. There is currently no licensed, human vaccine for CHIKV but insight into neutralizing viral epitopes is imperative for directed vaccine design so we hoped to learn more about neutralizing CHIKV epitopes and the molecular basis of neutralization.

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Chapter II

Development of a highly protective combination monoclonal antibody therapy

against Chikungunya virus

Development of a highly protective combination monoclonal antibody therapy against Chikungunya virus

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ABSTRACT

Chikungunya virus (CHIKV) is a mosquito-transmitted alphavirus that causes global epidemics of a debilitating polyarthritis in humans. As there is a pressing need for the development of therapeutic agents, we screened 230 new mouse anti-CHIKV monoclonal antibodies (MAbs) for their ability to inhibit infection of all three CHIKV genotypes. Four of 36 neutralizing MAbs (CHK-102, CHK-152, CHK-166, and CHK-263) provided complete protection against lethality as prophylaxis in highly susceptible immunocompromised mice lacking the type I IFN receptor (*Ifnar$^{-/-}$*) and mapped to distinct epitopes on the E1 and E2 structural proteins. CHK-152, the most protective MAb, was humanized, shown to block viral fusion, and require Fc effector function for optimal activity *in vivo*. In post-exposure therapeutic trials, administration of a single dose of a combination of two neutralizing MAbs (CHK-102 + CHK-152 or CHK-166 + CHK-152) limited the development of resistance and protected immunocompromised mice against disease when given 24 to 36 hours before CHIKV-induced death. Selected pairs of highly neutralizing MAbs may be a promising treatment option for CHIKV in humans.

AUTHOR SUMMARY

Chikungunya virus (CHIKV) is a mosquito-transmitted alphavirus that causes outbreaks of polyarthritis in humans, and is currently a threat to spread to the United States due to the presence of its mosquito vector, *Aedes albopictus*. At present, there is no licensed human vaccine or therapeutic available to protect against CHIKV infection. The primary goal of this study was to develop an antibody-based therapeutic agent against CHIKV. To do this, we developed a panel of 230 new mouse anti-CHIKV MAbs and tested them for their ability to neutralize infection of different CHIKV strains in cell culture. We identified 36 MAbs with broad neutralizing activity, and then tested several of these for their ability to protect immunocompromised *Ifnar*$^{-/-}$ mice against lethal CHIKV infection. In post-exposure therapeutic trials, administration a single dose of a combination of two neutralizing MAbs limited the development of resistance and protected *Ifnar*$^{-/-}$ mice against disease even when given just 24 to 36 hours before CHIKV-induced death. Analogous protection against CHIKV-induced arthritis was seen in a disease model in wild type mice. Our data suggest that pairs of highly neutralizing MAbs may be a therapeutic option against CHIKV infection.

INTRODUCTION

Chikungunya virus (CHIKV) infection causes a severe febrile illness in humans that is characterized by a debilitating polyarthritis, which can persist for months and cause significant morbidity [1,2]. There are three genotypes of CHIKV: Asian, East/Central/South African (ECSA), and West African [3-5], with 95.2 to 99.8% amino acid identity [4]. The CHIKV strains from the recent epidemics belong to the ECSA genotype and have affected millions in Africa and the Indian subcontinent [3,6]. Imported cases in the United States and outbreaks in Europe highlight the threat of CHIKV to developed countries [7]. Currently, there are no approved vaccines or therapeutics for CHIKV [8].

CHIKV is an enveloped alphavirus of the *Togaviridae* family that enters cells via receptor-mediated internalization and a low pH-triggered type II membrane fusion event in early endosomes. The mature virion is comprised of three structural proteins: a nucleocapsid protein and two glycoproteins, E1 and E2, where E2 functions in attachment to cells and E1 participates 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 [9]. X-ray crystallographic structures of the precursor pE3-E2-E1, mature E2-E1, and E1 proteins [10-13] have elucidated the architecture of the glycoprotein shell. The E1 ectodomain consists of three domains. Domain I (DI) is located 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 lie 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

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structure on the top of the spike. The mature E2 protein 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.

Mouse models have been developed for CHIKV infection. Newborn outbred and inbred mice are vulnerable to severe CHIKV infection with viral replication observed in muscle, joint, and skin [14,15]. Adult mice with defects in type I interferon signaling (*Ifnar*$^{-/-}$ mice) develop lethal disease, with muscle, joint, and skin appearing as the primary sites of infection [15]. CHIKV infection of juvenile C57BL/6 mice by a subcutaneous route results in metatarsal foot swelling with histological evidence of arthritis, tenosynovitis and myositis [16,17].

Passive transfer of MAbs or immune sera can protect animals against infection of alphaviruses including Sindbis (SINV), Semliki Forest (SFV), and Venezuelan equine encephalitis (VEEV) viruses [18-25]. Immune □-globulin from human donors in the convalescent phase of CHIKV infection exhibited neutralizing activity *in vitro* and had partial therapeutic efficacy in *Ifnar*$^{-/-}$ and neonatal wild type mice when administered up to 24 hours after infection [26]. Although mouse and human MAbs that neutralize CHIKV infection have been reported [27,28], their post-exposure efficacy against lethal infection *in vivo* has not been clearly established [29].

Here, we investigated the molecular basis of antibody-mediated neutralization of CHIKV using a panel of 230 newly generated, cloned MAbs. CHK-152 protected mice against CHIKV-induced mortality and disease. The inclusion of a second MAb (CHK-166 or CHK-102) prevented the emergence of viral resistance and extended the treatment window in *Ifnar*$^{-/-}$ mice up to 24 to 36 hours prior to death of the animals. Our results

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suggest that combination therapy with selected neutralizing MAbs has potential for treatment of CHIKV infection in humans.

RESULTS

Generation of MAbs. We generated a panel of neutralizing MAbs against CHIKV as a first step towards a possible therapy in humans. We infected adult C57BL/6 mice deficient for interferon regulatory factor 7 (*Irf7*^{-/-}) with 10^4 PFU of the La Reunion 2006 OPY-1 strain of CHIKV (CHIKV-LR); these mice were boosted with CHIK virus-like particles [30], soluble recombinant CHIKV E2 protein, or live CHIKV-LR. We immunized *Irf7*^{-/-} rather than wild type (WT) mice, as CHIKV replicated to higher titers, induced stronger neutralizing antibody responses, yet did not cause lethal infection in these innate immune-deficient animals ([31], and data not shown). We screened four independent myeloma cell-splenocyte fusions for binding of hybridoma supernatants to CHIKV-LR infected cells (**Fig S1**) and cloned 230 CHIKV-specific MAbs for further analysis (**Table S1** in **Text S1**). Using a single endpoint neutralization assay, we identified 36 MAbs with inhibitory activity against infection of CHIKV-LR in BHK21-15 cells (data not shown).

Neutralizing activity. To assess the inhibitory potential of our anti-CHIKV MAbs against the homologous CHIKV-LR and representative strains from the Asian and West African genotypes (RSU1I and bH35respectively), we performed focus reduction neutralization tests (FRNTs) on Vero cells. We determined the concentration of MAb that reduced the number of foci of infection by 50 or 90% (EC50 and EC90 values, **Fig 1A and B**, **and Table 1**). CHK-152 was the most strongly neutralizing MAb we identified; 3 and 15 ng/ml of this MAb prevented 50 and 90% of CHIKV infection against all three CHIKV genotypes (**Fig 1C**). Ten other MAbs inhibited CHIKV infection with EC50 values of <10 ng/ml against all three genotypes, and many others inhibited all three

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strains similarly, with a few exceptions. For example, CHK-9 failed to neutralize the Asian strain to the same extent as the West African or La Reunion (ECSA genotype) strains (**Fig 1D**), whereas CHK-151 inhibited infection of the Asian strain better than the others (**Table 1**). Also, for reasons that are unclear, some neutralizing MAbs (e.g., CHK-143, CHK-264, and CHK-269) were incapable of inhibiting all viruses (EC90 > 10,000 ng/ml) in this assay, even at high MAb concentrations.

We speculated that some MAbs might show cell type-dependent neutralization if they blocked attachment to cell type-specific factors. To test this hypothesis, we assessed MAb neutralization of CHIKV-LR infection in cells of another species, NIH 3T3 mouse fibroblasts (**Table 1**). For most MAbs, the EC50 values were comparable to those achieved with Vero cells. However, two MAbs (CHK-96 and CHK-176) showed a 12 to 250-fold reduction ($P < 0.05$) in neutralizing activity on NIH 3T3 compared to Vero cells; although further study is warranted, these MAbs may block a step in the entry pathway that varies among different cell types.

Prophylaxis studies. To evaluate whether neutralizing MAbs protect against CHIKV infection *in vivo*, we initially used a stringent test model: prevention of lethal infection in immunodeficient *Ifnar*^{-/-} C57BL/6 mice. One hundred micrograms of 14 different MAbs with strong, modest, or poor neutralizing activity were administered to *Ifnar*^{-/-} mice one day prior to CHIKV-LR infection. As seen previously [15], all *Ifnar*^{-/-} mice died by day 4 after infection when treated with saline or a negative control MAb (**Fig 2A**, and data not shown). Strongly neutralizing (e.g., CHK-102, CHK-152, and CHK-263) and one moderately inhibitory (CHK-166) MAb protected 100% of mice from lethal infection ($P < 0.0001$). In comparison, and somewhat surprisingly, CHK-95, a

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potently neutralizing MAb of the same IgG2c isotype, protected only 12% of mice from death. The other MAbs tested conferred intermediate levels of protection (**Fig 2A**). Thus, although several strongly neutralizing MAbs prevented against lethal CHIKV infection in *Ifnar*$^{-/-}$ mice, *in vitro* neutralization activity *per se* did not directly correlate with protection. To define the relative potency of the four MAbs that completely prevented lethal disease, we administered a lower (10 µg) dose. Whereas CHK-152 and CHK-263 still protected most mice from lethal infection, CHK-102 and CHK-166 protected to a lesser degree or only prolonged survival (**Fig 2B**). Consistent with their ability to protect against lethal infection, passive transfer of CHK-102, CHK-152, CHK-166, and CHK-263 MAbs all markedly reduced viral loads in serum, spleen, liver, muscle, and brain at 48 hours after infection relative to a non-binding isotype control (DENV1-E98) MAb (**Fig 2C-G**). The level of protection afforded by CHK-102, CHK-152, CHK-166, and CHK-263 MAbs, however, did not correlate directly with their binding strength to CHIKV surface glycoproteins (**Fig S2**).

Although a stringent test of MAb protection, CHIKV-infected *Ifnar*$^{-/-}$ mice do not develop the arthritis observed in humans. To evaluate this, we utilized a WT C57BL/6 mouse model in which inoculation of CHIKV into the footpad results in localized swelling and induction of arthritis and fasciitis within the foot and ankle [16,17], although infection does not cause lethality. Pretreatment of mice with either 100 µg of CHK-102 or CHK-152 completely protected against CHIKV-induced swelling, compared to control animals, which developed clinically apparent swelling (data not shown). While CHIKV infected control animals developed inflammatory arthritis in the ankle and foot, CHK-102 or CHK-152 MAb treated animals had normal appearing joint tissues (**Fig 2H**).

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Mechanism of neutralization. Antibody neutralization of enveloped viruses can occur by inhibiting attachment, internalization, and/or fusion [32,33]. To determine how many of our most protective MAbs inhibited infection in cell culture, we performed pre- and post-attachment neutralization assays [34,35]. Anti-CHK MAbs were incubated with CHIKV before or after virus binding to cells, and infection was measured. As expected, all MAbs efficiently neutralized infection when pre-mixed with virus (**Fig 3A**). While CHK-102, CHK-152, CHK-166, and CHK-263 also inhibited CHIKV infection when added after virus adsorption to the cell surface, suggesting that at least part of their blocking activity was at a post-attachment step, differences in the extent of neutralization were noted in this context for several MAbs. CHK-152 completely neutralized all CHIKV virions without a resistant fraction when added post-attachment. When studies were repeated with eight other neutralizing MAbs that showed pre-exposure protection *in vivo*, no other MAb inhibited infection completely when added after virus adsorption to the cell. As expected, an isotype control MAb (DENV1-E98) and a non-neutralizing anti-CHK MAb (CHK-84) had no inhibitory effects in this assay (**Fig S3**).

Blockade of viral fusion. Since CHK-152 neutralized infection efficiently at a post-attachment step, we investigated whether it blocked fusion using a viral fusion from without (FFWO) assay [36]. CHIKV was adsorbed to Vero cell monolayers on ice and then treated with MAbs. Fusion at the plasma membrane was triggered after a brief exposure to low pH buffered medium at 37°C. Subsequently, cells were incubated in the presence of 20 mM NH_4Cl to prevent CHIKV fusion via canonical endosomal pathways. As expected, at 14 hours after initial treatment, CHIKV infection was not observed when adsorbed virus was incubated at neutral pH (**Fig 3B**). In comparison, in the absence of

MAb or in the presence of a control MAb, a short exposure of cell surface-adsorbed virus to acidic pH resulted in infection and CHIKV-antigen positive cells. Notably, CHK-152 completely inhibited ($P < 0.0001$) plasma membrane fusion and infection, whereas other anti-CHIKV neutralizing MAbs showed significant yet incomplete inhibition in this assay (**Fig 3B and C**). These studies suggest that CHK-152 efficiently neutralizes infection by preventing the structural changes on the virion necessary for viral fusion with host cell membranes.

We utilized a model liposome fusion assay with pyrene-labeled virus [37,38] to confirm these results. Pyrene-labeled CHIKV was pre-incubated with different concentrations of MAb, mixed with liposomes at 37°C, and fusion was triggered by addition of a low-pH buffer [37]. In the absence of MAb or in the presence of 10 nM (1.5 μg/ml) of a non-binding control MAb, fusion was complete within seconds of acidification. In contrast, pre-incubation of virus with increasing doses of CHK-152 inhibited fusion (**Fig 3D and E**). Thus, CHK-152 can block low-pH-induced fusion of virus with liposomes.

The effector functions of CHK-152 contribute to protection *in vivo*. To define additional mechanisms by which our most strongly protective MAb (CHK-152) conferred protection *in vivo*, we generated a chimeric mouse-human CHK-152 (ch-CHK-152) as well as an aglycosyl variant (ch-CHK-152 N297Q) that lacks the ability to engage C1q or Fc-γ receptors; this mutation does not affect the ability to bind the neonatal Fc receptor (FcRn) or half-life of antibody in mouse serum [39]. The affinity of ch-CHK-152 and ch-CHK-152 N297Q binding to purified pE2-E1 was measured by surface plasmon resonance (SPR) and compared to the parent murine MAb. Notably, ch-CHK-152, ch-

CHK-152 N297Q, and the murine CHK-152 all had similar affinity (K_D of 3 to 4 nM) (**Fig 4A** and data not shown) and neutralizing activity in cell culture (**Fig 4B**). As expected, ch-CHK-152 N297Q failed to bind efficiently to soluble Fc-γ receptors or C1q (**Fig 4C**).

We transferred ch-CHK-152 and ch-CHK152 N297Q to *Ifnar*^{-/-} mice prior to infection. Although high doses (100 µg) of ch-CHK-152 and ch-CHK-152 N297Q provided similar protection against CHIKV infection (data not shown), lower doses (10 µg) of the aglycosyl variant were less protective; whereas 62% of the mice receiving ch-CHK152 N297Q survived, all *Ifnar*^{-/-} mice given ch-CHK-152 MAb remained alive (**Fig 4D**, $P < 0.05$). When parallel studies were performed with WT C57BL/6 mice and MAb was administered 18 hours after infection, ch-CHK-152 N297Q also provided less protection against arthritis compared to ch-CHK-152 (**Fig 4E**). These data suggest that the Fc effector interactions contribute to the potency of CHK-152 in mice.

Humanization of CHK-152. We humanized CHK-152 as a first step towards a MAb therapeutic (see Supplemental Methods). The affinity for pE2-E1 and neutralizing activity of the hu-CHK-152 were similar to mouse CHK-152 (**Fig S4A and B**). Hu-CHK-152 also protected *Ifnar*^{-/-} mice ($P > 0.0001$) when a single dose (10 or 100 µg) was administered one day before infection (**Fig S4C**).

Therapeutic studies. To define the therapeutic potential of our most protective MAbs, a single dose (100 µg) was administered to *Ifnar*^{-/-} mice 24 hours after CHIKV infection (**Fig 5A**). Whereas CHK-152 and 166 protected 58% and 63% of mice from death, respectively ($P < 0.0001$), CHK-263 and CHK-102 had less activity although both MAbs increased the median survival time (7 days versus 4 days with the control DENV1-

E98 MAb, $P < 0.0006$). Administration of CHK-152 at 12 or 18 hours post infection also protected WT mice from CHIKV-induced swelling and arthritis (**Fig 5B and Fig 4E**).

We next tested the activity of combinations of the most protective neutralizing MAbs in *Ifnar*$^{-/-}$ mice. Remarkably, administration of 50 µg each (100 µg total dose) of CHK-102 + CHK-152, CHK-263 + CHK-152, or CHK-166 + CHK-152 at 24 hours post infection completely prevented mortality in all animals (**Fig 5A,** $P < 0.0001$ for MAb combinations). This observation was not true for all MAb combinations, as administration of 50 µg each of CHK-102 + CHK-263 provided substantially less protection with a 14% survival rate. We then performed a more stringent test in which 100 µg each (200 µg total) of our most protective combinations was delivered as a single dose at 48 hours post-infection (**Fig 5C**). Treatment with CHK-102 + CHK-152 or CHK-166 + CHK-152 protected 62% of the *Ifnar*$^{-/-}$ mice ($P < 0.003$) and the combination of CHK-263 + CHK-152 functioned almost as well, with 50% of animals surviving ($P < 0.03$). To define the limits of protection in *Ifnar*$^{-/-}$ mice, which all succumb to CHIKV between days 3 and 4, therapy was initiated at 60 and 72 hours after infection. At 60 hours after infection, *Ifnar*$^{-/-}$ mice receiving 250 µg each of CHK-102 + CHK-152 or CHK-166 + CHK-152 had survival rates of 28 and 71%, respectively (**Fig 5D**, $P = 0.03$ and $P = 0.004$). Nonetheless, when combination therapy was given at 72 hours after infection, a time when overt disease was present, no survival benefit was conferred. Thus, combination MAb therapy is superior to monotherapy in protecting against lethal CHIKV infection in highly immunocompromised mice.

