Tissue-Restricted Chikungunya Virus Is Attenuated in Mice and Protective Against Virulent Virus Challenge

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Chikungunya virus (CHIKV) is a mosquito-transmitted arthritogenic alphavirus that has reemerged to produce devastating epidemics of fever, rash, polyarthralgia, and polyarthritis. During natural infection in the mammalian host, CHIKV replicates in a variety of cell types, including keratinocytes, endothelial cells, macrophages, and skeletal muscle. The contribution of CHIKV replication in discrete cell types to pathogenesis is unclear. To understand the role of CHIKV tropism in pathogenesis, target sequences corresponding to various tissue-specific miRNAs were engineered into the genome of clinical CHIKV isolate SL15649. Replication kinetics of these viruses were assessed alongside mismatch-control strains engineered to contain synonymous mutations in miR-target sequences to alleviate miRNA-mediated restriction. A CHIKV strain incapable of replication in skeletal muscle, SKE, and its mismatch control, SKE MM, were found to replicate with anticipated kinetics *in vitro*. *In situ* hybridization of limb sections from infected C57BL/6 mice using a CHIKV RNA-specific probe showed diminished SKE replication in myofibers in the interosseous muscle of the left rear foot adjacent to the site of inoculation, though both SKE and SKE MM replicated comparably in connective tissue that does not express the restrictive miRNA. Mice infected with SKE displayed diminished hind limb swelling and inflammation and necrosis of the interosseous muscle compared to mice infected with SKE MM, despite comparable titers in musculoskeletal tissues at days 1, 3, and 7 post-inoculation. Additionally, SKE infection was associated with decreased production of IL-6, IL-1β, IP-10, and

TNFα, as well as diminished infiltration of $CD4_+$ T cells into the interosseous muscle. Treatment of mice with an antibody directed against the IL-6 receptor led to diminished footpad swelling in mice infected with SKE MM, reducing swelling to levels seen in mice infected with SKE. Additionally, vaccination with SKE protected mice against disease following challenge with WT SL15649. These data suggest that IL-6, which is released following infection of myofibers, is a critical mediator of CHIKV-induced inflammation and may represent a therapeutic target to alleviate CHIKV disease. Additionally, incorporation of skeletal muscle-specific miR-target sequences into the CHIKV genome may constitute a new method for developing live-attenuated vaccines.

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Preface

I am grateful for the financial support that made this dissertation possible, including the public health service awards Chikungunya Virus Replication and Pathogenesis (R01 AI123348) and the Molecular Microbial Persistence and Pathogenesis training program (T32 AI049820). Additionally, I am thankful for funding provided by the Vira I. Heinz endowment awarded to Dr. Dermody that has supported much of my research.

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I am grateful to past and present members of the Dermody lab. Science can be hectic, but every day was better when working alongside such caring and brilliant people. In particular, I would like to thank Alison Ashbrook, my rotation mentor, whose friendship and encouragement made me feel so welcome and capable during my early days in lab. Additionally, I am endlessly grateful to Nicole McAllister and Danica Sutherland for their constant friendship and support. The laughter and memories we've shared have brightened even the worst science days. I would also like to thank past and present members of the CHIKV team, including Adam Brynes, Adaeze Izuogu, James Martin, and Krishnan Raghunathan, for thoughtful discussions each and every time I have hit a wall with my research. Finally, I would like to thank Pavithra Aravamudhan, Pamela Brigleb, Chris Lee, Gwen Taylor, and Kelly Urbanek for so frequently being willing to let me distract them into meaningless conversation when my mind needed a break from work.

I also would like to thank Tem Morrison, who was kind enough to host me in his lab for five months at the University of Colorado while I learned how to conduct animal studies. My time in his lab provided incredible training, and I am thankful to have had the opportunity to work with the thoughtful and talented team he has assembled. In particular, I thank Mary McCarthy, Nicholas May, Kathryn Carpentier, and Bennett Davenport, who all were unbelievably generous with their time and expertise and made me feel so welcome.

Members of my thesis committee have been integral in providing useful ideas and feedback that have enhanced my training and the quality of my thesis research. I thank Carolyn Coyne, William Klimstra, Kathryn Torok, and John Williams. I have greatly enjoyed my committee meetings, where your generous support and constructive comments have improved my training. I would also like to thank the PMI graduate student coordinator, Kristin DiGiacomo, as well as the rest of the PMI leadership team for their tireless work in helping the program run smoothly.

Finally, I would like to thank my family. I truly stand on the shoulders of giants. To my mother, Jennifer Loftis, you were my first, and most important, role model. I grew up in awe of your capacity for compassion, selflessness, and sacrifice and I have spent my life trying to be emulate you. I am lucky in so many ways, but I am luckiest to be your son. To my father, James Lentscher, you taught me patience, humor, and the indomitable nature of the human spirit. You are sorely missed, but I am so grateful for your life, love, and everything you taught me. To my sister, Allesandra Lentscher, you have been my closest friend and confidant. Without your love and torment, I would not be the person I am today. And to my partner, Brandon Pickell, you are my greatest supporter. Your determination to live life to the fullest inspires me to do the same. I cannot imagine being on this journey without you.