Functional interaction of MAbs. To begin to understand the basis for enhanced *in vivo* activity, we assessed whether CHK-152 and selected MAbs could bind

simultaneously to the CHIKV virion. We developed a competition ELISA in which virions were captured by a mouse MAb (CHK-65), and then incubated with increasing concentrations of CHK-102, CHK-152, CHK-166, or CHK-263 mouse MAbs. After washing, hu-CHK-152 MAb was added, and binding was assessed. While pre-bound mouse CHK-152 competed against hu-CHK-152 binding as expected, CHK-102, CHK-166, and CHK-263 minimally competed hu-CHK-152 binding (**Fig S5A**), suggesting their epitopes largely were distinct. However, addition of CHK-102, CHK-166, or CHK-263 failed to augment the inhibitory activity of CHK-152 when neutralization was measured in cell culture (**Fig S5B**), as no synergy was observed.

Neutralization escape mutants. To identify epitopes targeted by the therapeutic MAbs, we generated escape mutants in cell culture. After sequential virus passage under CHK-102, CHK-152, CHK-166, or CHK-263 selection, CHIKV became resistant to neutralization by these MAbs (**Fig 6A-D**). We assessed whether the escape variants generated in the presence of one MAb remained sensitive to neutralizationby the other MAbs. The CHK-152 escape variant was neutralized efficiently by CHK-102, CHK-166, and CHK-263 (**Fig 6B, Table S2** in **Text S1,** and data not shown), and analogously the CHK-166 escape variant was inhibited by CHK-102, CHK-152, and CHK-263 (**Fig 6C,** and data not shown). In contrast, CHK-102 and CHK-263 escape variants reciprocally were resistant, suggesting their epitopes were the same or overlapping (**Fig 6A and D**); however, CHK-102 and CHK-263 escape variants remained sensitive to neutralization by CHK-152 and CHK-166. Notably, selection with combinations of MAbs (e.g., CHK-102 + CHK-152) failed to produce escape variants despite several independent attempts (data not shown).

To identify the mutations that conferred resistance, we sequenced plaque-purified escape variants (**Table 2,** *top*). Six of eight sequences from CHK-102 escape variants contained an L210P mutation in the E2 protein; the remaining two sequences had a G209E mutation in E2. For CHK-152 resistant variants, all sequences (9 of 9) contained a D59N mutation in E2 and two contained a second A89E substitution in E2. For CHK-263, 3 of 4 escape variants had a K215E change in E2, whereas 1 of 4 had mutations in E2 at G209E. All escape variants (14 of 14) of CHK-166 had a single K61T mutation in the E1 protein.

To verify the amino acid changes that conferred MAb resistance *in vitro*, we introduced several of these substitutions into a chimeric SFV-GFP-CHIKV cDNA comprised of SFV non-structural genes, a GFP reporter gene, and the CHIKV structural genes (T. Lin, K. Dowd, and T. Pierson, unpublished results). Parental and SFV-GFP-CHIKV with single amino acid mutations were analyzed for neutralization by CHK-102, CHK-152, CHK-166, and CHK-263 (**Fig 6E-H**). Consistent with our sequencing results, viruses encoding mutations in E2-G209 and E2-L210 were resistant to CHK-102, changes in E2-D59 conferred resistance to CHK-152, substitutions in E1-K61 resulted in resistance to CHK-166, and mutation of E2-G209 and E2-K215 caused resistance to CHK-263. However, introduction of E2-A89E (which was present in 2 of 9 clones) failed to affect the neutralizing activity of CHK-152.

In addition to selecting escape variants in cell culture, we harvested organs from the few mice that became ill after infection despite single MAb treatment (**Table 3,** *bottom*). In these moribund *Ifnar*^{-/-} mice, CHIKV was present in the brain and muscle but absent from the spleen or liver (data not shown). This *in vivo*-derived virus was tested for

MAb resistance and sequenced. For mice receiving a 10 µg dose of CHK-102 as prophylaxis, resistant variants with a L210P mutation in E2 were obtained. For mice receiving CHK-263 or CHK-102 at 24 hours post infection, resistant viruses with a G209E mutation in E2 were identified. None of the animals that were pre-treated with 10 µg of CHK-166 developed escape mutants, as the virus harvested from all 3 mice tested retained sensitivity to CHK-166 (data not shown). However, in one animal receiving CHK-166 at 24 hours post infection, a single resistant virus with a G64S substitution in the E1 gene was recovered (**Fig S6**). For mice receiving a 10 µg dose of hu-CHK-152 as prophylaxis, partially resistant viruses with N231D and K233E mutations in E2 were isolated and confirmed by reverse genetics using the chimeric SFV-GFP-CHIKV infectious clone (**Fig S7**). In comparison, when CHK-152 was given as a therapeutic, a single mutation at D59N in E2 was obtained in 4 of the 5 mice tested, with a K233T mutation in virus from the remaining animal. For animals treated at 48 hours with combination MAb therapy, all recovered viruses remained sensitive to CHK-152 yet showed partial resistance to CHK-102 or CHK-166 (**Fig S8**). Mutations in E2 (N332I, CHK-166 + CHK-152) were identified. Comparison of 140 available E1 and E2 sequences from historical and circulating CHIKV strains in a public database (http://www.viprbrc.org/) revealed nearly complete conservation of the residues in which escape mutants were selected: E1-K61, 100%; E1-G64, 100%; E2-D59, 100%; E2-G209, 100%; E2-L210, 99.3%; E2-K215, 100%; E2-N231, 100%; and E2-K233, 99.3%.

To define spatially the location of the amino acids that conferred resistance to our highly protective MAbs, these residues were mapped onto the existing CHIKV protein crystal structures [10] (**Fig 6I,** *left*). Amino acids that conferred neutralization escape to

CHK-102 and CHK-263 were located in the B domain of E2. The residues that modulated CHK-152 neutralization mapped to the A domain of E2. In contrast, CHK-166 recognized amino acids on DII of E1, adjacent to the fusion loop. All amino acids that conferred neutralization escape appear solvent accessible and highly exposed when docked onto the E2-E1 spike (**Fig 6I**, *right*).

DISCUSSION

We set out to identify MAbs with the greatest therapeutic activity against CHIKV in mice as a first step toward generating an immunotherapy for humans. Thirty-six MAbs with neutralizing activity against CHIKV-LR were identified, the majority of which also inhibited infection of strains corresponding to the two heterologous CHIKV genotypes. Although all fourteen of the selected anti-CHIKV MAbs improved outcome in vulnerable *Ifnar*^{-/-} mice, only four of these (CHK-102, CHK-152, CHK-166, and CHK-263) completely prevented lethality when administered as prophylaxis. CHK-152 provided the greatest benefit as post-exposure therapy, although by itself, the window of treatment activity was limited in the *Ifnar*^{-/-} mouse model. While addition of a second MAb (CHK-102, CHK-166, or CHK-263) failed to enhance CHK-152 neutralization *in vitro*, it limited the development of viral resistance *in vitro* and *in vivo*. Remarkably, combinations of CHK-102 + CHK-152 or CHK-166 + CHK-152 protected *Ifnar*^{-/-} mice against mortality even when a single dose was administered 24 to 36 hours prior to the death of untreated or isotype control MAb-treated animals.

In comparison to the highly therapeutic activity of 0.5 mg of CHK-152 + CHK-166, a single 25 mg dose of immune IgG purified from a convalescent human subject protected only 50% of *Ifnar*^{-/-} mice when administered 24 hours after CHIKV infection [26]. The administered dose of neutralizing antibody likely is critical to post-exposure treatment of CHIKV infection because of the high viral burden [14,16,17,40]. A high viral load impacts therapeutic activity of antibodies as it (a) increases the chance for pre-existing or selected resistant variants to emerge through quasispecies [28,41]; and (b) results in a low relative fractional occupancy of binding to any individual virion, which

allows antibodies recognizing key epitopes to fall below their stoichiometric threshold of neutralization [42]. Although there is extensive literature on the protective efficacy of MAbs or immune sera against alphavirus infection [18-25], no prior study has demonstrated reduced CHIKV-induced mortality with MAbs. Although a recent study showed that combination post-exposure therapy with two human anti-CHIKV MAbs (5F10 and 8B10, 250 µg each at + 8 h) prolonged survival of AG129 (*Ifnar*^{-/-} x *Ifngr*^{-/-}) mice by ten days, they failed to prevent lethal infection [29]; the basis of this treatment failure remains unclear but could reflect the lower neutralizing potency of the MAbs (compared to CHK-152), rapid emergence of resistant mutants, or the relative susceptibility of the immunocompromised mouse host. In comparison, a neutralizing MAb (UM 5.1) administered two days after SFV infection completely protected immuocompetent BALB/c mice [43].

Why were some combinations of two MAbs effective *in vivo*? (a) Pairs of MAbs may show neutral, additive, or synergistic effects on neutralization. Positive antiviral effects could occur through cooperative binding or by trapping CHIKV in conformations that makes it less competent to bind a receptor or fuse with host membranes. Nonetheless, when we added increasing concentrations of CHK-102, CHK-166, or CHK-263 to CHK-152, we failed to observe synergy. (b) Certain MAb combinations could prevent the emergence of resistance due to the low frequency of two escape mutations occurring simultaneously in a single replication cycle. Although we could readily select for neutralization escape against a single MAb *in vitro* and *in vivo*, we failed to isolate resistant mutants against CHK-152 when two MAbs (e.g., CHK-102 + CHK-152) were combined. However, some viruses from moribund animals treated with combination

MAb therapy showed reduced sensitivity (up to 200-fold) to the other MAb (e.g., CHK-102) in the pair. In comparison, when mice were treated with a combination of 50 µg each of CHK-102 + CHK-263, we failed to observe the same survival benefit that was conferred by the combinations of CHK-102, CHK-166, or CHK-263 with CHK-152. Since CHK-102 and CHK-263 appear to share overlapping footprints on domain B of E2, this particular MAb combination may fail to prevent the rapid emergence of escape mutants relative to others targeting distinct epitopes on E1 and E2 proteins. (c) Combinations of MAbs could select for resistant viruses that have reduced fitness [44], and thus are less pathogenic *in vivo*. Virulence studies with CHIKV encoding selected single and double mutations are planned to evaluate this possibility.

We localized the epitopes of our four highly protective MAbs using neutralization escape selection, sequencing, and reverse genetics. CHK-152, which blocked viral fusion, mapped to the wings of the A domain on E2, a result that we recently confirmed by cryo-electron microscopic analysis of CHK-152 Fab-virus particle complexes [45]. This epitope also was identified as a recognition site for neutralizing MAbs against VEEV [46] and SINV [47]. CHK-166, which was the least neutralizing (EC50 of ~100 ng/ml) of our highly protective MAbs mapped to an epitope in domain II of the E1 protein, adjacent to the highly conserved fusion loop. While anti-E1 MAbs against SINV and VEEV that protect or neutralize infection have been described [46,48,49], none have been characterized against CHIKV. A neutralizing human MAb (8B10) against CHIKV was reported with possible reactivity against E1, although further analysis revealed that it bound to the E1/E2 heterodimer [27,28]. CHK-102 and CHK-263 mapped to residues within the B domain on E2. A related epitope also was identified in mapping studies of

strongly neutralizing antibodies against Ross River virus [50], SINV [51,52], VEEV [46,53,54], and CHIKV [10,28]. The B domain on E2 comprises an important antigenic domain that is under selective pressure for antibody neutralization [41]. It serves as a cap to the fusion loop on E1 and because of its location at the tip of the heterodimeric spike [10,11] may contribute to attachment of cellular receptors.

In summary, we identified combinations of MAb pairs that were highly effective as post-exposure therapeutic agents. These findings are consistent with recent studies showing enhanced post-exposure efficacy of MAb combinations against Ebola [55], influenza A [56] and rabies [57] viruses. Our most promising pair of MAbs mapped to distinct epitopes, limited the generation of resistance, blocked multiple stages of the viral entry pathway, and protected *Ifnar*$^{-/-}$ mice against mortality even when administered 60 hours after infection. CHK-152 was humanized as a first step towards a possible therapeutic for humans and demonstrated similar efficacy compared to the parent murine MAb. Tailored combinations of potently neutralizing MAbs show promise to prevent or treat infection by CHIKV, and likely other pathogenic alphaviruses in humans. Ultimately, a more detailed kinetic analysis of CHIKV infection in humans and determination of a treatment window relative to symptom onset is warranted to establish whether combination MAb therapy can prevent or mitigate acute or chronic and persistent infection and joint disease.

METHODS

Cells and viruses. Vero, Vero76 (ATCC), BHK21-15, and NIH 3T3 mouse fibroblast cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% or 15% (for 3T3 cells) fetal bovine serum (FBS) (Omega Scientific). C6/36 *Aedes albopictus* cells were grown in Leibovitz-15 medium supplemented with 10% FBS at 27°C. The infectious clones of CHIKV La Reunion 2006 OPY-1 (strain 142, CHIKV-LR) and CHIKV-GFP (strain 145) were gifts from S. Higgs (Manhattan, KS) [58]. CHIKV-RSU1 and CHIKV-IbH35 were gifts of R. Tesh, (Galveston, TX). Infection studies of WT mice used the SL15649 strain of CHIKV, which was generated from an infectious clone [17]. The S27 African prototype CHIKV strain was a gift from Dr. S. Günther (Bernhard-Nocht-Institute for Tropical Medicine, Germany) and isolated from a patient in Tanzania in 1953.

Ethics statement. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committee at the Washington University School of Medicine (Assurance Number: A3381-01) and the University of North Carolina (A3410-04). Dissections and footpad injections were performed under anesthesia that was induced and maintained with ketamine hydrochloride and xylazine, and all efforts were made to minimize suffering.

Generation of chimeric SFV-CHIKV. Chimeric SFV-CHIKV virus was generated by complementation of a double sub-genomic DNA-launched SFV replicon "backbone" plasmid (pSFV-GFP-BB) with the structural genes of CHIKV as described

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recently for WNV [59]. The vectors and methods will be described in detail elsewhere (TY Lin, K. Dowd, and T. Pierson, in preparation). To generate SFV-CHIKV, a DNA fragment encoding WT or mutant CHIKV structural genes was ligated into the pSFV-GFP-BB plasmid and transfected directly into HEK-293T cells using Lipofectamine LTX. The source of CHIKV structural genes was a sub-cloning vector pCHIKV-struct: mutations were introduced into this vector using site-directed mutagenesis and fully sequenced. Virus was harvested at 48, 72, or 96 hours after transfection, filtered, and stored at -80°C.

CHIKV protein. The CHIKV E2 ectodomain (residues S1-E361) and pE2-E1 (E3-E2-E1: residues S1-R64 of E3, S1-E161 of E2, and Y1-Q411 of E1 including a $(GGGS)_4$ polylinker between E2 and E1) of the CHIKV-LR strain were amplified from the infectious cDNA clone using high-fidelity Phusion PCR (Thermo Scientific). The E2 ectodomain was cloned into pET21a, expressed in *E. coli*, and purified using an oxidative refolding protocol followed by size-exclusion column purification using fast protein liquid chromatography [60]. pE2-E1 was cloned into the mammalian expression vector pHLsec (Invitrogen) with a C-terminal octa-histidine tag and modified to express the Epstein–Barr virus EBNA-1 protein for enhanced protein expression. pE2-E1 was expressed in serum-free HEK-293F suspension cells and purified by Ni-NTA agarose affinity (Qiagen) and Superdex 200 gel filtration chromatography.

MAb generation. *Irf7*^{-/-} mice were infected and boosted with 10^4 PFU of CHIKV-LR and, depending on the experiment, given a final intravenous (i.v.) boost with CHIKV virus-like particles [30], 25 µg of E2 protein, or 2 x 10^5 PFU of CHIKV-LR three days prior to fusion with myeloma cells. Hybridomas secreting antibodies that

reacted with CHIKV-GFP-infected BHK21-15 cells were identified by flow cytometry and cloned by limiting dilution. MAbs were isotyped by ELISA (Pierce), adapted for growth under serum-free conditions, and purified by protein G affinity and size exclusion chromatography. All MAbs were screened initially with a single endpoint neutralization assay using neat hybridoma supernatant (~10 µg/ml), which was incubated with 100 FFU of CHIKV-LR for one hour at 37°C. MAb-virus complexes were added to BHK21-15 cell monolayers in 6-well plates. After 90 minutes, cells were overlaid with 1% (w/v) agarose in Modified Eagle Media (MEM) supplemented with 4% FBS. Plates were fixed with 10% formaldehyde in PBS 48 hours later, stained with crystal violet, and plaques were counted. The V_H and V_L sequence of neutralizing MAbs CHK-102, CHK-152, CHK-166, and CHK-263 were amplified from hybridoma cell RNA by a 5' RACE procedure **Table S3** in **Text S1**).

Chimerization of MAbs. The generation of a chimeric mouse-human CHK-9 and CHK-152 with mouse V_H and V_L and human IgG1 constant regions was performed as described previously [60]. A point mutation that abolishes FcγR and C1q binding (N297Q) was introduced by QuikChange mutagenesis (Stratagene). Recombinant antibodies were produced after transfection of HEK-293T cells, harvesting of supernatant, and purification by protein A affinity chromatography.

Infection of mice. (a) Immunocompromised mice. *Ifnar*^{-/-} mice were bred in pathogen-free animal facilities of the Washington University School of Medicine and infection experiments were performed in A-BSL3 facilities with the approval of the Washington University Animal Studies Committee. For prophylaxis studies, MAbs were administered by i.p. injection to 6 to 8 week-old *Ifnar*^{-/-} mice one day prior to s.c.

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infection in the footpad with 10 FFU of CHIKV-LR. For therapeutic studies, 10 FFU of CHIKV-LR was delivered 24, 48, 60, or 72 hours prior to administration of a single dose of individual or combinations of MAbs. To monitor viral burden *in vivo*, mice were treated with a single 100 CHIKV isotype control MAb one day before infection with 10 FFU of CHIKV-LR. Animals were sacrificed two days later for virological analysis. After extensive perfusion with PBS, organs were harvested, weighed, homogenized and virus was titered by focus-forming assay. **(b) Immunocompetent mice**. Four to six week-old C57BL/6 mice were infected s.c. in the footpad with 100 PFU of CHIKV SL15649 in 10 µl of PBS as described previously [17]. Some animals received 100 µg of MAb in 500 µl of PBS via an i.p. route before or after infection. Mice were monitored daily for footpad swelling. At 10 days after infection, mice were sacrificed and sections prepared from decalcified hind limbs [17] for histopathological analysis. All CHIKV studies with WT mice were performed under A-BSL-3 conditions and in accordance with approved protocols following University of North Carolina guidelines.

Neutralization assays. Serial dilutions of MAb were incubated with 100 FFU of CHIKV for one hour at 37°C. MAb-virus complexes were added to cells in 96-well plates. After 90 minutes, cells were overlaid with 1% (w/v) methylcellulose in Modified Eagle Media (MEM) supplemented with 4% FBS. Plates were harvested 18 to 24 hours later, and fixed with 1% PFA in PBS. The plates were incubated sequentially with 500 ng/ml of ch-CHK-9 and horseradish peroxidase (HRP)-conjugated goat anti-human IgG in PBS supplemented with 0.1% saponin and 0.1% BSA. CHIKV-infected foci were visualized using TrueBlue peroxidase substrate (KPL) and quantitated on an ImmunoSpot

5.0.37 macroanalyzer (Cellular Technologies Ltd). Non-linear regression analysis was performed, and EC50 values were calculated after comparison to wells infected with CHIKV in the absence of antibody.

Pre- and post-attachment neutralization assays. 96-well tissue culture plates were coated with 100 µl of poly-L lysine and seeded with 3×10^4 Vero cells/well overnight. For pre-attachment assays, dilutions of MAb were prepared at 4ºC in DMEM with 2% FBS and pre-incubated with 100 FFU of CHIKV-LR for one hour at 4ºC. MAb-virus complexes were added to pre-chilled Vero cells for one hour at 4ºC. Non-adsorbed virus was removed with three washes of DMEM and adsorbed virus was allowed to internalize during a 37ºC incubation for 15 minutes. Cells were overlaid with 1% (w/v) methylcellulose in MEM supplemented with 4% FBS. The post-attachment assay was performed similarly, except that an equivalent amount of CHIKV was adsorbed first onto Vero cells for one hour at 4ºC. After removing free virus, dilutions of MAb were added to the virus-adsorbed cells for one hour at 4ºC. Virus was allowed to internalize and cells were overlaid with methylcellulose as described above. Nineteen hours later, the plates were harvested and analyzed for antigen-specific foci as described above.

Fusion inhibition assays. (a) Fusion from without assay. Virus fusion with the plasma membrane was assessed using a fusion from without (FFWO) assay [36]. Vero cells were seeded in 96-well plates, washed once with Binding medium (RPMI 1640, 0.2% BSA, 10 mM HEPES pH 7.4, and 20 mM NH_4Cl) at 4ºC, and incubated for 15 minutes at 4ºC. CHIKV-LR (MOI of 15) was prepared in Binding medium and added to cells for one hour at 4ºC, and then free virus was removed. Subsequently, DMEM containing 2% FBS with or without CHIKV-specific or control MAbs (50 µg/ml) was

added to cells for one hour at 4ºC. FFWO was induced by the addition of pre-warmed fusion media (RPMI 1640, 0.2% BSA, 10 mM HEPES, and 30 mM succinic acid at pH 5.5) for two minutes at 37ºC. In parallel wells, control media (RPMI 1640, 0.2% BSA, 10 mM HEPES at pH 7.4) was added for 2 minutes at 37ºC to ensure that infection occurred only through pH-dependent plasma membrane fusion. Medium was removed and cells were incubated in DMEM supplemented with 5% FBS, 10 mM HEPES, and 20 mM NH_4Cl (pH 7.4); NH_4Cl prevented secondary infection through endosomal fusion pathways. Cells were detached 14 hours later, fixed with 1% PFA in PBS for 8 minutes, and permeabilized with 0.1% (w/v) saponin detergent solution. Cells were incubated sequentially with ch-CHK-9 and Alexa 647 conjugated goat anti-human IgG secondary antibody (Invitrogen). Infection was evaluated on a FACSArray flow cytometer (Becton-Dickinson) and analyzed using FlowJo software. **(b) Liposomal fusion assay**. Pyrene-labeled CHIKV (S27 African strain) was recovered from supernatants of infected Vero76 cells cultured for 48 hours in the presence of 15 µg/ml 16-(1-pyrenyl)hexadecanoic acid (Invitrogen) as described [37]. Fusion of pyrene-labeled CHIKV with liposomes was monitored continuously in a Fluorolog 3-22 fluorometer (BFi Optilas), essentially as described [37]. Pyrene-labeled CHIKV and an excess of liposomes were mixed in a final volume of 665 µl in 5 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4. Fusion was triggered by the addition of 35 µl 0.1 M MES, 0.2 M acetic acid, which achieved a pH of 4.7. For the antibody inhibition experiments, pyrene-labeled CHIKV was incubated with increasing concentrations of CHIKV-152 or isotype control IgG2a MAb (MAb 0031, R&D systems) for 10 minutes at 37°C prior to mixing with liposomes.

SPR. The binding of human FcγR and C1q to ch-CHK-152 and ch-CHK-152 (N297Q) was analyzed by SPR using a BIAcore 3000 biosensor (GE Healthcare Life Sciences). MAbs were captured (~900 RU) after flowing over immobilized F(ab)'2 fragments of goat anti-human F(ab)'2 specific IgG on a CM-5 sensor chip. Binding experiments were performed in HBS-EP buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% P20 surfactant). Binding of CD16A and CD64 (as monomeric soluble FcγR), CD32A (as dimeric soluble FcγR-aglycosylated Fc fusion), and C1q (Sigma-Aldrich) was analyzed at a single concentration. The FcγR and C1q were injected for 60 sec at a flow rate of 30 µl/min then allowed to dissociate over 2 minutes. Affinity measurements of CHK-152 MAbs for pE2-E1 were performed by SPR in HBS-EP buffer. Ch-CHK-152, ch-CHK-152 N297Q, hu-CHK-152 and mouse CHK-152 were captured (~300 RU) after flowing over immobilized F(ab)'2 fragments of goat anti-human or anti-mouse Fc specific IgG. Purified pE2-E1 was injected at concentrations of 0, 6.25, 12.5, 25, 50, and 100 nM, at a flow rate of 30 µl/min for 120 sec, and then allowed to dissociate over 2 minutes. Regeneration of capture surfaces was performed by pulse injection of 10mM glycine pH 1.5. Binding curve at the zero concentration of pE2-E1 was subtracted from each experimental curve as a blank. Data were analyzed using BIAevaluation 4.1 software. Kinetic constants, k_a and k_d, were estimated by global fitting analysis of the association/dissociation curves to the 1:1 Langmuir interaction model.

Escape mutant selection. CHIKV-LR (1.2 x 10^5 FFU) was incubated with 25 µg/ml of MAbs for one hour at 37ºC. Virus-MAb complexes were added to Vero cells and infection proceeded for 24 hours. At each passage, half of the supernatant was mixed (1:1) with 50 µg/ml of the selection MAb for one hour at 37ºC. These complexes were

added to a new monolayer of Vero cells for 2 hours, and the procedure was repeated from 3 to 6 times depending on the selection MAb. Individual MAb-resistant viral plaques were picked and virus was grown in Vero cells in the presence of 10 µg/ml of MAb for 24 hours. RNA was isolated from cells using an RNeasy kit (Qiagen) and cDNA was made with random hexamers using the Superscript III Reverse Transcriptase kit (Invitrogen) and amplified by PCR with primers flanking the structural genes (**Table S4**). The PCR product was sequenced using ten overlapping primer sets (**Table S4**).

Mapping of mutations onto the CHIKV p62-E1 crystal structure. Figures were prepared using the atomic coordinates of CHIKV pE2-E1 (RCSB accession number 3N44) using the program CCP4MG[61].

Statistical analysis. For survival analysis, Kaplan-Meier survival curves were analyzed by the log-rank test. For growth kinetics and neutralization an unpaired T-test or analysis of variance was used to determine significance. These analyses were assessed using Prism software (GraphPad software). The protective effects of ch-CHK-152 versus ch-CHK-152 N297Q in wild type C57BL/6 mice were analyzed by the Kruskal-Wallace test with Bonferroni correction using the agricolae package of R (R Development Core Team, 2010. Foundation for Statistical Computing, Vienna, Austria).

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FIGURE LEGENDS

Figure 1. Profile of neutralizing MAbs against CHIKV. A. Examples of MAb neutralization as judged by a reduction in the number of FFU using the Biospot Macroanalyzer. Rows 2 to 12 going across represent decreasing (3-fold) concentrations of CHK-152 or the negative control DENV1-E98 MAb. Column 1 shows infection in the absence of MAb. **B.** Increasing concentrations of CHK-95, CHK-102, CHK-166, CHK-187, or CHK-263 were mixed with 100 to 150 FFU of CHIKV-LR for one hour at 37°C and Vero cells were infected. Neutralization was determined by FFU assay. **C-D**. CHK-152 (**C**) or CHK-9 (**D**) was mixed with CHIKV-LR (East, Central and South African genotype), CHIKV-RSUI (Asian genotype), or CHIKV IbH35 (West African genotype) for one hour at 37°C and Vero or NIH 3T3 cells were infected as indicated. Neutralization was determined by FFU assay. Data in this Figure is pooled from three independent experiments performed in duplicate or triplicate. All error bars represent the standard deviations.

Figure 2. **Efficacy of anti-CHIKV MAb prophylaxis**. **A**. Six to eight week-old *Ifnar*$^{-/-}$ C57BL/6 mice were passively transferred 100 µg of the indicated MAbs via an i.p. injection one day before infection with 10 FFU of CHIKV-LR via a s.c. route. The percentage and number of surviving mice were as follows: DENV1-E98 (0%, 0 of 9), CHK-88 (62.5%; 5 of 8), CHK-95 (12.5%; 1 of 8), CHK-98 (28.6%; 2 of 7), CHK-102 (100%; 8 of 8), CHK-124 (75%; 6 of 8), CHK-151 (87.5%; 7 of 8), CHK-152 (100%; 8 of 8), CHK-155 (85.7%; 6 of 7), CHK-165 (28.6%; 2 of 7), CHK-166 (100%; 8 of 8), CHK-175 (75%; 6 of 8), CHK-187 (50%; 4 of 8), CHK-263 (100%; 8 of 8), or CHK-266 (0%; 0 of 8). MAbs italicized in red in the Figure provided 100% protection. **B**. *Ifnar*$^{-/-}$ mice were passively transferred 10 µg of MAb via an i.p. injection one day before

infection with 10 FFU of CHIKV-LR via a s.c. route. The percentage and number of surviving mice were as follows: DENV1-E98 (0%; 0 of 7), CHK-102 (12.5%; 1 of 8), CHK-152 (83%; 10 of 12), CHK-166 (0%; 0 of 12), or CHK-263 (73%; 8 of 11). For (**A**) and (**B**) the survival curves were constructed from data of at least two independent experiments. All anti-CHK MAbs provided statistically significant protection in the percentage of surviving animals or mean survival time compared to the control DENV1-E98 MAb ($P < 0.05$). **C-G**. Viral burden in MAb-treated *Ifnar*$^{-/-}$ mice. Animals were passively transferred 100 µg of the indicated MAbs (CHK-102, CHK-152, CHK-166, CHK-263, or isotype control DENV1-E98) via an i.p. injection one day before infection with 10 FFU of CHIKV-LR via a s.c. route. Two days later, viremia (**C**) and tissues (**D**, spleen; **E**, liver; **F**, muscle; and **G**, brain) were harvested and infectious virus was titrated by focus-forming assay. Results are pooled from two independent experiments ($n = 4$ mice per group). The dashed line indicates the limit of detection of the assay and the solid bar indicates the median values. All viral burden results with CHK-102, CHK-152, CHK-166, and CHK-263 were statistically different ($P < 0.02$) from those obtained with DENV1-E98, as analyzed by the Mann-Whitney test. **H.** Four week-old female WT C57BL/6 mice were sham-treated or administered 100 µg of CHK-102 or CHK-152 via an i.p. route. 24 hours later, mice were infected with 100 PFU of CHIKV-SL 15649 and at day 10, virus-induced pathology in the foot and ankle joint was assessed. (*Outer left*) Sham-infected, (*middle left*) CHIKV infected and sham-treated, (*middle right*) CHIKV-infected and CHK-102 treated, and (*outer right*) CHIKV infected and CHK-152 treated. Shown are representative images after hematoxylin and eosin staining from at least 3

mice per group at 100x magnification. Yellow and green arrows indicate regions of inflammation or normal joints, respectively.

Figure 3. **Mechanism of neutralization by CHIKV MAbs. A.** Pre- and post-attachment inhibition assays. Vero cells were pre-chilled to 4ºC and 100 FFU of CHIKV-LR was added to each well for one hour. After extensive washing at 4^oC, the indicated MAbs were added for one hour at 4ºC, and then the FRNT protocol was completed (*black lines*, *Post*). In comparison, a standard pre-incubation FRNT with all steps performed at 4ºC is shown for reference. Virus and MAb are incubated together for one hour at 4^oC, prior to addition to cells (*red lines, Pre*). Data shown are representative of three experiments performed in duplicate with error bars representing standard deviation. **B-C**. FFWO assay. CHIKV was incubated with Vero cells at 4^oC to allow virus attachment. Free virus was removed after washing and 50 µg/ml of the indicated MAbs (including DENV1-E98, a negative control MAb) were added at 4^oC. Viral fusion at the plasma membrane was induced after a brief exposure to a low pH buffer. After pH normalization, cells were cultured for 14 hours in the presence of NH_4Cl to inhibit infection through the endosomal pathway. Cells were analyzed for infection by staining with an anti-E2 MAb. Representative histograms are shown (**B**) and the data was pooled from four independent experiments for statistical analysis (**C**). For simplicity of display, not all of the MAbs included in the summary graph are shown by flow cytometry analysis. Asterisks indicate values that are statistically different ($P < 0.05$) from the control MAb. Error bars represent standard deviations. Note low pH-triggered viral fusion at the plasma membrane is an inefficient process with only 10 to 20% of cells becoming infected even when a high MOI was used. **D-E.** Viral membrane fusion with liposomes. Fusion of

pyrene-labeled CHIKV was measured at pH 4.7 (37°C) using liposomes consisting of phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, and cholesterol in a molar ratio of (1/1/1/1.5), as described in the Methods. (**D**) Curve a, no MAb; curve b, 0.1 nM CHK-152; curve c, 1 nM CHK-152; curve d, 10 nM CHK-152. (**E**) Extent of fusion (average value between 50 to 60 seconds post acidification) at increasing concentrations of MAb. Black bars, CHK-152; white bar, isotype control (MAb 0031, only included at 10 nM concentration). All fusion measurements were performed at least three independent times.

Figure 4. **The effector functions of CHK-152 contribute to protection *in vivo*. A**. Comparison of binding of ch-CHK-152 and agylocsyl ch-CHK-152 N297Q to pE2-E1, as measured by surface plasmon resonance. A single representative sensogram is shown for each MAb. The experimental curves (*colored lines*) were fit using a 1:1 Langmuir analysis (*dashed lines*), after double referencing, to determine the kinetic parameters presented in the Table immediately below. **B**. Comparison of neutralizing activity of murine CHK-152, ch-CHK-152, and ch-CHK-152 N297Q, as measured by FRNT on Vero cells. **C**. Comparison of binding of ch-CHK-152 and ch-CHK-152 N297Q to FcγR (CD16A, 500 nM; CD32A, 100 nM; and CD64, 100 nM) or C1q (50 nM), as measured by surface plasmon resonance. **D**. Comparison of pre-exposure protective activity of ch-CHK-152 and ch-CHK-152 N297Q. *Ifnar*^{-/-} mice were administered via an i.p. injection 10 µg of ch-CHK-152 and ch-CHK-152 N297Q one day before infection with 10 FFU of CHIKV-LR via a s.c. route. Mice were monitored for survival for 21 days after infection. The survival curves were constructed from data of at least two independent experiments and the number of animals for each antibody ranged

from 8 to 10 per group. ch-CHK-152 provided statistically greater protection than ch-CHK-152 N297Q ($P < 0.05$). **E.** Five week-old WT C57BL/6 mice were infected with 100 PFU of CHIKV in the left rear footpad and either sham-treated, or treated with 100, 50, or 25 µg of ch-CHK-152 (*left panel*) or ch-CHK-152 N297Q (*right panel*) at 18 hours post infection. Mice were scored daily for virus-induced footpad swelling, where a score of 0 = no swelling, 1 = mild swelling where the top of the foot is slightly raised, 2 = moderate swelling with the entire top of foot raised, and 3 = severe swelling involving both the top and bottom of the foot. Scores are the mean values for 7 to 8 mice per treatment group and are representative of three independent experiments. Ch-CHK-152 mediated protection was significantly greater than ch-CHK-152 N297Q on days 7, 8, and 9 post infection for the 100 µg antibody dose, and at day 7 post infection for the 50 µg dose, as determined by the Kruskal-Wallace test with Bonferroni correction ($P < 0.05$). No statistically significant differences between ch-CHK-152 and ch-CHK-152 N297Q were observed with the 25 µg dose. Of note, we observed a reproducible decrease in clinical score on day 5 in many animals. This reflects the biphasic pattern of swelling: during the first 3 to 4 days, swelling is due to edema, whereas after day 5, it is due to inflammatory cell infiltration into the foot and ankle.