1.0 Introduction

1.1 Thesis Overview

Virus cell and tissue tropism is a critical determinant of viral pathogenesis. The capacity of a virus to replicate in a particular cell or tissue is often an essential precursor to development of disease at that site. Susceptibility of a cell or tissue to virus infection can be influenced by a variety of factors including expression of cell-surface attachment factors and receptors required by the virus for attachment and entry, production of host restriction factors capable of limiting various stages of virus replication, and a variety of other barriers encountered by viruses as they traverse different host tissues. Elucidating determinants of viral tropism and the effects of replication in specific cells on pathogenesis is critical both to understanding how viruses cause disease and to designing safe and effective therapeutics and vaccines.

Chikungunya virus (CHIKV) is an Old World alphavirus that has caused documented outbreaks of rheumatic disease since its isolation and characterization in 1953 (1-3). The virus causes periodic bouts of endemic disease in Sub-Saharan Africa and Southeast Asia. Recent emergence of the virus into naïve populations in the Western Hemisphere has resulted in a massive epidemic causing over 2 million cases of CHIKV disease. Globalization and climate change have led to more individuals coming into contact with mosquito vectors capable of virus transmission and illustrate a growing need for the development of interventions to limit the burden of CHIKV disease. However, no specific therapeutics or vaccines have achieved licensure. This is due in part to limited knowledge about the molecular basis of CHIKV pathogenesis and a significant gap in our current understanding of the role of CHIKV tropism in the development of disease.

In Chapter I, I review CHIKV epidemiology, virus replication, means of dissemination and tropism in the mammalian host, pathogenesis and disease progression, and the current state of therapeutic and vaccine development. In Chapter II, I describe the design, recovery, and *in vitro* characterization of CHIKV strains with limited replicative capacity at discrete sites in the mammalian host designed to parse apart the contribution of CHIKV replication at these specific sites to pathogenesis. In Chapter III, I describe the pathogenesis of a CHIKV strain with limited replicative capacity in skeletal muscle cells in a mouse model of CHIKV disease. Additionally, I highlight a new target for therapeutic intervention. In Chapter IV, I describe current plans for development of this skeletal muscle-restricted strain as a vaccine candidate due to its attenuation in mice. Finally, in Chapter V, I summarize my thesis studies and discuss future studies for the continuation of this work. Collectively, research described in this dissertation answers critical questions about the influence of CHIKV tropism in pathogenesis. Importantly, this research has yielded both a new therapeutic target and a new method of CHIKV attenuation that may foster development of new CHIKV vaccines.

1.2 Alphaviruses

Alphaviruses are members of the only genus of the *Togaviridae* family, many of which are clinically relevant human pathogens. The family is comprised of 31 members organized into 8 antigenic complexes (Figure 1-1) traditionally categorized into Old World and New World constituents based on their capacity to cause arthritogenic or encephalitic disease, respectively (4). This geographic nomenclature has become outdated as regions of the globe experiencing endemic disease of many of these viruses have expanded significantly due to population shifts and an

increase in host range of associated vectors. Transmission of alphaviruses occurs through the bite of hematophagous invertebrates, most commonly mosquitoes, though some members can be transmitted by ticks, lice, and cliff swallow bugs (5, 6). These viruses infect a variety of vertebrate hosts, including humans (7). Humans represent a dead-end host for all alphaviruses except CHIKV, which is capable of eliciting high enough viral titer in serum to allow transmission through a human-mosquito-human cycle (8). The significant clinical illness caused by many of these viruses, combined with the growing threat of emergence, expansion, and epidemics, has established a critical need to investigate the mechanisms by which they cause disease.

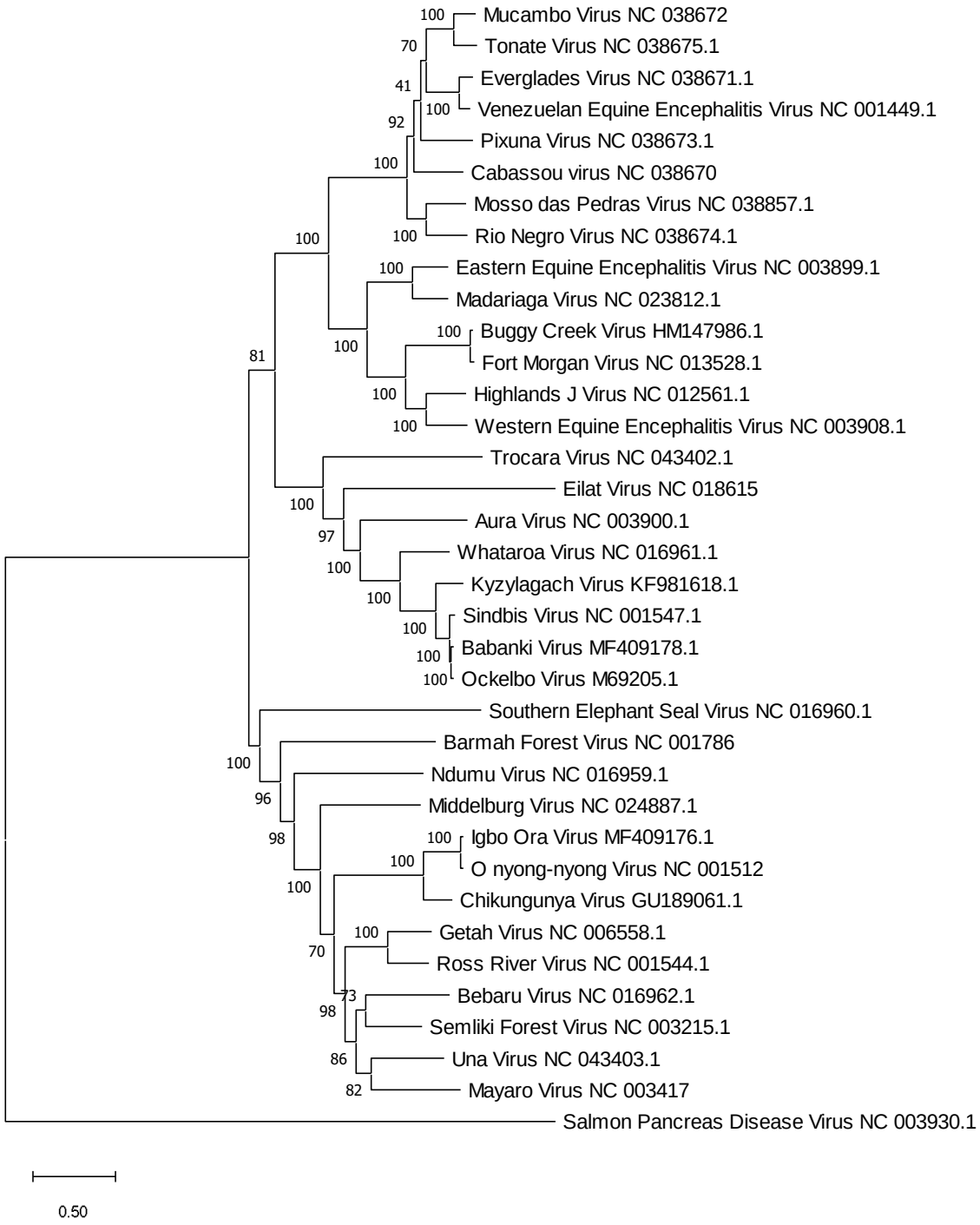

Figure 1-1. Phylogeny of alphaviruses.

Phylogenetic analysis of members of family *Togaviridae* based on complete coding region in GenBank. Evolutionary history inferred using the Maximum Likelihood and General Time Reversible model. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the

branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X (9).

1.3 Chikungunya Virology

CHIKV is an arthritogenic alphavirus of the Semliki Forest virus (SFV) antigenic complex (7). The virus was first isolated from an individual with febrile illness in Tanganyika, now Tanzania, in 1952 (1-3), although CHIKV has likely been circulating since at least 1779 with associated epidemics misattributed to dengue virus (DENV) (10). The name chikungunya is derived from the Kimakonde language and translates to "that which bends up," describing the clinical syndrome observed in infected individuals (1). CHIKV virions are structurally similar to other alphaviruses, being comprised of a small (~70 nm in diameter) enveloped shell encapsidating a single-stranded, positive-sense RNA molecule of ~12 kb (11, 12). The RNA genome resembles a host messenger RNA with a 5' 7-methylguanosine cap and a 3' poly-A tail (12). The first two-thirds of the genome encode the first open reading frame (ORF), which is translated to produce the nonstructural proteins responsible for replication of the viral RNA (13). The latter one-third of the genome encodes the second ORF from which the structural proteins, including the E1 and E2 glycoproteins that are incorporated into the virus envelope, are transcribed and translated (13). In total, CHIKV encodes 10 proteins that mediate replication in host cells (Table I-1).

Table 1-1. Chikungunya virus proteins.

Protein	Size (aa)	Function
Nonstructural protein cassette		
nsP1	535	Methyltransferase and guanylyltransferase activity that caps viral RNA; sole membrane anchor for replicase complex
nsP2	798	N-terminal NTPase, helicase, and RNA triphosphatase activities; C-terminal cysteine protease activity responsible for processing of nonstructural polyprotein
nsP3	530	Phosphoprotein important for minus-strand synthesis; contains macro domain and SH3-binding regions; unknown functions likely mediated through host protein interactions
nsP4	611	RNA-dependent RNA polymerase (RdRp); putative terminal transferase activity
Structural protein cassette		
Capsid	261	Encapsidates genomic RNA to form nucleocapsid core; carboxyl domain is an autocatalytic serine protease
E3	64	N-terminal domain is uncleaved leader peptide of E2; may help shield fusion peptide in E1 during egress
E2	423	Mediates binding to receptors and attachment factors on the cell membrane; major target of neutralizing antibodies
6K	61	Leader peptide for E1; putative ion channel; may enhance particle release
TF	76	Transframe protein resulting from ribosomal frameshifting; shares N-terminus with 6K; putative ion channel; may enhance particle release; expression prevents synthesis of E1
E1	439	Type II fusion protein; mediates fusion of viral envelope and cellular membrane via fusion peptide

Table adapted with permission from (14).

1.4 Chikungunya Fever: Symptoms and Pathogenesis

CHIKV is the causative agent of chikungunya fever (CHIKF). The virus is transmitted to humans through the bite of an infected mosquito, after which the average incubation period is 2-4 days followed by a sudden onset of disease with no prodromal phase (15). Typical acute disease manifestations include headache, high fever, myalgia, nausea, rash, and, most characteristically, a severe, often debilitating polyarthralgia and polyarthritis (16). CHIKV infection is often self-limiting, and clinical symptoms resolve after 7-10 days in most cases.