Figure 5. **Therapeutic efficacy of anti-CHIKV MAbs. A.** *Ifnar*^{-/-} mice were passively transferred via an i.p. injection 100 µg of DENV1-E98, CHK-102, CHK-152, CHK-166, or CHK-263 or 50 µg each of CHK-102 + CHK-152, CHK-166 + CHK-152, CHK-263 + CHK-152, or CHK-102 + CHK-263 at 24 hours after CHIKV infection. **B.** Five week-old WT C57BL/6 mice were infected with 100 PFU of CHIKV in the footpad and either sham-treated, or treated with 100 or 50 µg of CHK-152 at 18 hours post

infection. Virus induced pathology in the foot and ankle joint was assessed by histopathological analysis at day 10 post-infection. (*Left*) CHIKV-infected, sham-treated, (*middle*) CHIKV-infected, CHK-152 (100 µg) treated at + 18 hours, and (*right*) CHIKV-infected, CHK-152 (50 µg) treated at + 18 hours. Shown are representative images after hematoxylin and eosin staining from 3 mice per group at 100x magnification. Yellow and green arrows indicate regions of inflammation or normal joints, respectively. **C**. *Ifnar*^{-/-} mice were passively transferred via an i.p. injection 200 µg of DENV1-E98 or 100 µg each of CHK-102 + CHK-152, CHK-166 + CHK-152, or CHK-263 + CHK-152 at 48 hours after CHIKV infection. **D**. *Ifnar*^{-/-} mice were passively transferred via an i.p. injection 500 µg of DENV1-E98 or 250 µg each of CHK-102 + CHK-152 or CHK-166 + CHK-152 at 60 hours after CHIKV infection. For **A**, **C**, and **D** the survival curves were constructed from data of at least two independent experiments. The number of animals for each antibody ranged from 8 to 10 per group, with the exception of CHK-102 + CHK-263, which was performed with 7 mice only. Statistically significant differences in protection compared to DENV1-E98 are described in the text.

Figure 6. **Characterization and mapping of neutralization escape mutants. A-D**. FRNT assay with bulk virus obtained after three to six passages under selection of (**A**) CHK-102, (**B**) CHK-152, (**C**) CHK-166, or (**D**) CHK-263 on Vero cells. Bulk virus also was tested for infectivity in the presence of the non-selecting MAbs. Results are representative of two to three independent experiments performed in triplicate. **E-H**. Confirmation of resistant phenotype with SFV-CHIKV-GFP containing the indicated single engineered point mutations. Serial dilutions of MAb were incubated with chimeric SFV-CHIKV virus (WT or mutant stocks) for one hour at room temperature. MAb-virus

complexes were added to Vero cells plated in 96-well plates and incubated at 37°C. After 8 hours cells were trypsinized, fixed, and the number of GFP-positive, infected cells was assessed by flow cytometry. Curves are representative of 2 to 3 independent experiments. **I**. Epitope mapping of anti-CHIKV MAbs on the crystal structure of the mature envelope glycoprotein complex (PDB code 3N44). (*Left*) The domains on E2 (cyan) and E1 (gold) are indicated, and the fusion loop on E1 (E1 FL) is delineated. Amino acid residues of neutralizing MAbs were determined by escape selection, sequencing, and reverse genetic confirmation. CHK-102 and CHK-263 recognize the B domain on E2, CHK-152 recognizes a residue on the wings of the A domain on E2, and CHK-166 recognizes an amino acid in domain II of E1 proximal to the conserved fusion loop. (*Right*) The mature envelope glycoprotein docked onto the trimer conformation (PDB code 2XFB) that is present on the virion. E3, E2, and E1 and the escape residues are colored as in the left panel. Neutralization escape residues are readily accessible on the top of the trimer, distal to the viral membrane.

Table 1. Inhibitory activity of neutralizing anti-CHIKV MAbs

MAb	CHIKV-LR EC50 ng/ml (CI) Vero	CHIKV-LR EC90 ng/ml (CI) Vero	CHIKV-LR EC50 ng/ml (CI) 3T3	CHIKV-RSU1 EC50 ng/ml (CI) Vero	CHIKV-RSU1 EC90 ng/ml (CI) Vero	CHIKV-IbH35 EC50 ng/ml (CI) Vero	CHIKV-IbH35 EC90 ng/ml (CI) Vero
CHK-9	36 (31-43)	882 (612-1271)	37 (27-51)	3712 (2636 - 5229)	**>10,000**	68 (54-87)	1478 (885-2469)
CHK-11	1356 (1049-1753)	**>10,000**	4680 (3691-5932)	215 (150-308)	**>10,000**	1141 (570-2284)	>10,000
CHK-48	16 (14-20)	430 (304-607)	32 (17-61)	**8** (7-11)	220 (121-399)	**7** (5-9)	359 (193-669)
CHK-65	**7** (6-9)	587 (330-1047)	25 (15-43)	**5** (4-6)	265 (164-431)	**4** (3-5)	147 (80-271)
CHK-77	48 (41-56)	576 (407-817)	91 (53-155)	17 (14-20)	368 (250-543)	27 (22-32)	444 (291-677)
CHK-88	**5** (3-6)	422 (230-776)	12 (7-21)	**2** (2-3)	190 (106-343)	**2** (2-3)	211 (104-431)
CHK-95	**4** (3-5)	156 (90-271)	**6** (4-10)	**6** (5-8)	411 (246-686)	**2** (1-3)	176 (76-404)
CHK-96	40 (33-50)	8457 (5115-13982)	**>10,000**	95 (65 - 139)	**>10,000**	19 (14-26)	4486 (2126-9466)
CHK-98	155 (130-183)	5337 (3630-7846)	394 (220-706)	21 (16-27)	2022 (950-4304)	103 (80-131)	7923 (4494-13968)
CHK-102	14 (11-17)	351 (227-543)	30 (17-53)	**5** (4-7)	1318 (532-3261)	**6** (5-7)	104 (68-161)
CHK-105	19 (16-23)	1156 (773-1728)	94 (59-150)	11 (9-14)	927 (563-1528)	11 (9-13)	645 (433-960)
CHK-112	11 (9-13)	235 (162-339)	12 (8-19)	**8** (6-11)	2116 (1072-4174)	**4** (3-5)	115 (76-176)
CHK-124	**4** (4-6)	101 (66-	**10** (5-22)	**3** (2-4)	228 (129-	**3** (2-3)	96 (55-167)

		156)			402)		
CHK-140	9 (8-11)	219 (137-348)	56 (32-98)	8 (6-11)	721 (345-1508)	5 (4-6)	83 (55-125)
CHK-142	9 (7-11)	369 (238-572)	32 (21-48)	7 (5-10)	731 (321-1668)	4 (3-5)	108 (66-177)
CHK-143	34 (23-51)	>10,000	277 (76-1010)	6 (5-9)	3456 (1332-8965)	12 (7-23)	3094 (728-13143)
CHK-151	6883 (3467-13665)	>10,000	>10,000	261(154-440)	>10,000	5784 (2785-12015)	>10,000
CHK-152	2 (2-2)	10 (9-12)	1 (1-2)	3 (2-3)	15 (11-22)	1 (1-2)	6 (5-8)
CHK-155	5 (5-7)	110 (73-166)	7 (6-8)	4 (4-5)	93 (61-142)	2 (2-3)	52 (32-86)
CHK-164	3523 (2904-4274)	>10,000	4395 (3045-6342)	1637 (1182-2265)	>10,000	2366 (1582-3538)	>10,000
CHK-165	9725 (6275-15070)	>10,000	>10,000	1817 (111-2972)	>10,000	>10,000	>10,000
CHK-166	154 (116-205)	8604 (4459-16604)	202 (98-418)	40 (30-52)	2175 (1195-3959)	82 (59-114)	2576 (1234-5379)
CHK-175	6 (5-8)	423 (285-626)	5 (3-10)	3 (3-4)	343 (199-593)	4 (3-5)	662 (295-1489)
CHK-176	806 (616 - 1055)	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
CHK-180	140 (104-186)	7615 (3907-14843)	168 (74-384)	49 (38-63)	2588 (1465-4570)	79 (58-108)	1597 (802-3180)
CHK-187	11 (9-12)	524 (355-772)	17 (8-34)	7 (6-8)	140 (97-203)	5 (4-7)	186 (91-383)
CHK-189	4325 (3172-5897)	>10,000	3974 (2119-7451)	951 (589-1538)	>10,000	3809 (1856-7816)	>10,000
CHK-262	4934 (3938-6181)	>10,000	3740 (2488-5623)	4622 (3376-6328)	>10,000	3306 (2049-5336)	>10,000
CHK-263	5 (5-7)	136 (93-197)	2 (2-4)	4 (3-5)	48 (32-73)	2 (2-2)	51 (36-74)

CHK-264	18 (13-23)	**>10,000**	**6** (4-10)	**9** (7-14)	1019 (430-2419)	**11** (7-16)	5962 (2099-16932)
CHK-265	**8** (7-9)	207 (148-289)	**6** (4-8)	**5** (4-6)	143 (88-234)	**5** (4-6)	201 (103-389)
CHK-266	2187 (1642-2913)	**>10,000**	1500 (1093-2059)	**>10,000**	**>10,000**	1330 (814-2173)	**>10,000**
CHK-267	18 (14-23)	1063 (628-1799)	**3** (3-5)	**9** (7-11)	452 (262-780)	14 (6-34)	3694 (468-29151)
CHK-268	24 (19-30)	934 (567-1540)	10 (7-14)	22 (17-29)	1212 (641-2289)	10 (8-13)	468 (278-789)
CHK-269	136 (104-178)	**>10,000**	32 (18-57)	50 (33-76)	**>10,000**	47 (33-69)	3834 (1617-9090)
CHK-270	14 (10-21)	9302 (3777-22912)	N.D.	14 (10-18)	669 (340-1317)	**8** (6-11)	748 (363-1537)

Neutralizing activity was determined by FRNT on Vero or NIH 3T3 cells with increasing concentrations of purified MAbs and 100 FFU of the indicated CHIKV strains corresponding to different genotypes (CHIKV-LR, East, Central, and South African; CHIKV-RSUI, Asian and, IbH35, West African). The data were derived from three independent experiments performed in duplicate or triplicate. The inhibitory concentrations of MAb that reduced infected foci by 50% (EC50) and 90% (EC90) were calculated by nonlinear regression analysis and are expressed as ng/ml of antibody. In parenthesis, immediately below the EC50 and EC90 values, are confidence intervals (CI). Bold red indicates that the EC50 or EC90 value was greater than the highest concentration (10,000 ng/ml) of MAb used. Bold blue indicates an EC50 or EC90 value of less than 10 ng/ml, which reflects a highly neutralizing MAb for a given cell type or

virus strain. N.D. indicates not determined. Of note, NIH 3T3 cells are less permissive than Vero cells (1 FFU on NIH 3T3 cells = 35 FFU on Vero cells).

Table 2. *In vitro* selection of viruses resistant to MAb neutralization

MAb	Mutation[a]	# of plaque picks
CHK-102	E2: L210P	6 of 8
CHK-102	E2: G209E	2 of 8
CHK-152	E2: D59N	9 of 9
CHK-152	E2: A89E[b]	2 of 9
CHK-166	E1: K61T	14 of 14
CHK-263	E2: K215E	3 of 4
CHK-263	E2: G209E	1 of 4

[a]*In vitro* selection for neutralization escape variants was performed by passaging CHIKV-LR in the presence of 25 µg/ml of the indicated MAbs. Resistant virus was isolated at passage 3 (CHK-102, CHK-152, and CHK-263) or passage 6 (CHK-166), plaque purified, and sequenced.

[b]The A89E mutant was identified after sequencing of CHK-152 escape mutants in cell culture, but was determined to be insignificant for CHK-152 neutralization by reverse genetic analysis (see **Fig 6F).**

Table 3. *In vivo* selection of viruses resistant to MAb neutralization

MAb	Condition	EC50 (ng/ml) (Parent➔mutant)	Mutation[a]
CHK-102	- 24 h, 10 µg	11➔ >10,000	E2: L210P
CHK-102	+ 24 h, 100 µg	11➔ >10,000	E2: G209E
CHK-152	-24 h, 10 µg	2➔ 10,000	E2: N231D E2: K233E
CHK-152	-24 h, 10 µg	2➔ 3,000	E2: K233E
CHK-152	+ 24 h, 100 µg	2➔ >10,000	E2: D59N
CHK-152	+ 24 h, 100 µg	2➔ >10,000	E2: K233T
CHK-166	+ 24 h, 100 µg	170➔>10,000	E1: G64S
CHK-263	+ 24 h, 100 µg	5➔ >10,000	E2: G209E
CHK-166 + CHK-152	+48 h, 250 µg	CHK-166: 170➔ 540 CHK-152: 2➔ 2.6	E2: N332I

[a]*In vivo* selection for resistant virus was performed by administering the indicated individual or combinations of MAbs before (-24 hours) or after (+ 24 or 48 hours) CHIKV-LR infection. Resistant virus was isolated directly from tissues (leg and brain), and cDNA was prepared by reverse transcription and sequenced. The change in neutralizing activity of the bulk virus recovered from tissue is highlighted by the differences in EC50 values.

Figure 1

a Pre-exposure treatment, 100 µg MAb

b Pre-exposure treatment, 10 µg MAb

c

Sham-infected   CHKV-infected   CHKV-infected + CHK-102   CHKV-infected + CHK-152

Figure 2

Figure 3

Figure 4

a

MAb	k-assoc 1/Ms	k-dissoc 1/s	K_D nM
ch-CHK-152	1.3×10^5	4.0×10^{-4}	3.1
ch-CHK152 (N297Q)	1.3×10^5	5.1×10^{-4}	3.9

a Therapy, + 24 hours, 100 μg

c Therapy, + 48 hours, 200 μg

d Therapy. + 60 hours, 500 μg

- CHK-102+ CHK-152 (50 μg + 50 μg)
- CHK-166 + CHK-152 (50 μg + 50 μg)
- CHK-263 +CHK-152 (50 μg + 50 μg)
- CHK-166 (100 μg)
- CHK-152 (100 μg)
- CHK-263 (100 μg)
- CHK-102 (100 μg)
- DENV1-E98 (100 μg)

- CHK-102 + CHK-152 (100 μg + 100 μg)
- CHK-263 + CHK-152 (100 μg + 100 μg)
- CHK-166 + CHK-152 (100 μg + 100 μg)
- DENV1-E98 (200 μg)

- CHK-102 + CHK-152 (250 μg + 250 μg)
- CHK-166 + CHK-152 (250 μg + 250 μg)
- DENV1-E98, 500 μg

b

No treatment   CHK-152, 100 μg, +18h   CHK-152, 50 μg, +18h

Figure 5

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Figure 6

SUPPORTING INFORMATION

Figure S1. Screening of hybridoma supernatants for binding to CHIKV-infected cells.

Figure S2. Binding kinetics of CHK-MAbs to pE2-E1.

Figure S3. Pre- and post-attachment neutralization assays.

Figure S4. Construction and efficacy of humanized CHK-152.

Figure S5. Interaction of neutralizing MAbs.

Figure S6. Selection of escape E1-G64S escape mutant in vivo against CHK-   166.

Figure S7. Confirmation of neutralization escape mutants selected *in vivo*.

Figure S8. Relative resistance of CHIKV recovered from mice after treatment with combination MAb therapy.

Table S1. List of anti-CHIKV MAbs.

Table S2. Cross-neutralization of infection by wild type and mutant SFV-CHIKV infection with anti-CHIKV MAbs.

Table S3. List of V_H and V_L sequences of CHK-102, CHK-152, CHK-166, and CHK-263 mouse MAbs.

Table S4. Primers used for sequencing and amplifying the structural genes of CHIKV-LR 2006-OPY1.

Text S1. Supplemental Methods.

Figure S1. Screening of hybridoma supernatants for binding to CHIKV-infected cells. Hybridoma supernatants were incubated with CHIKV-GFP infected BHK21 cells and tested for immunoreactivity by flow cytometry. Shown are examples of a negative control MAb (DENV3-E2), three 'hits' (later named as CHK-102, CHK-117, and CHK-130), and a negative supernatant (5E3). The y-axis shows GFP staining associated with the reporter gene that is translated from the subgenomic promoter of CHIKV, and the x-axis shows staining of the tested mouse MAb. Double-positive cells were considered 'hits' in the screen. The result is representative of many different MAbs performed in the original screen.

Figure S2. Binding kinetics of CHK-MAbs to pE2-E1. Binding curves and kinetic parameters of pE2-E1 binding to mouse CHK-102, CHK-152, CHK-166, and CHK-263 MAbs. A single representative sensogram is shown for each MAb. The experimental curves (*colored lines*) were fit using a 1:1 Langmuir analysis (*dashed lines*), after double referencing, to determine the kinetic parameters presented immediately below.

Figure S3. Pre- and post-attachment neutralization assays. Vero cells were pre-chilled to 4ºC and 100 FFU of CHIKV-LR was added to each well for one hour at 4 ºC. After extensive washing at 4ºC, the indicated MAbs (CHK-48, CHK-65, CHK-95, CHK-112, CHK-124, CHK-142, CHK-155, CHK-175, CHK-84 and DENV1-E98) were added for one hour at 4ºC, and then the FRNT protocol was completed (*black lines*, *Post*). In comparison, a standard pre-incubation FRNT with all steps performed at 4ºC is shown for reference. Virus and MAb are incubated together for one hour at 4ºC, prior to

addition to cells (*red lines, Pre*). Data shown are representative of three experiments performed in duplicate with error bars representing standard deviation.