A number of atypical disease manifestations have been ascribed to CHIKV, occurring mainly in vulnerable populations. In particular, neonates, individuals over the age of 65, and immunocompromised patients are prone to more severe disease following CHIKV infection (16). Atypical symptoms include dermatological disease, hemorrhage, hepatitis, myocarditis, nephritis, neurological disorders, ocular disease, and pancreatitis (17-22). Vertical transmission of CHIKV can occur during childbirth and results in severe illness in the majority of cases (23). Children also are subject to more severe disease, though mainly due to dermatological and neurological complications (24-27).

CHIKV disease is often misattributed to DENV and more recently Zika virus (ZIKV), but there are some differences in disease presentation. Unlike DENV and ZIKV in which the majority of infections are asymptomatic (28, 29), CHIKV infection results in disease in as many as 95% of infected individuals (30). Additionally, a hallmark of CHIKV disease is persistent, recurring arthralgias observed in as many as 60% of symptomatic patients (31). Persistent arthralgias are usually symmetrical and affect primarily the distal joints of the limbs, including the ankles, knees, wrists, and small joints of the hands and feet (16). While infectious virus cannot be recovered from patient tissues during the chronic phase of infection, CHIKV antigen has been detected in synovial

macrophages and muscle progenitor cells from patient biopsies taken during the chronic phase (32, 33). Risk factors for progression to chronic disease include advanced age, pre-existing joint pain or incidence of osteoarthritis, and high viral load during the acute phase (34).

Studying molecular mechanisms of CHIKV disease in humans is challenging, although a few studies have provided insight into the immune responses elicited during both the acute and chronic phases of disease. Acute infection is associated with robust activation of the innate immune system, leading to production of elevated interferon (IFN)-α and IFN-γ (32, 35). A variety of cytokines and chemokines are elevated during CHIKV infection in humans (Table I-2), with severe disease being linked to increased production of IL-1β, IL-6, CXCL10 (IFN-γ-induced protein 10; IP-10), CCL2 (monocyte chemoattractant protein 1; MCP-1), CXCL9 (monokine induced by IFN-γ; MIG), and regulated on activation, normal T cell expressed and secreted (RANTES) (35-37). Additionally, flow cytometric analysis of PBMCs from infected humans has demonstrated an increase in activation of dendritic cells, NK cells, B cells, and $CD4^+$ and $CD8^+$ T cells (32, 38).

Table 1-2. Soluble mediators elevated during CHIKV infection.

Disease	Acute	Severe	Chronic
Proinflammatory cytokines			
IFN-α	+		+
IFN-γ	+		+
IL-1β		+	+
IL-2	+		
IL-2R	+		
IL-6	+	+	+
IL-7	+		
IL-8			+
IL-12	+		+
IL-15	+		
IL-17	+		+
IL-18	+		
TNFα			+
Antiinflammatory cytokines			
IL-1Ra	+		+
IL-4	+		
IL-10	+		+
Chemokines			
G-CSF	+		
GM-CSF			+
IP-10	+	+	
MCP-1	+	+	+
MIG		+	
MIP-1α	+		+
MIP-1β	+		+
Growth factors			
bFGF	+		

Table adapted with permission from (14).

Study of CHIKV pathogenesis using animal models has afforded additional insights into mechanisms underlying disease development. In mice, a robust IFN response is required for control of CHIKV disease, and mice lacking the receptor for type I IFN rapidly succumb to infection (39). Additionally, many of the same proinflammatory cytokines and chemokines increased in humans are upregulated in mice, and serum levels of IL-5, IL-12, IP-10, MCP-1, and MIG correlate with the degree of joint swelling in mice (40, 41). Treatment of mice with bindarit, an inhibitor of MCP-1 synthesis, diminishes arthritis, myositis, and bone erosion following CHIKV infection, indicating a pathogenic role for macrophages (42, 43). Subcutaneous inoculation in the footpad results in infiltration of a variety of cells into the inoculated foot and biphasic swelling, which peaks during both the innate and adaptive phase (41, 44). Dendritic cells, macrophages, neutrophils, and NK cells infiltrate tissues during the innate phase of infection (44-47). Swelling in mice is promoted by activation of the inflammasome, and specific inhibition of NLR family pyrin containing 3 (NLRP3) reduces inflammation and abrogates osteoclastogenic bone loss and myositis (48). During the adaptive phase, B cells and $CD4^+$ and $CD8^+$ T cells infiltrate the joint (41, 44). Of these, $CD4^+$ T cells contribute most to disease, as there is diminished swelling following infection of mice lacking these cells (49). Interestingly, although $CD8^+$ T cells are activated during infection in humans and mice, they are not required for clearance of CHIKV in mice (50). Instead, CHIKV clearance is mediated by macrophages and antibody responses (41, 51).

1.5 Evolution and Epidemiology

Phylogenetic and antigenic analyses of CHIKV strains have led to the hypothesis that CHIKV originated in central Africa (52). In this area, CHIKV is maintained in a sylvatic life cycle between nonhuman primates and forest-dwelling *Aedes* species mosquitoes (*A. furcifer*, *A. africanus*, among others) (53-55). In Africa, the virus has diverged into two principal lineages, termed West African (WA) and East/Central/South African (ECSA) (8). Prior to 1951, the ECSA lineage was imported to Thailand (56) and began circulating in an urban transmission cycle, diverging into a distinct Asian lineage (Figure 1-2) (52, 57). In contrast to the sylvatic cycle in Africa, the peridomestic mosquitoes *A. aegypti* and *A. albopictus* are the only known vector species capable of transmitting CHIKV in Asia (52).