Figure S4. Construction and efficacy of humanized CHK-152. We amplified the cDNA encoding the heavy (VH) and light (VL) variable domains from the hybridoma cellular RNA and grafted the complementarity determining regions onto the human VH1-18 and human Vκ-L6 backbones. The resulting humanized VH and VL were combined with human γ1 and κ constant regions, fused to an IgG signal sequence, expressed in 293T cells and purified (data not shown). **A.** Binding curves and kinetic parameters of pE2-E1 binding to mouse CHK-152 and hu-CHK-152. A single representative sensogram is shown for each MAb. The experimental curves (*colored lines*) were fit using a 1:1 Langmuir analysis (*dashed lines*), after double referencing, to determine the kinetic parameters presented in the Table immediately below. **B**. Neutralization studies with mouse CHK-152 and hu-CHK-152. Neutralizing activity was determined by FRNT assay on Vero cells. Samples were performed in duplicate and the experiment is one representative of three. **C.** Pre-exposure protective activity of hu-CHK-152. *Ifnar*^{-/-} mice were passively transferred via an i.p. injection 10 or 100 µg of mouse hu-CHK-152 one day before CHIKV infection. Mice were monitored for survival for 21 days after infection. The survival curves were constructed from data of at least two independent experiments and the number of animals for each antibody ranged from 8 to 10 per group.

Figure S5. Interaction of neutralizing MAbs. A. Virion capture ELISA and competition of MAb binding. 96-well plates were coated with 5 µg/ml of CHK-65 MAb. Plates were washed, blocked and 3×10^6 FFU of CHIKV 181-25 was captured. Subsequently, plates were incubated with the indicated anti-CHK mouse MAbs (CHK-

102, CHK-152, CHK-166, or CHK-263) or controls (no MAb, PBS; irrelevant MAb, WNV E28) for one hour. After washing, plates were incubated sequentially with 125 ng/ml hu-CHK-152 and biotin-labeled goat anti-human secondary antibody. After washing and incubation with HRP-conjugated streptavidin, plates were developed and emission (450 nm) was read using an iMark microplate reader (Bio-Rad). Results are representative of three independent experiments, each performed in triplicate. **B**. Neutralizing activity of MAb combinations. Increasing concentrations of individual MAbs (CHK-102, CHK-152, CHK-166, and CHK-263) or combinations of MAbs (CHK-102 + CHK-152, CHK 102 + CHK-263, CHK-152 + CHK-166, or CHK-152 + CHK-263) were mixed with 100 FFU of CHIKV-LR for one 1 hour at 37°C and Vero cells were infected. Neutralization was determined by FFU assay. Data is representative of three independent experiments performed in duplicate.

Figure S6. Selection of escape E1-G64S escape mutant *in vivo* against CHK-166. *Ifnar*^{-/-} mice were infected with CHIKV and 24 hours later given a single 100 µg dose of CHK-166 therapy. Virus was recovered from the contralateral leg and brain from one moribund mice and the structural genes were sequenced. All 2 of 2 viral isolates recovered showed a single point G64S mutation in the E1 gene. This isolate was tested subsequently for neutralization by CHK-102 (EC50 of 161 ng/ml), CHK-152 (EC50 of 2 ng/ml), CHK-166 (EC50 > 10,000 ng/ml) and CHK-263 (25 ng/ml). Data is the average of two independent experiments performed in triplicate.

Figure S7. Confirmation of neutralization escape mutants selected *in vivo*. Confirmation of resistant phenotype selected with CHK-152 *in vivo* using SFV-CHIKV-GFP containing the indicated single engineered point mutations. Serial dilutions of CHK-

152, CHK-102, and CHK-263 were incubated with chimeric SFV-CHIKV virus (WT or mutant stocks) for one hour at room temperature. MAb-virus complexes were added to Vero cells plated in 96-well plates and incubated at 37°C. After 8 hours cells were trypsinized, fixed, and the number of GFP-positive, infected cells was assessed by flow cytometry. Curves are representative of 2 independent experiments.

Figure S8. Relative resistance of CHIKV recovered from mice after treatment with combination MAb therapy. *Ifnar*$^{-/-}$ mice were infected with CHIKV and 48 hours later given a single dose of combination MAb (CHK-102 + CHK-152 or CHK-166 + CHK-152) therapy. Virus was recovered from the contralateral leg and/or brain from the few moribund mice and the structural genes were sequenced. Two viral isolates showed differences in neutralization patterns that corresponded to amino acid substitutions (see **Table 2**). Neutralization analysis of these viruses recovered from animals treated with (*left*) CHK-102 and CHK-152 or (*right*) CHK-166 and CHK-152 and tested against the respective MAbs. A comparison with the parent virus is shown. The curves are representative of two independent experiments performed in triplicate, and error bars indicate standard deviations.

Table S1. List of anti-CHIKV MAbs. All MAbs listed were cloned successfully by limiting dilution. Isotypes were assigned based on a commercial assay. MAbs CHK-1 to CHK-51 were produced from mice receiving a final boost with CHIKV VLP; MAbs CHK-52 to CHK-145 were produced from mice receiving a final boost with recombinant E2 protein; and MAbs CHK-146 to CHK-270 were produced from mice receiving a final boost with either recombinant E2 protein or infectious CHIKV-LR. Binding to soluble E2 or pE2-E1 expressed in bacteria or mammalian cells was determined by ELISA. N.D.

indicates not determined. Yes, reflects binding that yielded an O.D. value of > 1.1; Weak, indicates binding with an O.D. value of > 0.8 and < 1.1; Background binding (irrelevant MAb control) had an O.D. value of 0.2.

Table S2. Cross-neutralization of infection by wild type and mutant SFV-CHIKV infection with anti-CHIKV MAbs. CHIKV escape variants were selected in the presence of the indicated neutralizing MAbs. After sequencing of escape variants, the indicated amino acid substitutions were engineered into an infectious SFV-GFP-CHIKV chimeric virus for analysis of resistance or sensitivity to neutralization. Viruses denoted as "resistant" were not neutralized appreciably by the indicated MAbs, whereas viruses marked "sensitive" showed no greater than a 2-fold difference in EC50 values compared to the parent virus. Results are from two to four independent dose-response experiments performed in duplicate with nine serial dilutions of each MAb.

Table S3. List of V_H and V_L nucleotide and corresponding amino acid sequences of CHK-102, CHK-152, CHK-166, and CHK-263 mouse MAbs.

Table S4. Primers used for sequencing and amplifying the structural genes of CHIKV-LR 2006-OPY1.

SUPPLEMENTAL METHODS

Humanization of CHK-152. To construct a humanized version of CHK-152, a cDNA encoding the CDR from the V_H and V_L variable domains were amplified from hybridoma cell RNA by a 5' RACE procedure and grafted onto the homologous human V_H (1-18) and Vκ (L-6) backbones. The resulting humanized V_H and V_L were combined with human γ1 and κ constant regions, fused to an IgG signal sequence and inserted into a pCI-neo cassette to construct the heavy and light chain expression plasmids.

Competition ELISA Polystyrene 96-well plates were coated overnight at 4°C with 5 µg/ml of CHK-65 MAb in sodium carbonate buffer (pH 9.3). Plates were washed three times in PBS with 0.02% Tween 20 and blocked for one hour at 37°C with PBS, 2% BSA, and 0.02% Tween 20. 3 X 10^6 FFU of CHIKV 181-25 (gift of R. Tesh, Galveston, TX) was captured, and plates were washed four times with PBS with 1% BSA and incubated with murine MAbs for one hour. After washing, plates were incubated with 125 ng/ml hu-CHK-152 in PBS with 1% BSA. Plates were washed and biotin-labeled goat anti-human secondary antibody (Jackson Labs) was added for one hour. After washing and incubation with HRP-conjugated streptavidin (Vector Laboratories), plates were developed with tetramethylbenzidine substrate (Dako). The reaction was stopped with the addition of 2 N H_2SO_4, and emission (450 nm) was read using an iMark microplate reader (Bio-Rad).

Table S1. List of anti-CHIKV MAbs.

MAb[a]	Isotype	E2[b]	MAb	Isotype	E2	MAb	Isotype	E2	MAb	Isotype	E2
CHK-1	IgG2c	No	CHK-59	IgG2c	Yes	CHK-127	IgG1	Yes	CHK-206	IgG1	No
CHK-2	IgG2c	No	CHK-60	IgG2c	Yes	CHK-129	IgG2c	Yes	CHK-207	N.D.	N.D.
CHK-3	IgG2c	weak	CHK-61	IgG2c	Yes	CHK-130	IgG2b	Yes	CHK-208	IgM	No
CHK-4	IgG2c	Yes	CHK-62	IgG2c	Yes	CHK-131	IgG2c	Yes	CHK-209	N.D.	N.D.
CHK-5	IgG2b	Yes	CHK-63	IgG2b	Yes	CHK-133	IgG2b/c	Yes	CHK-210	N.D.	N.D.
CHK-6	IgG2c	Yes	CHK-64	IgG2c	Yes	CHK-134	IgG1	Yes	CHK-211	N.D.	N.D.
CHK-7	IgG3	weak	CHK-65	IgG2c	Yes	CHK-135	IgG2c	Yes	CHK-212	N.D.	N.D.
CHK-8	IgG2c	No	CHK-66	IgG2c	Yes	CHK-138	IgG2c	Yes	CHK-213	IgG2c	No
CHK-9	IgG2c	Yes	CHK-67	IgG2c	Yes	CHK-140	IgG2c	Yes	CHK-214	N.D.	N.D.
CHK-10	IgG2c	No	CHK-68	IgG2c	Yes	CHK-141	IgG2c	Yes	CHK-215	IgM/IgG2b	No
CHK-11	IgG2c	Yes	CHK-69.A	IgG1	Yes	CHK-142	IgG2c	Yes	CHK-216	N.D.	N.D.
CHK-12	IgG2c	No	CHK-69.B	IgG2c	Yes	CHK-143	IgG3	No	CHK-217	IgM	No
CHK-13	IgG1	Yes	CHK-70	IgG2c	Yes	CHK-144	IgG2c	Yes	CHK-218	N.D.	Yes
CHK-14	IgG2c	Yes	CHK-71	IgG2c	Yes	CHK-145	IgG2c	Yes	CHK-219	N.D.	N.D.
CHK-15	IgM	No	CHK-72	IgG2c	Yes	CHK-147	IgG2b	Yes	CHK-220	N.D.	N.D.

CHK-16	N.D.	N.D	CHK-73	IgG2c	Yes	CHK-148	IgG2c	Yes.	CHK-222	IgG1	No
CHK-17	IgG2c	Yes	CHK-74	IgG2c	Yes	CHK-149	IgG2c	Yes	CHK-223	IgM	No
CHK-18	IgG2c	No	CHK-75	IgG1	Yes	CHK-150	IgG2b	Yes	CHK-224	N.D.	N.D.
CHK-19	IgG2c	No	CHK-76	IgG1	Yes	CHK-151	IgG2c	Yes	CHK-225	IgM	No
CHK-20	N.D.	N.D	CHK-77	IgG2c	Yes	CHK-152	IgG2c	No	CHK-226	IgM	No
CHK-21	IgM	No	CHK-78	IgG2b	Yes	CHK-153	N.D.	N.D.	CHK-227	N.D.	N.D.
CHK-22	IgG2b	No	CHK-79	IgM/IgG1	Yes	CHK-154	N.D.	N.D.	CHK-228	N.D.	N.D.
CHK-23	N.D	N.D	CHK-81	IgG2c	Yes	CHK-155	IgG2c	Yes	CHK-229	IgG1/IgM	No
CHK-24	IgG1/IgM	No	CHK-82	IgG2c	Yes	CHK-156	IgG2b	Yes	CHK-230	IgG1	No
CHK-25	IgG2c	Yes	CHK-83	IgG2c	Yes	CHK-157	IgG2b	Yes	CHK-231	IgG2c	No
CHK-26	N.D	N.D	CHK-84	IgG2c	Yes	CHK-158	IgG2c	Yes	CHK-232	IgM	No
CHK-27	N.D	No	CHK-85	IgG2c	Yes	CHK-159	IgG2c	Yes	CHK-233	IgM	No
CHK-28	N.D	No	CHK-86	IgG2c	Yes	CHK-160	IgG2c	Yes	CHK-234	IgG1	No
CHK-29	N.D	No	CHK-87	IgG1	Yes	CHK-161	IgG2c	Yes	CHK-235	IgG1	No
CHK-30	IgM	No	CHK-88	IgG2c	Yes	CHK-162	IgG2c	Yes	CHK-237	IgM	No
CHK-31	N.D	No	CHK-89	IgG2b	Yes	CHK-163	IgG2c	Yes	CHK-238	IgM	No
CHK-	N.D	No	CHK-	IgM	No	CHK-	IgG2c	Yes	CHK-	IgM	weak

32			90			164			239		
CHK-33	IgG2c	No	CHK-91	IgG2c	Yes	CHK-165	IgG2c	Yes	CHK-240	IgM	No
CHK-34	IgM	No	CHK-92	IgG2c	No	CHK-166	IgG2c	No	CHK-241	N.D.	N.D.
CHK-35	N.D	No	CHK-94	IgG2c	Yes	CHK-168	IgG2c	Yes	CHK-242	N.D.	N.D.
CHK-36	N.D	Yes	CHK-95	IgG2c	Yes	CHK-169	IgG2c	Yes	CHK-243	N.D.	Yes
CHK-37	N.D	No	CHK-96	IgG2c	Yes	CHK-170	IgG1	No	CHK-244	IgG2c	Yes
CHK-38	IgG2c	No	CHK-97	IgG2c	Yes	CHK-172	IgG2c	Yes	CHK-245	N.D.	weak
CHK-39	N.D	N.D	CHK-98	IgG2c	Yes	CHK-173	IgG2c	Yes	CHK-246	IgG2c	Yes
CHK-40	IgG3	No	CHK-99	IgG2c	Yes	CHK-174	IgG2b	Yes	CHK-247	IgG2c	Yes
CHK-41	N.D	N.D	CHK-101	IgG2b	Yes	CHK-175	IgG2c	Yes	CHK-248	N.D.	N.D.
CHK-42	N.D	N.D	CHK-102	IgG2c	Yes	CHK-176	IgG2c	No	CHK-249	N.D.	N.D.
CHK-43	N.D	N.D	CHK-103	IgM	No	CHK-177	IgG2c	Yes	CHK-250	N.D.	N.D.
CHK-44	N.D	N.D	CHK-104	IgG2c	Yes	CHK-178	IgG2c	Yes	CHK-251	IgG2c	Yes
CHK-45	N.D	N.D	CHK-105	IgG1	Yes	CHK-179	IgG2c	Yes	CHK-256	IgM	No
CHK-46	IgG3	No	CHK-106	IgG2c	Yes	CHK-180	IgG2c	Yes	CHK-261	N.D.	No
CHK-47	N.D	N.D	CHK-107	IgG2c	Yes	CHK-181	IgG1	Yes	CHK-262	IgG2c	Yes
CHK-48	IgG2c	Yes	CHK-108	N.D.	No	CHK-182	N.D.	No	CHK-263	IgG2c	Yes

CHK-49	N.D	No	CHK-109	IgG1	Yes	CHK-186	IgG2c	Yes	CHK-264	IgG2c	Yes
CHK-50	N.D	N.D	CHK-110	IgG2c	Yes	CHK-187	IgG2c	Yes	CHK-265	IgG2c	Yes
CHK-51	N.D	N.D	CHK-112	IgG2c	Yes	CHK-188	IgG2c	Yes	CHK-266	IgG2c/IgG1	Yes
CHK-52	IgG1	No	CHK-114	IgG2c	No	CHK-189	IgG2c	Yes	CHK-267	IgG2c	Yes
CHK-53	IgG2c	Yes	CHK-117	IgG2c	Yes	CHK-190	IgG2c	Yes	CHK-268	IgG2c	Yes
CHK-54	IgG2c	Yes	CHK-119	IgG2c	Yes	CHK-191	IgG2c	Yes	CHK-269	IgG2b	No
CHK-55	IgG2c	Yes	CHK-122	IgM	No	CHK-193	IgG2c	Yes	CHK-270	IgG2c	Yes
CHK-56	IgG2c	Yes	CHK-124	IgG2c	Yes	CHK-201	IgG1	Yes			
CHK-57	IgG2c	Yes	CHK-125	N.D.	No	CHK-204	IgG2b	Yes			
CHK-58	IgG2c	Yes	CHK-126	N.D.	No	CHK-205	IgG2b	No			

Table S2. Cross-neutralization of infection by wild type and mutant SFV-CHIKV infection with anti-CHIKV MAbs

Selecting MAb	CHIKV variant	Test MAb	Test MAb	Test MAb	Test MAb
		CHK-102	**CHK-152**	**CHK-166**	**CHK-263**
CHK-102	E2 L210P	**Resistant**	Sensitive	N.D.	**Resistant**
CHK-102	E2 G209E	**Resistant**	Sensitive	N.D.	**Resistant**
CHK-152	E2 D59N	Sensitive	**Resistant**	N.D.	Sensitive
CHK-152	E2 A89E	Sensitive	Sensitive	N.D.	Sensitive
CHK-166	E1 K61T	N.D.	N.D.	**Resistant**	N.D.
CHK-166	E1 P74A	N.D.	N.D.	Sensitive	N.D.
CHK-263	E2 K215E	**Resistant**	Sensitive	N.D.	**Resistant**

Table S3. V$_H$ and V$_L$ nucleotide and protein sequences of protective anti-CHIKV MAbs

A. CHK-102

CHK-102-V$_H$

<u>atgggatggagctgtatcatgttcttcctcctgtcaggaactgcaggtgtccaatcc</u>CAGGTTCAGCTGCAGCAG
TCTGGGGCTGAGCTGGTGAAGCCTGGGGCCTCAGTGAAGATTTCCTGCAAAA
CTTCTGGCTACGCATTCAGTAGTTTCTGGATGCACTGGGTGAAGCAGAGGCCT
GGAAAGGGTCTTGAGTGGATTGGACAGATTTATCCTGGAGATGGTGATACTA
ACTATAACGGAAAGTTCAAGGACAAGGCCACACTGACTGCAGACAAATCCTC
CAACACAGCCTACATGCAGCTCACCAGCCTGACCTCTGAGGACTCTGCGGTC
TATTTCTGTGCAAGAAACTTACTTTTTGACTACTGGGGCCAAGGCACCACTCT
CACAGTCTCCTCA

<u>MGWSCIMFFLLSGTAGVQS</u>QVQLQQSGAELVKPGASVKISCKTSGYAFSSFWMH
WVKQRPGKGLEWIGQIYPGDGDTNYNGKFKDKATLTADKSSNTAYMQLTSLTS
EDSAVYFCARNLLFDYWGQGTTLTVSS

CHK-102-V$_L$

GAGATCCTGATGACTCAGTCTCCAGCCATCCTGTCTGTGAGTCCAGGAGAAA
GAGTCAGTTTCTCCTGCAGGGCCAGTCAGAGCATTGGCTCAAACATACACTG
GTATCAGCAAAGAACAAATGGTTCTCCAAGGCTTCTCATAAAGTATGCCTCT
GAGTCTATCTCTGGGATCCCTTCCAGGTTTAGTGGCAGTGGGTCAGGGACAG
ATTTTACTCTTAGCATCAACAGTGTGGAGTCTGAAGATATTGCAGATTATTAC
TGTCAACAGAATAATATCTGGCCATTCACGTTCGGCTCGGGGACAAAGTTGG
AAATAAAG

EILMTQSPAILSVSPGERVSFSCRASQSIGSNIHWYQQRTNGSPRLLIKYASESISGI
PSRFSGSGSGTDFTLSINSVESEDIADYYCQQNNIWPFTFGSGTKLEIK

B. CHK-152

CHK-152-V$_H$

<u>atgggatggagctgtatcatcctcattttggtagcagcagctacaggtgtccactcc</u>CAGGTCCAGCTGCAGCAG
CCTGGGGCTGCGCTTGTGAAGCCTGGGGCTTCAGCGATGATGTCCTGCAAGG
CTTCTGGCTACACCTTCACCAGCTACTGGATAACCTGGGTGAAGCAGAGGCC

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TGGACAAGGCCTTGAATGGATTGGGGACATTTACCCTGGTACTGGTCGAACT
ATCTACAAGGAGAAGTTCAAGACCAAGGCCACACTGACTGTAGACACATCCT
CCAGCACAGCCTTCATGCAGCTCAACAGCCTGACATCTGAGGATTCAGCGGT
CTATTACTGTGCAAGAGGCTACGGTAGTCCTTACTATGCTTTGGACTACTGGG
GTCAAGGAACCTCAGTCACCGTCTCCTCA

MGWSCIILILVAAATGVHSQVQLQQPGAALVKPGASAMMSCKASGYTFTSYWIT
WVKQRPGQGLEWIGDIYPGTGRTIYKEKFKTKATLTVDTSSSTAFMQLNSLTSED
SAVYYCARGYGSPYYALDYWGQGTSVTVSS

CHK-152-V$_L$

atggagacagacacaatcctgctatgggtgctgctgctctgggttccaggctccactggtGACATTGTGCTGACCC
AATCTCCAGCTTCTTTGGCTGTGTCTCAAGGGCAGAGGGCCACCATCTCCTGC
AAGGCCAGCCAAAGTGTTGATTATGATGGTGATAGTTATGTGAACTGGTACC
AACAGAAACCAGGACAGTCACCCAAACTCCTCATCTATGATGCATCCAATCT
AGAATCTGGGATCCCAGCCAGGTTTAGTGGCAGTGGGTCTGGGACAGACTTC
ACCCTCAACATTCATCCTGTGGAGGAAGAGGATGTTGCAACCTATTACTGTCA
GGAAAGTAATGAGGATCCTCGGACGTTCGGTGGAGGCACCAAGCTGGAAATC
AAA

METDTILLWVLLLWVPGSTGDIVLTQSPASLAVSQGQRATISCKASQSVDYDGDS
YVNWYQQKPGQSPKLLIYDASNLESGIPARFSGSGSGTDFTLNIHPVEEEDVATY
YCQESNEDPRTFGGGTKLEIK

C. CHK-166

CHK-166-V$_H$

atgaacttgggggctcagcttgattttccttgtccttgttttaaaaggtgtccagtgtGAAGTGAGGCTGGTGGAGT
CTGGGGGAGGCTTAGAGCAGCCTGGAGGGTCCCTGAAACTCTCCTGTGCAGC
CTCTGGATTCACTTTCAGTGACTATTTCATGTATTGGGTTCGCCAGACTCCAG
AGAAGAGGCTGGAGTGGGTCGCATATATTAGTAATGGTGGTATTAGTACCTT
TTATTCAGACGCTGTTAAGGGCCGATTCACCATCTCCAGAGACAATGCCAGG
AACACCCTATACCTACAAATGAGTCGTCTGAAGTCTGAGGACACAGCCATAT
ATTACTGTGTAAGACAGGTCTACGGTCAGGGCTACTTTGACTACTGGGGCCA
AGGCACCACTCTCGCAGTCTCCTCA

MNLGLSLIFLVLVLKGVQCEVRLVESGGGLEQPGGSLKLSCAASGFTFSDYFMY
WVRQTPEKRLEWVAYISNGGISTFYSDAVKGRFTISRDNARNTLYLQMSRLKSE
DTAIYYCVRQVYGQGYFDYWGQGTTLAVSS

CHK-166-V_L

atggattttcaggtgcagattttcagcttcctgctaatcagtgcctcagtcataatgtccagaggaCAAATTGTTCTCAT
CCAGTCTCCAGCGATCATGTCTGCGTCTCTAGGGGAACGGGTCACCATGACC
TGCACTGCCAGCTCAAGTGTAAGTTCCAGTTACTTGCACTGGTACCAGCAGA
AGCCAGGATCCTCCCCCAAACTCTGGATTTATAGTTCATTCAGCCTGGCTTCT
GGAGTCCCAGCCCGGTTCAGTGGCAGTGGATCTGGGACCTCTTACTCTCTCAC
AATCAGCACCATGGAGGCTGAAGATGCTGCCACGTATTACTGCCACCAGTAT
TTGCGTTCCCCGTGGACGTTCGGTGGAGGCTCCAAGCTGGAAATCAAA

MDFQVQIFSFLLISASVIMSRGQIVLIQSPAIMSASLGERVTMTCTASSSVSSSYLH
WYQQKPGSSPKLWIYSSFSLASGVPARFSGSGSGTSYSLTISTMEAEDAATYYCH
QYLRSPWTFGGGSKLEIK

D. CHK-263

CHK-263-V_H

atggaatggcctttgatctttctcttcctcctgtcaggaactgcaggtgtccaatccCAGGTTCAGCTGCAGCAGT
CTGGGGCTGAGCTGGTGAAGCCTGGGGCCTCAGTGAAGATTTCCTGCAAAGC
TTCTGGCTACGCATTCAGTAGCTACTGGATGAACTGGGTGAAGCAGAGGCCT
GGAAAGGGTCTTGAGTGGATTGGACAGATTTATCCTGGAGATGGTGATACTA
ACTACAACGGAAAGTTCAAGGGCAAGGCCACACTGACTGCAGACAAATCCTC
CAGCACAGCCTACATGCAGCTCAGCAGCCTGACCTCTGAGGACTCTGCGGTC
TATTTCTGTGCAAGAGGAGGTCTAACTATTGACTACTGGGGCCAAGGCACCA
CTCTCACAGTCTCCTCA

MEWPLIFLFLLSGTAGVQSQVQLQQSGAELVKPGASVKISCKASGYAFSSYWMN
WVKQRPGKGLEWIGQIYPGDGDTNYNGKFKGKATLTADKSSSTAYMQLSSLTS
EDSAVYFCARGGLTIDYWGQGTTLTVSS

CHK-263-V_L

atggtatccacacctcagttccttggacttatgctttttggatttcagcctccagaggtGATATTGTACTGACTCAG
TCTCCAGCCACCCTGTCTGTGACTCCAGGAGATAGCGTCAGTCTTTCCTGCAG
GGCCAGCCAAAGTATTAGCGACAACCTACACTGGTATCAACAAAAATCACAT
GAGTCTCCAGGGCTTCTCATCAAGTATGCTTCCCAGTCCATCTCTGGGATCCC
CTCCAGGTTCAGTGGCAGTGGATCAGGGACAGATTTCACTCTCAGTATCAAC
AGTGTGGAGACTGAAGATTTTGGAATGTATTTCTGTCAACAGAGTAACAGCT
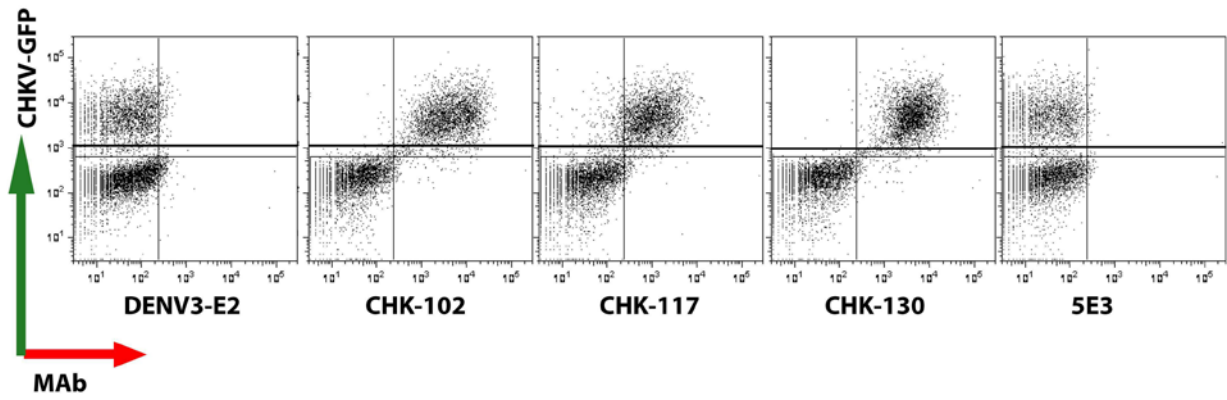GGCCGTACACGTTCGGAGGGGGGACCAAGCTGGAAATAAAA

<u>MVSTPQFLGLMLFWISASRG</u>DIVLTQSPATLSVTPGDSVSLSCRASQSISDNLHW
YQQKSHESPGLLIKYASQSISGIPSRFSGSGSGTDFTLSINSVETEDFGMYFCQQSN
SWPYTFGGGTKLEIK

The V$_H$ and V$_L$ variable domains were amplified from hybridoma cell RNA by a 5'
RACE procedure. Underlined nucleotides and amino acids correspond to the signal
sequences, when obtained.

Table S4. Primers used for sequencing and amplifying the structural genes of CHIKV-LR 2006-OPY1.

Sequencing Primer	5'-Sequence-3'
1: 5'-8248F	GTCTTAGGAGGAGCTAATGAAGGAG
2: 5'-8573F	CCACAAGACCATACTTAGCTCACTGTCC
3: 5'-8912F	CATGTACGCACCCATTTCACC
4: 5'-9224F	CGGTCACCAATCACAAAAAGT
5: 5'-9500F	CCGTGCCGACTGAAGGG
6 5'-9802F	GCTAAAGCGGCCACATACC
7: 5'-10101	CACTTTGGAGCCAACACTATCG
8: 5'-10389	GCTCCGCGTCCTTTACCA
9: 5'-10676	CGGTACACGTGCCATACTCTCAGG
10: 5'-11017	GCTGAGATAGAAGTTGAAGGGA

PCR Primer	5'-Sequence-3'
5'-8248F	GTCTTAGGAGGAGCTAATGAAGGAGCCCGT
5'-11359R	GTGTGTCTCTTAGGGGACACATATACCTTCATACTT

Supplementary Figure 1

Supplementary Figure 2

a

CHK-152 | hu-CHK-152

RU / Time (s)

MAb	k-assoc 1/Ms	k-dissoc 1/s	K_D nM
CHK-152	1.2×10^5	5.2×10^{-4}	4.3
hu-CHK-152	1.3×10^5	5.9×10^{-4}	4.5

b

Relative Infection vs Log_{10} [MAb] $\mu g/mL$

● Mouse CHK-152
■ hu-CHK-152

c

Percent survival vs Days after infection

── hu-CHK-152 10 μg
── hu-CHK-152, 100 μg
── DENV1-E98, 100 μg

Supplementary Figure 3

a

Competition of binding of ch-CHK-152 to CHKV

- WNV E28
- PBS
- CHK-166
- CHK-102
- CHK-263
- CHK-152

b

CHK-102 + CHK-152
- CHK-102
- CHK-152
- CHK-102 + CHK-152

CHK-102 + CHK-263
- CHK-102
- CHK-263
- CHK-102 + CHK-263

CHK-152 + CHK-166
- CHK-152
- CHK-166
- CHK-152 + CHK-166

CHK-152 + CHK-263
- CHK-152
- CHK-263
- CHK-152 + CHK-263

Supplementary Figure 4

95

Neutralization of CHKV
in vivo **escape mutants**
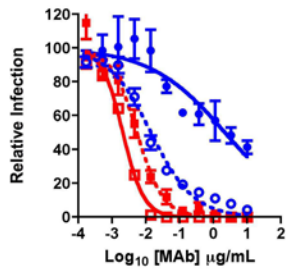
- ● Tx: CHK-102 + CHK-152; Neutralization: CHK-102
- ■ Tx: CHK-102 + CHK-152; Neutralization: CHK-152
- ○ CHKV parent; Neutralization: CHK-102
- □ CHKV parent; Neutralization: CHK-152
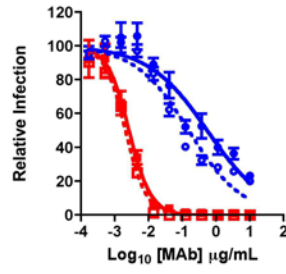
- ● Tx: CHK-166 + CHK-152; Neutralization: CHK-166
- ■ Tx: CHK-166 + CHK-152; Neutralization: CHK-152
- ○ CHKV parent; Neutralization: CHK-166
- □ CHKV parent; Neutralization: CHK-152

Supplementary Figure 5

CHK-166 in vivo escape mutant (E1-G64S)

- ● CHK-102
- ■ CHK-152
- ▲ CHK-166
- ▼ CHK-263

Figure S6

Figure S7

Figure S8

Chapter III

Conclusions and Future Directions

Conclusions

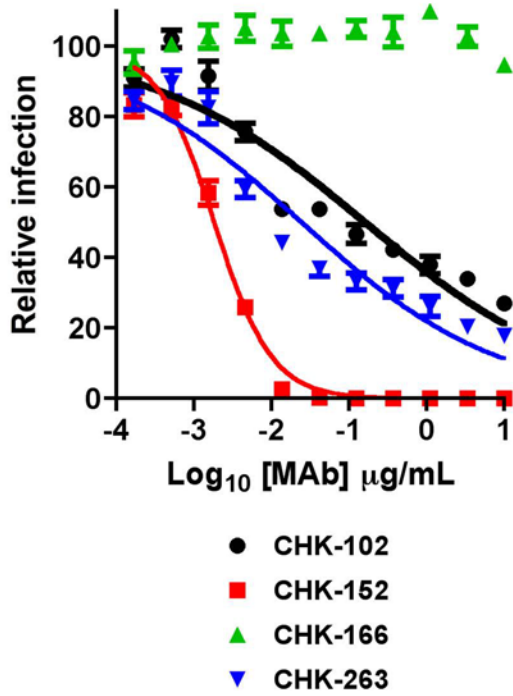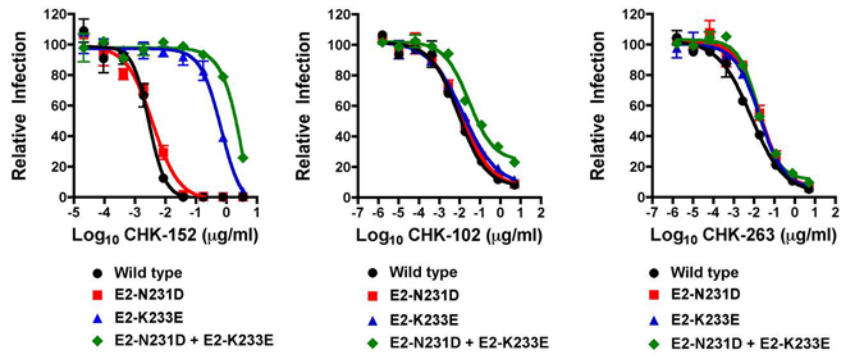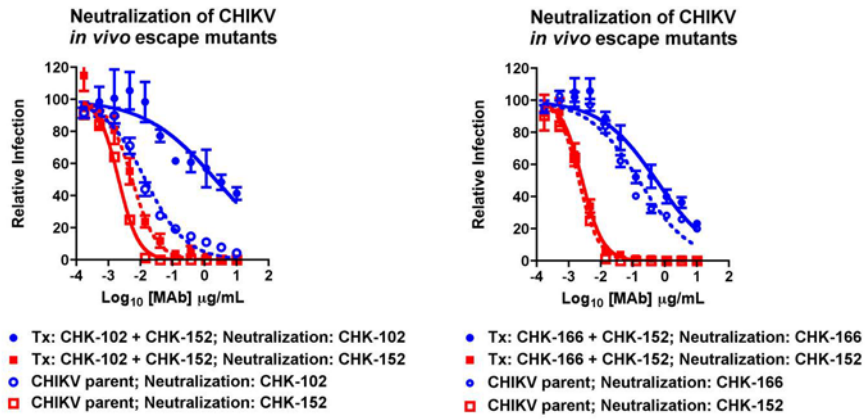In this study, we have identified, cloned, and begun characterization of 230 novel CHIKV MAbs produced from mice immunized with the epidemic strain, CHIKV-LR 2006 OPY1. Thirty-six of these MAbs are neutralizing; over half of them potently inhibit strains representing all three distinct genotypes of CHIKV. Many of these MAbs were protective in both a lethal and arthritis model of CHIKV infection and four MAbs (CHK-102, CHK-152, CHK-166 and CHK-263) prophylactically protected all *IFNAR$^{-/-}$* mice against death. We sought to define the functional epitopes of highly neutralizing anti-CHIKV MAbs and to identify correlates of humoral protection. To functionally map protective MAbs, we selected for escape mutants *in vitro* and also isolated mutant CHIKVs *in vivo*.