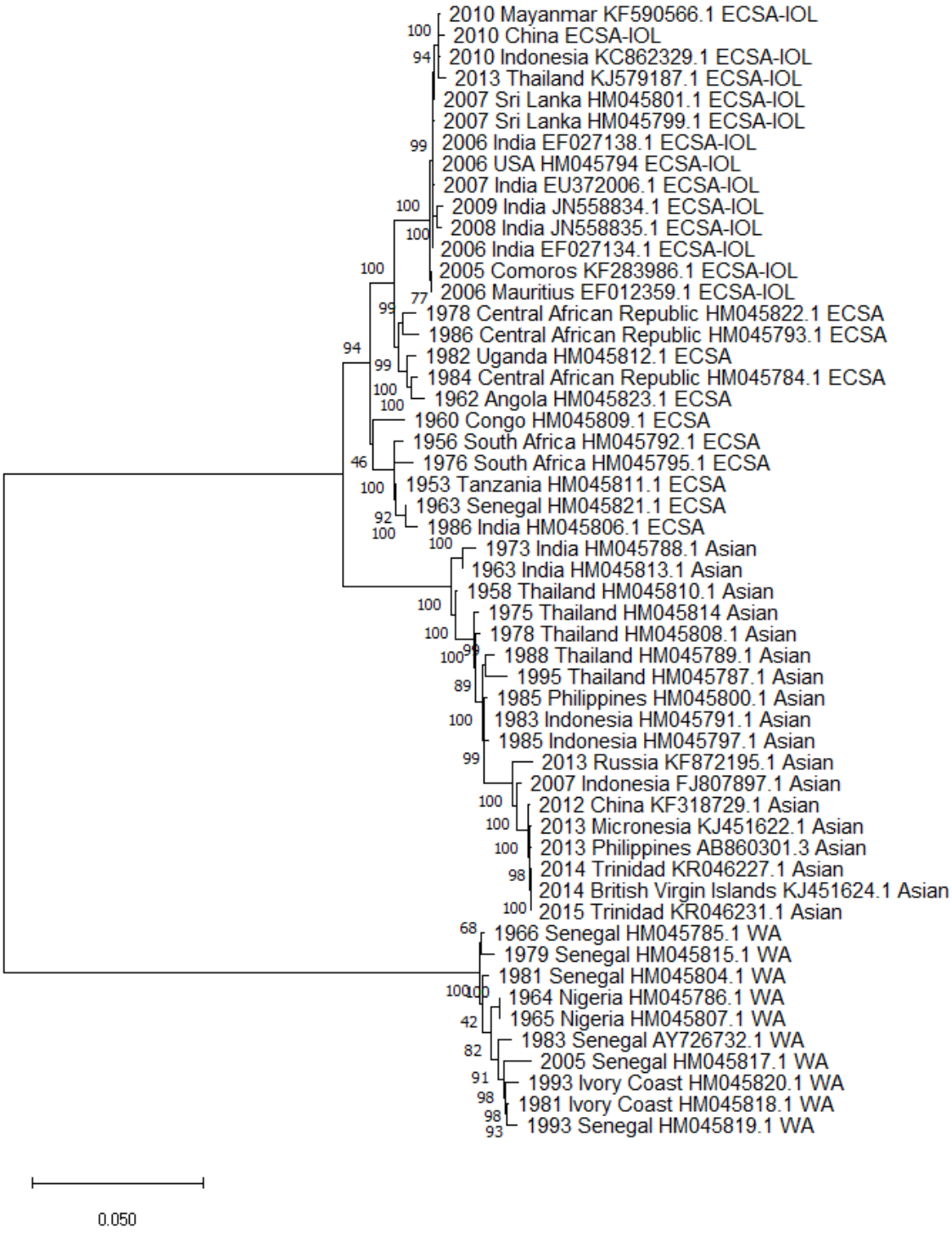

Figure 1-2. Phylogeny of CHIKV strains.

Phylogenetic analysis of members of family *Togaviridae* based on complete coding region in GenBank. Evolutionary history inferred using the Maximum Likelihood and General Time Reversible model. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the

branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X (9).

The three clades of CHIKV have caused numerous bouts of endemic disease in Sub-Saharan Africa and Southeast Asia since their initial discovery (Figure 1-3). CHIKV gained global recognition as an international health threat following two notable recent epidemics. In 2004, an ECSA-lineage virus reemerged in Kenya and expanded to nearby regions including Comoros in 2005, the Seychelles, Madagascar, Mauritius, and La Reunion Island in 2005-2006, and in India in 2006-2007 (58-63). This epidemic led an estimated 6 million cases (16, 64). Some virus isolates during this epidemic acquired an adaptive mutation (E1-A226V) that enhanced vector competence in *A. albopictus* mosquitoes, which exhibit a broader geographic range. This mutation does not compromise the capacity for spread by *A. aegypti* (65). Enhanced transmission by *A. albopictus* due to this mutation was found to require epistatic interactions (E1-98A and E2-211T), which have not been observed in Asian-lineage strains and thus constrain the capacity of viruses from this lineage to be transmitted by *A. albopictus* (65, 66). In these outbreaks, infection resulted in incapacitating disease that was unexpectedly associated with an increased mortality rate, indicating that epidemic strains of CHIKV might also be mutating in such a way as to enhance virulence (24, 67, 68). These cases illustrate the potential for CHIKV to adapt to new vectors as well as its epidemic potential upon emergence into naïve populations.