We characterized the first MAb targeting the E1 glycoprotein of CHIKV (CHK-166); although this MAb was not as potently neutralizing *in vitro*, it was still highly effective *in vivo*. It binds to domain II of E1, adjacent to the conserved fusion loop. CHK-102 and CHK-263 target domain B of E2; escape mutants selected against one of these two MAbs were always reciprocally resistant. Combination therapy with CHK-102 and CHK-263 was not more effective than monotherapy, further supporting our hypothesis that MAb therapy targeting different epitopes is protective because it prevents the emergence of escape mutants. We determined that our most potent MAb, CHK-152, inhibits fusion of CHIKV with lipid membranes and targets domain A of E2. Although neutralization alone can protect the majority of mice when an aglycosyl N297Q variant of CHK-152 (lacking the ability to engage effector functions) is administered, it is still more protective when effector functions are recruited, especially at lower doses of MAb. While CHK-152 neutralization was not enhanced by CHK-102, CHK-166 or CHK-263 *in vitro*, its protective capacity was certainly augmented *in vivo*. Indeed, combination

monoclonal antibody therapy with MAbs that target different epitopes on E1 and E2 was protective against CHIKV-induced death in *IFNAR⁻ᐟ* mice.

With the globalization of diseases and spread of mosquito vectors, the need for development of a CHIKV vaccine has increased. Currently, no licensed human vaccine exists for CHIKV and mosquito control is the only reliable means of preventing infection. Since sera from convalescent CHIKV infected patients or CHIK virus-like particle (VLP) vaccinated primates can prevent and cure an otherwise lethal infection in *IFNAR⁻ᐟ* mice [1,2], we believe an epitope-directed vaccine may be possible. This study leaves us with many exciting questions and directions, some of which we have begun to address.

Future Directions

There are 29 different alphaviruses distributed throughout the world; the Semliki Forest virus antigenic complex consists of CHIKV and seven other related alphaviruses [3]. Many are endemic in Africa and Asia, where CHIKV epidemics have been reported for centuries. CHIKV is serologically most closely related to O'nyong'nyong virus (ONNV), which causes a similar debilitating, arthritic fever. In fact, the term "O'nyong'nyong" describes a weakening of the joints in the Nilotic language of Uganda where an epidemic began in the late 1950s and spread of many neighboring countries, affecting about two million people [4]. ONNV and CHIKV are similar genetically and share 72% nucleotide homology and 87% amino acid homology [5] but phylogenetic analysis indicates that these two viruses probably diverged thousands of years ago [6]. Additionally, ONNV is the only alphavirus transmitted by *Anopheles* mosquitoes, which are widely known as the vector for transmission of malaria-causing *Plasmodium* species [7]. Mayaro virus causes a disease indistinguishable from that caused by ONNV, but has been found only in South America, or in travelers returning from this region [6,8].

Based on the antigenic and phylogenetic relatedness of many of these alphaviruses to CHIKV (**Figure 1**), it is possible that some of our CHIKV MAbs cross-react with or even neutralize some of these other alphaviruses. We began to study this by growing stocks of ONN, Ross River, Semliki Forest, Mayaro, Una, Getah, Bebaru, Middleburg, Barmah Forest, Sindbis and Venezuelan equine encephalitis viruses. We tested for cross-reactivity between the 36 neutralizing CHIKV MAbs and two non-neutralizing MAbs against the eleven alphaviruses by flow cytometry, as described in the methods. We also tested another strain of CHIKV, CHIKV-Ross, representing the ECSA genotype prior to the emergence of La Reunion epidemic strain, which is known to contain many mutations in the structural genes. All of the MAbs, including

the non-neutralizing MAbs CHK-84 and CHK-141 cross-reacted with CHIKV-Ross (**Figure 2**). CHK-266 was the only MAb tested that failed to bind to one of the strains of CHIKV; it showed no reactivity to CHIKV-RSU1, the Asian strain, and also did not cross-react to any of the other alphaviruses. The epitope for this MAb appears to be restricted to the African lineage of CHIKVs and will be interesting to explore further with mapping studies.

Seventeen of the neutralizing MAbs strongly cross-reacted (50-100% of infected cells bound MAb as judged by flow cytometry) and ten moderately cross-reacted (10-50% of infected cells bound MAb) with ONNV. As demonstrated previously, CHK-263 and CHK-102 are both potent, protective E2 domain B MAbs, as determined by functional mapping experiments; however, CHK-102 strongly cross-reacts with ONNV but CHK-263 does not recognize ONNV. Neither of these MAbs binds to any of the other alphaviruses tested. It will be interesting to further explore how the epitopes for these two MAbs differ since they both neutralize all three strains of CHIKV (LR, RSU1 and IbH35) with comparable potency and completely protect IFNAR$^{-/-}$ mice prophylactically against CHIKV-LR infection at the 100 µg dose. However, CHK-263 is more protective than CHK-102; this is apparent when lower doses are used. Perhaps the CHIKV epitope specificity of this MAb contributes to its enhanced protective potential. Neutralization of ONNV should be explored by performing neutralization assays with the MAbs that cross-react with this virus. This may lend insight into the functional significance of shared epitopes between these two related alphaviruses.

Just a few of the CHIKV neutralizing MAbs cross-reacted with seven or more of the alphaviruses tested: CHK-166, CHK-180, CHK-187 and CHK-265 **(Figure 2)**. Cross-reactivity with many such diverse viruses suggests that these four MAbs target an epitope that is conserved amongst the alphaviruses. From escape mutant analysis we know that CHK-166 binds to a highly

conserved region of domain II of E1. Further studies into CHK-180, CHK-187 and CHK-265 may define other highly conserved alphavirus epitopes. Neutralization experiments performed by our collaborators (Ted Pierson, NIH, unpublished data) demonstrate that CHK-65, CHK-77, CHK-88 and CHK-124 also neutralize Semliki Forest virus (SFV) in a dose-dependent fashion. They did not test CHK-187, which is the only other strongly cross-reactive MAb against SFV. These results are exciting because all of the MAbs tested that efficiently cross-reacted to SFV also neutralized infection. This suggests that it may be promising to test the other alphaviruses that strongly bind CHK MAbs for neutralization potential.

Cross-reactivity appears to correlate with the genetic relatedness of the alphaviruses. Middleburg and Barmah Forest viruses are assigned to two separate antigenic complexes and displayed only limited cross-reactivity with these CHK-MAbs. Only two MAbs, CHK-96 and CHK-98, moderately cross-react with Venezuelan equine encephalitis virus (VEEV), a more distantly related New World Alphavirus.

It also will be informative to functionally map all of the neutralizing anti-CHIKV MAbs and develop a more global structural picture of neutralization. We have begun working on this by using an NNN codon mutagenesis strategy. Primer pairs incorporating NNN at the amino acid of interest will be designed, so there will be 64 primer pairs per amino acid selected. Theoretically speaking, to completely map these antibodies we would need to make NNN mutations for each codon of the 1,100 amino acids in E1, E2 and E3 by designing and using an NNN primer set for every amino acid. We can target candidate amino acids by choosing those that fulfill certain characteristics including high conservation amongst alphaviruses, variation among CHIKV strains, or surface exposed or hydrophilic residues based on existing crystal and cryo-electron microscopy structures [9,10]. It is possible that rare MAbs inhibit infection by binding to regions

that are exposed only at certain times in the viral replication cycle, such as homo-trimerization or the removal of the E2-Domain B cap from the fusion loop. The relevance of these regions in neutralization may be missed if only surface exposed residues are selected, so it may be prudent to revisit mapping of any MAbs that were unaffected by the amino acids initially mutated.

In preliminary experiments, we screened 38 anti-CHIKV MAbs against CHIKV encoding a point mutation (see methods) that conferred resistance to CHK-152 or CHK-166 (E2: D59N and E1: K61T, respectively); these mutant viruses were tested first since CHK-152 and CHK-166 were the most protective *in vivo*. We were interested in determining whether any of the other MAbs in our repertoire require either of these residues and perhaps share an epitope with these potent MAbs. While none of the 38 MAbs (besides CHK-152) were incapable of neutralizing CHIKV-D59N, CHK-180 and CHK-269 were unable to neutralize CHIKV-K61T, even at a high MAb concentration of 10 µg/mL. This indicates that residue K61T also is important for efficient neutralization by CHK-180 and CHK-269. Since CHK-166 and CHK-180 have identical cross-reactivity and neutralization profiles, it is possible that they share a large portion of their epitope.

Further study into the antibody response of seropositive, asymptomatic individuals also is warranted. Most people that are infected with CHIKV develop acute symptoms of disease, including fever, arthralgia, and myalgia. However, small subsets of people have been identified on La Reunion and in Kerala, India, that are seropositive for CHIKV but failed to develop symptoms consistent with CHIKV disease [11,12]; this indicates that prevalence of asymptomatic, seropositive people is not regionally restricted. It will be interesting to investigate the quality and kinetics of the antibody response generated by asymptomatic individuals. It is possible that they produced highly neutralizing antibodies that, in conjunction with a rapid innate immune response, clear CHIKV before the infection became fully established. CHIKV spreads

widely in naïve populations; on the island of La Reunion over a third of the population (~300,000 people) was infected, so identifying seropositive, asymptomatic individuals should be straightforward [13-15]. It is also possible that asymptomatic individuals lack a necessary co-factor or receptor in particular cells (akin to a CCR5Δ30 mutation in patients exposed to HIV who do not develop disease), which may render the environment resistant to CHIKV infection. Another possible explanation for why a subset of people did not exhibit any apparent clinical symptoms of CHIKV disease is that they previously generated cross-reactive antibodies against a related alphavirus. In this case, upon CHIKV infection, memory B cells would re-activate, differentiate, and secrete antibodies that help to rapidly clear infection. A complete history of febrile, arthritic diseases and the persistence of previous joint pain in this population will be important to note. Nevertheless, it may be informative to understand how the antibody response of asymptomatic, seropositve people differed from those that became ill. Epitopes targeted by potent antibodies in the former may help inform vaccine design.

Examining differences between the antibody responses of patients that do or do not develop chronic arthritis also may be enlightening. Patient studies have correlated a more severe, acute disease with early, high viremia but this group also developed an early IgG3 response and completely cleared CHIKV infection without persistent arthralgia [16]. In fact, none of the patients producing early IgG3 (7- 10 days after the onset of infection) in this study developed persistent arthrlagias, but 30% of late IgG responders did [16]. While acute CHIKV is a painful and debilitating illness, it is usually self-limited and resolves within two weeks; arguably, the most significant morbidity occurs during the chronic stages, during which symptoms may linger and recur for months to years. While kinetics of the antibody response appear to contribute to disease pathogenesis or restriction, the quality of this response may be important. Further

investigation into epitopes targeted by late IgG responders that do not develop chronic illness may be informative. B cells from consenting, convalescent patients with a history of persisting arthralgias and with no chronic illness could be harvested and immortalized so that the neutralization potential of their antibody response and epitopes targeted by these MAbs can be studied.

CHK-152 or CHK-166 completely protected *IFNAR$^{-/-}$* mice prophylactically, but a lower dose of CHK-152 protected 75% of mice. A lower dose of CHK-166, however, failed to protect any mice, and they all died at 7 days post infection. Independently, CHK-152 and CHK-166 were partially protective when administered one day post CHIKV infection. We identified CHIKV escape mutants, including the E2-D59N mutation, in the leg muscle and brains of mice that died despite CHK-152 treatment, but only sequenced wild-type virus from tissues harvested from mice prophylactically treated with CHK-166. Tissue was harvested from one mouse that died despite CHK-166 therapeutic treatment, and we discovered a novel mutation that conferred complete resistance to neutralization by CHK-166, E1-G64S. When we selected for escape mutants to CHK-166 *in vitro,* all plaques sequenced featured the mutation E1-K61T; however, this mutation was not isolated *in vivo*. Combination therapy with CHK-166 and CHK-152 was completely protective earlier in the course of infection in *IFNAR$^{-/-}$* mice, but less effective when administered 24 to 36 hours before death. However, our sequencing studies from mice dying despite combination therapy never defined a double mutant (ie D59N + K61T) or a novel mutation conferring complete resistance to the respective MAbs. As such, we have begun to test the potential fitness costs of the E2-D59N, E1-K61T and E2-D59N + E1-K61T mutations on CHIKV. To assess this, we introduced these mutations into CHIKV-LR 2006 OPY1 infectious clone, to generate isogenic viruses. After generating passage 0 stocks by electroporation of

mammalian cells and passage 1 insect cell stocks, we have begun performing growth curves on mammalian and insect cells. We have not observed any difference in the growth of the D59N or K61T viruses, but studies are ongoing. We are being careful to maintain the cells in medium containing CHK-152, CHK-166 or both CHK-152 and CHK-166 to prevent reversion to the wild-type virus.

Fitness experiments also have been performed in mice, and while all three viruses (delivered to mice that were treated with the respective MAb to prevent reversion and to untreated mice) are lethal in the $IFNAR^{-/-}$ model, the mice infected with the double mutant and treated with CHK-152 and CHK-166 survived a few days longer before succumbing to death (data not shown). This suggests that *in vivo*, there may be a fitness cost to CHIKV for maintaining both these mutations. Collaboratively, with the Higgs laboratory, we are testing the effect of these three mutations on CHIKV fitness in their *Aedes* mosquito vector. Unlike other viruses, arboviruses undergo unique selective pressure that occurs with infection of vertebrate and invertebrate hosts. As such, fewer mutations are generally observed amongst alphavirus genomes than in other RNA viruses [17], presumably due to purification of genomic changes that yield fitness costs in a given species. We are interested in investigating whether these mutations that confer resistance to MAbs CHK-152 and CHK-166 are purified in mosquito populations; this has important implications for possible MAb-based therapy. It has previously been shown that *Aedes albopictus* midgut infectivity was enhanced by a single point mutation E1-A226V [18] and that aspartic acid (D) at amino acid 60 on E2 is an important determinant of *Aedes aegypti* and *Aedes albopictus* infectivity [19]. This latter mutation is directly adjacent to E2-D59N mutation that we are testing.

As another step towards a potential combination MAb therapy for patients, therapeutic treatment of non-human primates was performed by our collaborators (Dan Streblow, OHSU). These studies show a decrease in viremia and viral dissemination after treatment with CHK-152 and CHK-166. Thus, it is possible that there could be a future role of MAb based prophylaxis or therapy in target populations including pregnant women, infants, and patients with certain pre-existing co morbidities that are vulnerable to serious CHIKV-induced complications [20-23].

Materials and methods

Cross-reactivity of CHIKV antibodies. CHIKV MAbs were tested for cross-reactivity against a panel of non-CHIKV arthritogenic alphaviruses available from the World Arbovirus collection (generous gift of R. Tesh, Galveston, TX) and a single New World encephalitic alphavirus. This panel included the following viruses, which were passaged once in Vero76 or Vero T144 cells: Una (CO AR), Mayaro (BE H 407), Semliki Forest (M4862), Sindbis (AR339), Getah (AMM-2021), Barmah (K 10521), O'nyong-nyong (MP30), Middleburg (30037), Ross River (T48) and Venezuelan equine encephalitis (TC-83) viruses. Vero cells were infected with each virus (MOI of 0.01 for 12 to 24 hours, depending on the virus), harvested with HBSS supplemented with 3 mM EDTA, fixed with 1% PFA in PBS for 8 minutes, and permeabilized with 0.1% (w/v) saponin detergent solution in HBSS. Approximately 10^5 cells were incubated with different CHIKV MAbs (10 µg/mL solution) for 60 minutes on ice. After washing, cells were then incubated with 4 µg/mL of Alexa 647 anti-mouse IgG secondary antibody (Invitrogen), evaluated on a FACSArray flow cytometer (Becton-Dickinson) and analyzed using FlowJo software (Tree Star).

Cells and viruses. Vero cells and BHK21-15 cells were cultured in DMEM (Dulbecco's modified Eagle medium) supplemented with 5% or 10% fetal bovine serum, respectively, (FBS) (Omega Scientific), 10 mM HEPES, nonessential amino acids (Cellgro) and antibiotics (penicillin and streptomycin G) at $37^{o}C$ in a 5% CO_2 incubator. C6/36 *Aedes albopictus* cells were grown in Leibovitz-15 media (Gibco) supplemented with 10% FBS, 10 mM HEPES, and antibiotics (penicillin and streptomycin G) at $27^{o}C$. The infectious clone of CHIKV LR 2006 OPY-1 (strain 142) was a generous gift from S. Higgs (Galveston, TX). Plasmids containing the wild-type CHIKV structural genes or single point mutations, CHK struct- pDonor221, were a

generous gift from T. Pierson (NIAID). Single point mutations were introduced into the

infectious clone by ligating a SgrA1 (New England Biolabs) and Sfi1 (New England Biolabs)

double digested CHK struct- pDonor221fragment containing the respective mutation, into the

SgrA1 and Sfi1 double digested infectious clone. The double mutant D59N+K61T-142 was

created by performing a mutagenesis reaction on the D59N-142 plasmid with primers :

Forward - gtctccgtacgtgacgtgctgcggtacag
Reverse - ctgtaccgcagcacgtcacgtacggagac

To produce virus from infectious clones, plasmids were linearized and RNA was produced using

an SP6 DNA-dependent RNA polymerase transcription kit following the manufacturer's

instructions (mMessage kit; Ambion). CHIKV RNA was electroporated into BHK21-15 cells

and supernatant was harvested 28 hours later, aliquoted, and frozen at -80°C. C6/36 cells were

infected with this P0 CHIKV-LR at an MOI of 0.01, in the presence of 10 µg/mL of either CHK-

166 for K61T-142, CHK-152 for D59N-142 and 10 µg/mL of both CHK-166 and CHK-152 for

the D59N+K61T-142 double mutant. Supernatant was harvested 68- 72 hrs later. Virus was

titered on Vero cells by focus forming unit (FFU) assay.