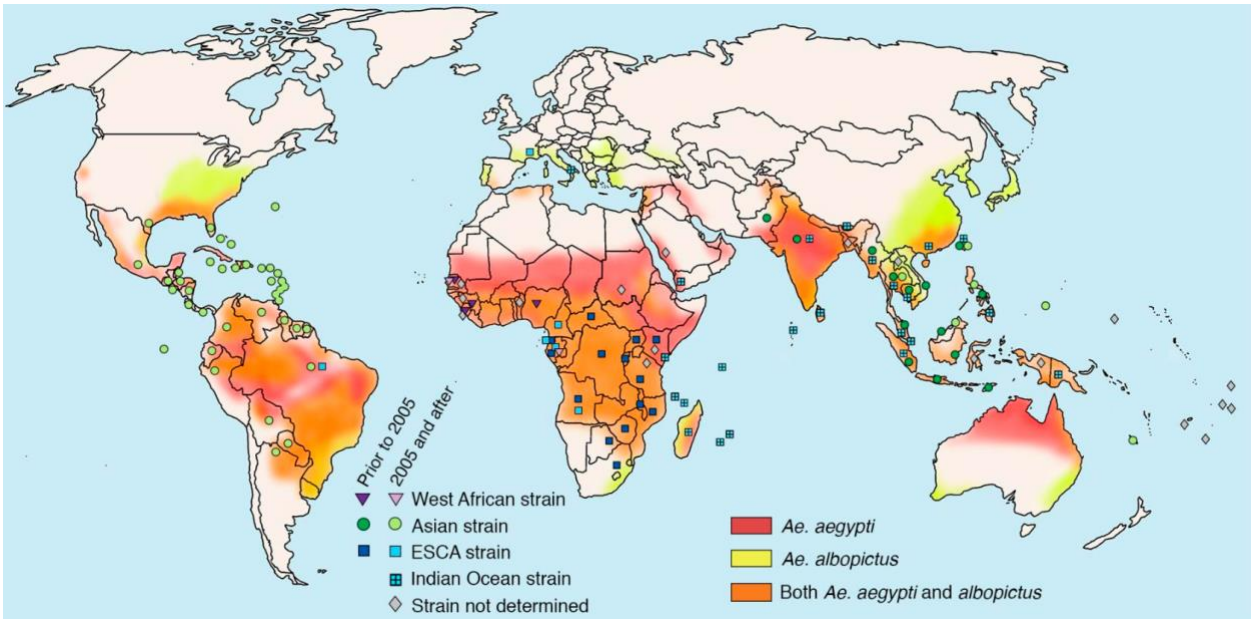

Figure 1-3. Global distribution of CHIKV and vectors.

Geographic distribution of two primary mosquito vectors is shown (*A. aegypti*, red; *A. albopictus*, yellow; both, orange). Areas where locally acquired cases of CHIKV have been documented are indicated by colored symbols representing different clades (WA, purple; Asian, green; ECSA, blue) is overlaid. Figure adapted with permission from (14).

A second notable epidemic began in December 2013 when the virus was identified in the Caribbean Island of French St. Martin, which was the first documented autochthonous (i.e., acquired locally and not imported) case of CHIKV in the Western Hemisphere (69, 70). The epidemic strain was of the Asian lineage, which limited transmission to the more range-restricted *A. aegypti* mosquitoes. Despite this limitation, the effects of the epidemic were significant and far-reaching. On average, the Pan-American Health Organization (PAHO) reported 55,700 new cases per month during the height of the epidemic from December 2013 to December 2017, totaling over 2.5 million total cases for this interval (71). According to the Centers for Disease Control and Prevention (CDC), there have been 4098 cases in the United States, of which 13 were acquired locally (72). This epidemic demonstrated that, although CHIKV has historically been isolated to

regions of the Eastern Hemisphere, with human travel, expansion of vector range, and adaptation of the virus to new vectors, the possibility of virus emergence into naïve populations is significant, and the resulting epidemics are devastating.

1.6 Replication Cycle

CHIKV uses host cell factors to complete its replication cycle (Figure 1-4). Virus attachment and entry into cells are mediated by the E1 and E2 glycoproteins that stud the virion surface. The E1 protein forms a heterodimeric complex with E2. Three of these heterodimers comprise the CHIKV spike, and 80 spikes form the icosahedral virion (11). Attachment to cells is a function of the E2 glycoprotein, as it is the primary target of neutralizing antibodies (73, 74). Identification of a bona fide receptors has been difficult for many members of the alphavirus genus. Many proteins have been implicated in the attachment of CHIKV to host cells, including lectin DC-SIGN, prohibitin 1 (PHB1), and T-cell immunoglobulin and mucin 1 (TIM-1) (75-78). Additionally, work from our lab and others has shown that glycosaminoglycans (GAGs) are important attachment factors, providing low-affinity interactions between CHIKV particles and the cell surface that then allow virus to engage an entry receptor in a high-affinity manner (79-81). Matrix remodeling associated 8 protein (Mxra8) is an entry receptor sufficient for binding and internalization of many arthritogenic alphaviruses, including CHIKV (82). Cryo-EM analysis of Mxra8 in complex with CHIKV virions indicates that the receptor interacts with multiple sites on the E2 protein, although the interaction interface is quite complex and also involves sites in E1 (83). The fact that some CHIKV strains are not fully dependent on Mxra8 for internalization into

cells as well as the lack of Mxra8 expression on some CHIKV target cells indicate the existence of other receptors.

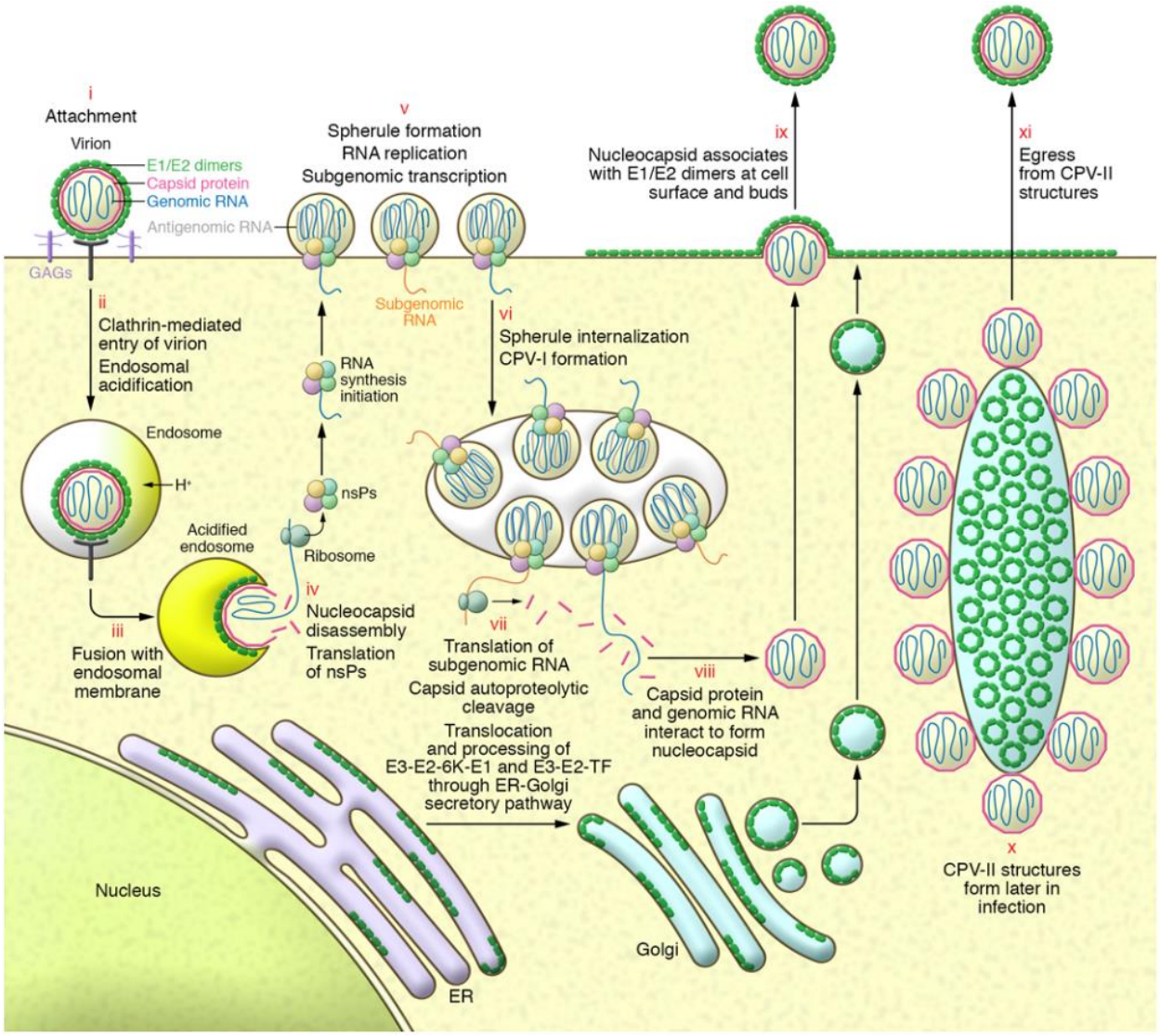

Figure 1-4. Replication cycle of CHIKV.

During infection, (i) CHIKV binds to attachment factors, including glycosaminoglycans, or receptors, including Mxra8, on the cell surface and (ii) is internalized via clathrin-mediated endocytosis. (iii) Acidification of the endosome results in fusion of the viral envelope with the endosomal membrane and (iv) release of the nucleocapsid into the cell cytoplasm, where the genomic RNA is translated to form the replicas. (v) Spherules form at the plasma membrane and house viral replication intermediates. (vi) Internalization of spherules results in formation of CPV-1 structures. (vii) Translation of subgenomic RNA produces the viral structural polyprotein. Capsid is liberated via autoproteolysis and E2/E1 transit through the secretory system for deposition in the plasma membrane. (viii) Capsid and genomic RNA form nucleocapsids in the cytoplasm. (ix) Nucleocapsids bud at the plasma membrane through glycoprotein-

rich regions to form progeny virions. (x) Formation of CPV-II structures occurs later in infection. (xi) Assembly of structural proteins in CPV-II structures promotes formation of mature virions and egress. Figure adapted with permission from (14).