Neutralization assays. MAb neutralization of CHIKV was assessed by an FFU assay.

Eleven three-fold serial dilutions of MAb (ranging from 10 µg/ml to ~1 ng/ml in 2% DMEM)

were incubated with ~10^2 FFU of CHKV for one hour at 37°C. MAb-virus complexes were

added to a monolayer of African monkey Vero cells (seeded overnight at 3 X 10^4 cells/well, in

96-well plates) in 5% DMEM. Cells were infected at 37°C for 90 minutes, and overlaid with 1%

(w/v) methylcellulose in Modified Eagle Media (MEM) supplemented with 4% FBS. Plates were

harvested 18 and fixed with 1% (w/v) paraformaldehyde in PBS. The plates were incubated

sequentially with 500 ng/ml of a chimeric CHK-9 MAb (chCHK9) and a 0.3 mg/ml solution of

HRP-conjugated goat anti-human IgG secondary antibody (Sigma) in PBS supplemented with 0.1% saponin and 0.1% BSA. CHIKV-infected foci were visualized using TrueBlue peroxidase substrate (KPL) and quantitated on an ImmunoSpot 5.0.37 macroanalyzer (Cellular Technologies Ltd). Non-linear regression analysis was performed, and EC50 values were calculated using Prism compared to wells infected with CHIKV in the absence of antibody.

Growth Curves Vero cells were seeded overnight at 6×10^4 cells/well in 12 well plates in 1mL of DMEM containing 5% FBS. C6/36 cell-derived CHIKV mutants or WT CHIKV were incubated with 10 µg/mL of CHK-166, CHK-152, both CHK-166 and CHK-152 or no MAb for 1 hour at 37°C. Media was removed from Vero cells and they were infected with 500 µL of inoculum for 1 hour at 37°C. After the hour, inoculum was removed and cells were washed with PBS. Media containing the respective MAb or no MAb was added back to the cells. Time points were harvested at 1, 12, 24 and 36 hours post-infection. Similarly, growth curves were performed on C6/36 cells with P0 BHK-derived CHIKVs and time points were harvested at 1, 24, 48 and 72 hours post-infection. Virus was titered by FFU assay. Experiments were performed in triplicate three times.

Mouse Experiments. *IFNAR*^{-/-} C57BL/6 mice were obtained from J. Sprent (Scripps Institute, San Diego CA), backcrossed ten times onto the C57BL/6 background, and bred in the pathogen-free animal facilities of Washington University School of Medicine. Experiments were performed with the approval of the Washington University Animal Studies Committee. To prevent mutant CHIKV reversion, some mice were treated pre-exposure with 100 µg of either CHK-152, CHK-166 or both CHK-152 and CHK-166. MAbs were administered by

intraperitoneal (IP) injection into 6 to 8 week old *IFNAR*$^{-/-}$ mice. Twenty-four hours later, mice

were inoculated in the footpad with 10 FFU of C6/36 cell derived D59N-142, K61T-142,

D59N+K61T-142, or CHIKV-LR in 50 µl of Hanks balanced salt solution (HBSS) supplemented

with 1% heat-inactivated FBS.

Acknowledgements

Figure Legends

Figure 1. This figure is borrowed from Powers, 2001 [6]. **Phylogenetic relationship of most Alphaviruses species, generated from partial E1 envelope protein sequences.** The open circle adjacent to a branch indicates hypothetical Old to New World introduction, and the closed circle indicates New to Old World introduction, assuming a New World origin; the open square indicates Old to New World introduction, and the closed square indicates New to Old World introduction, assuming an Old World origin of the nonfish Alphavirus clade.

Figure 2. Cross-reactivity of Alphaviruses with 38 anti-CHIKV MAbs. Vero76 cells were infected at an MOI of 0.01, fixed and permeabilized. These cells were stained with 10 µg/mL of the anti-CHIKV MAbs. The viruses are listed in order from least to most divergence from CHIKV-LR. (++) denotes strong binding, 50-100 % of cells stained with this MAb. (+) denotes moderate binding, 10-50% of cells stained with this MAb. (-) denotes no binding, <10% of cells bound this MAb. Immune sera refers to a 1:1000 dilution of serum harvested from mice that were immunized with CHIKV-LR.

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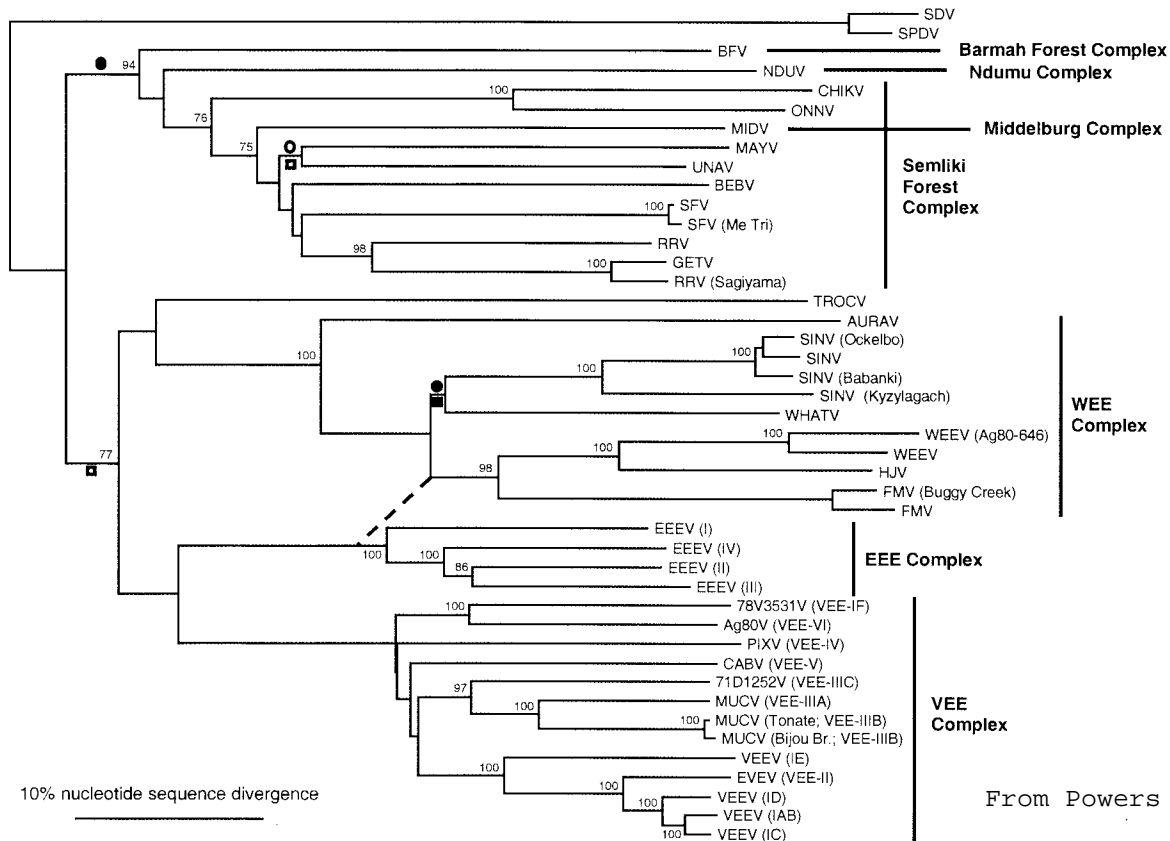
FIGURE 1

10% nucleotide sequence divergence

From Powers et al, 2001.

Figure 2 : Cross Reactivity with Related Alphaviruses

Legend: ■ 50-100% binding ■ 10-50% binding - < 10% binding

	CHK Ross	CHK RSUI	CHK West African	ONN	Ross River	Semliki Forest	Mayaro	Una	Getah	Bebaru	Middle-burg	Barmah Forest	Sindbis	VEEV
Immune sera	++	++	ND	++	+	+	++	+	+	+	+	++	ND	-
CHK 9	++	++	++	++	-	-	-	-	-	-	-	-	-	-
CHK 11	++	++	++	++	-	+	-	-	-	-	-	-	-	-
CHK 48	++	++	++	++	-	+	++	++	++	-	-	-	-	-
CHK 65	++	++	++	-	-	++	++	+	++	-	-	-	-	-
CHK 77	++	++	++	+	+	++	++	++	++	-	-	-	-	-
CHK 84	++	++	-	-	-	-	-	-	-	-	-	-	-	-
CHK 88	++	++	++	+	-	++	++	++	++	-	-	-	-	-
CHK 95	++	++	++	-	++	-	++	-	+	++	-	-	-	-
CHK 96	++	++	++	+	-	-	+	-	+	-	+	+	-	+
CHK 98	++	++	++	-	+	-	++	-	-	-	+	+	+	+
CHK 102	++	++	++	++	-	-	-	-	-	-	-	-	-	-
CHK 105	++	++	++	-	-	-	+	++	+	-	-	-	-	-
CHK 112	++	++	++	++	-	-	-	-	-	-	-	-	-	-
CHK 124	++	++	++	-	-	++	++	++	++	-	-	-	-	-
CHK 140	++	++	++	+	-	-	-	-	-	-	-	-	-	-
CHK 141	++	++	++	-	-	-	-	-	-	-	-	-	+	-
CHK 142	++	++	++	+	-	-	-	-	-	-	-	-	-	-
CHK 143	++	++	++	-	-	-	-	++	-	-	-	-	-	-

Figure 2 : Cross Reactivity with Related Alphaviruses , Part 2

	CHK Ross	CHK RSUI	CHK West African	ONN	Ross River	Semliki Forest	Mayaro	Una	Getah	Bebaru	Middle-burg	Barmah Forest	Sindbis	VEEV
CHK 151	++	++	++	++	-	-	-	-	-	-	-	-	-	-
CHK 152	++	++	++	+	-	-	-	-	-	-	-	-	-	-
CHK 155	++	++	++	++	-	-	-	-	-	-	-	-	-	-
CHK 164	++	++	++	++	-	-	-	-	-	-	-	-	-	-
CHK 165	++	++	++	++	-	-	-	-	-	-	-	-	-	-
CHK 166	++	++	++	++	+	-	++	-	+	++	++	++	+	
CHK 175	++	++	++	++	-	-	++	++	++	-	-	-	-	-
CHK 176	++	++	++	++	-	-	-	-	-	-	-	-	-	-
CHK 180	++	++	++	++	+	-	++	-	+	++	++	++	+	
CHK 187	++	++	++	+	++	++	++	++	++	+	-	-	-	-
CHK 189	++	++	++	++	-	-	-	-	-	-	-	-	+	-
CHK 262	++	++	++	++	-	-	-	-	-	+	-	-	-	-
CHK 263	++	++	++	-	-	-	-	-	-	-	-	-	-	-
CHK 264	++	++	++	++	-	-	++	++	++	-	-	-	-	-
CHK 265	++	++	++	+	++	+	++	++	++	+	-	-	-	-
CHK 266	++	-	++	-	-	-	-	-	-	-	-	-	-	-
CHK 267	++	++	++	+	-	-	-	-	-	-	-	-	-	-
CHK 268	++	++	++	+	-	-	+	-	-	-	-	-	-	-
CHK 269	++	++	++	+	-	-	++	-	-	-	-	-	-	-

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<u>**Education**</u>

August 2007 – present
   Washington University in St. Louis School of Medicine- MSTP
   PhD in Molecular Microbiology and Microbial Pathogenesis defended in September 2013
   MD/PhD candidate, anticipated degree in May 2015

August 2003 – May 2007
   Florida State University, Tallahassee, Florida- Undergraduate Education
   Bachelors in Science in Biochemistry with Honors in the Major
   Bachelors in Science in Chemistry
   Bachelors in Science in Biomedical Mathematics

   Summa Cum Laude with 218 credit hours

<u>**Research Experience**</u>

  July 2009 – September 2013

   Graduate Student in Laboratory of Michael Diamond, MD, PhD

   Developed a novel panel of 36 neutralizing monoclonal antibodies (MAbs) against Chikungunya
   Virus and further characterized their protective capacity in mouse models. We identified
   epitopes critical for neutralization for a number of these MAbs by developing escape mutants in
   vitro and determined that the most protective combinations in-vivo included 2 MAbs targeting
   different regions of the envelope glycoproteins. Our data suggest that pairs of highly
   neutralizing MAbs may be a therapeutic option against CHIKV infection. We sought to develop
   an antibody-based therapeutic agent against CHIKV and develop a better understanding of viral
   epitopes that can be targeted during vaccine development.

August 2003 – August 2007

   **Undergraduate Research Student and Howard Hughes Fellow in Laboratory of Michael
   Chapman, PhD**
   Made progress towards developing a stable mammalian cell line capable of supporting
   independent adeno-associated virus production. Through plasmid transfection, we incorporated
   the 5 adenovirus helper genes (E1A, E1B, E2A, E4 and VA RNA) into the HeLa cell genome,
   under inducible expression.

   A high resolution (1.2 A) transition state analog crystal structure of arginine kinase (ak) shows
   much dynamic motion. We investigated if any additional or different movements exist at the
   physiological temperature of 298K (2.1 A low-resolution data) compared to the 100K structure by

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first establishing the best fit between the 1.2 A ak structure and the 2.1 A x-ray diffraction data. Initial coordinate refinement and B- factors analysis did not uncover significant differences.

Honors and Awards

Spring 2013   ASCI/AAP Travel Award for Annual ASCI/AAP/APSA conference
2007-2008   Phi Kappa Phi Honor Society : National Marjorie Schoch Fellowship
Spring 2007   1st Place in Undergraduate Poster Competition at
                 American Chemical Society's Annual Florida Section Meeting
2006-2007   Honors in the Major Thesis Grant
2005-2006   Howard Hughes Medical Institute Undergraduate Research Fellowship
2005-2006   Biology Department John Mark Caffrey Memorial Scholarship
2004-2007   Charles A. Brautlecht Chemistry Scholarship

Research Presentations

Spring 2013   Gordon Research Conference: Cells and Viruses, Poster Presentation
Spring 2013   ASCI/AAP/APSA conference, Poster Presentation
Spring 2011   ASCI/AAP/APSA conference, Poster Presentation
Summer 2007   American Society for Virology conference, Poster Presentation
Spring 2007   American Chemical Society's Annual Florida Section Meeting, Poster
                 Presentation
Summer 2006   American Society for Virology conference, Poster Presentation
Spring 2006   American Chemical Society, Oral Presentation
Spring 2005   American Chemical Society, Oral Presentation

Publications

Papers:

Pal P, Dowd KA, Brien JD, Edeling MA, Gorlatov S, et al. (2013) Development of a Highly Protective Combination Monoclonal Antibody Therapy against Chikungunya Virus. PLoS Pathogens 9(4): e1003312. doi:10.1371/journal.ppat.1003312.

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Sun S, Xiang Y, Akahata W, Holdaway H, **Pal P**, et al. (2013) Structural analyses at pseudo atomic resolution of Chikungunya virus and antibodies show mechanisms of neutralization. Elife 2: e00435.

Patent Pending:

Diamond M, **Pal P**, Fremont D, Edeling M. <u>Recombinant Chikungunya virus soluble E2 protein</u>. 2014.

Recent Conference Reports:

Pal, Pankaj, Kim Dowd, James Brien, Iris Lee, Melissa Edeling, Daved Fremont, Mark Heise, Ted Pierson, Michael S. Diamond. Development of a Highly Protective Combination Monoclonal Antibody Therapy against Chikungunya Virus**.** Presented at Cells and Viruses Gordon Research Conference. May 5th-10th 2013.

Pal, Pankaj, Kim Dowd, James Brien, Iris Lee, Melissa Edeling, Daved Fremont, Sergey Gorlatov, Syd Johnson, Mark Heise, Ted Pierson, Michael S. Diamond. Development of a Highly Protective Combination Monoclonal Antibody Therapy against Chikungunya Virus**.** Presented at AAP/ASCI/APSA conference. April 26th-28th 2013.

Pal, Pankaj, Kim Dowd, James Brien, Iris Lee, Melissa Edeling, Daved Fremont, Sergey Gorlatov, Syd Johnson, Mark Heise, Ted Pierson, Michael S. Diamond. Generation and Characterization of Monoclonal Antibodies against Chikungunya Virus**.** Presented at AAP/ASCI/APSA conference. April 15th-17th 2011.

<u>**Extracurricular Activities**</u>

2012- 2010	Molecular Microbiology and Microbial Pathogenesis Program Student Representative
2011- 2008	Choreographer/Dancer/Organizer Medical School Class Show
2009- 2008	Choreographer/Cast Medical School Musical
2010-2008	Volunteer at Saturday Neighborhood Free Health Clinic
2010- 2009	Teaching Assistant for Medical Physiology
2009	Course Liaison for Infectious Diseases
2009	Course Liaison for Psychiatry
2009-2008	Co-leader of Washington University Chapter of Physicians for Human Rights
2008	Choreographed and Performed First Anatomy Memorial Dance