Following attachment to cells, CHIKV is internalized by clathrin-mediated endocytosis (84-86). Much of the process of CHIKV replication is inferred from studies of SFV and Sindbis virus (SINV). Fusion of SFV occurs via the viral E1 glycoprotein, which contains a type II fusion loop that is normally buried between domains of the E2 glycoprotein. The fusion loop becomes exposed following acid-dependent conformational changes in the trimeric spike, inserts into the endosomal membrane, and allows for extrusion of the nucleocapsid into the host cell (87-89). Containing both a cap and poly-A tail, the genome can immediately be translated in the cytoplasm to produce the nonstructural replicase polyprotein P1234 or, in the case of strains with an opal stop following the nsP3 gene, two polyproteins (P123 and P1234) (90). The nonstructural polyprotein is sequentially processed into its components through sequential proteolytic cleavage by nsP2 (91). The P123 polyprotein intermediate along with nsP4 uses the full-length genome as template to transcribe the negative-sense genome (92, 93). The nsPs only form a replicase capable of minus strand synthesis when they are expressed as a polyprotein (92). Full cleavage of the nonstructural polyprotein into individual nsPs leads to a switch from negative-sense genome synthesis to positive-sense synthesis, which is required both for production of additional genomic RNA for packaging into progeny virions and transcription of the subgenomic RNA that is translated into the structural polyprotein (92, 93). The structural polyprotein is introduced into the secretory pathway by a signal sequence present at the N-terminus of the E3 glycoprotein and cleaved into components (capsid, E3, E2, 6K, TF, and E1) by various proteases and subject to posttranslational modification as it transits the secretory pathway prior to deposition in the plasma membrane (94-

99). Nucleocapsids comprised of newly synthesized genomes and capsid protein form intracellularly and bud at viral glycoprotein-rich areas of the plasma membrane to form progeny virions.

CHIKV replication causes morphological changes in the host cell. During early stages of replication, the replicase inserts into the plasma membrane, an activity that is dependent on nsP1 and nsP3 (100, 101). Negative curvature of cellular membranes results in the formation of spherules, which house the replicating viral RNA and protect double-stranded RNA replication intermediates from detection by innate immune sensors (102). As the infectious cycle progresses, spherules are internalized into the cytoplasm to form large cytopathic vacuoles (CPV-I), which function in production of various viral RNAs (102, 103). Another type of large vacuole, cytopathic vacuoles II (CPV-II), forms later in infection, contains helical tubular arrays of viral glycoproteins that reflect the organization of timers on the envelope of mature virions, and may function in transport of structural components to sites of budding in the plasma membrane (104). The virus is highly cytopathic in many susceptible mammalian cells, although this cytopathic effect is not observed in mosquito cells (105). Overall, while much is known about alphavirus replication, critical knowledge gaps remain about how virus proteins interact with each other and host proteins and whether CHIKV uses the same strategies as other alphaviruses.

1.7 Tropism, Dissemination, and Animal Models

Early investigation into the tropism of chikungunya virus was performed mainly *in vitro* and led to the conclusion that CHIKV is capable of infecting a broad variety of cell types from many different species (85, 105-107). However, there is little evidence that CHIKV infects

numerous mammalian and avian species in nature (108-110). While the virus undoubtedly infects nonhuman primates, humans, and mosquitoes, the species tropism of CHIKV appears more constrained than cell culture experiments would indicate. What is clear, however, is that the virus exhibits a broad cellular tropism in the mammals it infects.

To understand mechanisms of tropism, dissemination, and pathogenesis in humans, chikungunya virus has been studied extensively using animal models. These include both immunocompetent and immunodeficient mice (111) as well as in nonhuman primates (112). The model used by our lab, in which 3-to-4-week-old C57BL/6 mice are inoculated subcutaneously in the left rear footpad, recapitulates many aspects of CHIKV disease in humans, including arthritis, myositis, tenosynovitis, and persistence (41, 44). This model, along with the rhesus macaque model of CHIKV disease, have been instrumental in enhancing our current understanding of CHIKV tropism and dissemination.

In humans, CHIKV is deposited into the skin following the bite of an infected mosquito. A number of cells in the skin have been implicated as potential sites of CHIKV replication. Dermal fibroblasts represent the primary target of CHIKV in the skin, being highly susceptible both *in vitro* and *in vivo* (85, 113, 114). Keratinocytes and skin-resident macrophages also have been implicated as potential sites of replication. Conflicting reports exist regarding the capacity of CHIKV to infect human keratinocytes *in vitro* (82, 115, 116). In mice, keratinocytes appear to be susceptible to CHIKV infection only in the absence of IFN regulatory factors 3 and 7 (IRF3/7) (117). The virus is capable of infection and even persistence in macrophages (85, 118), but infection of these cells does not result in significant production of progeny virions (85), and no study has determined whether different macrophage subsets are differentially susceptible to infection.

Following primary replication in the skin, the virus disseminates via lymphatics to the local draining lymph node (119) prior to introduction into the vasculature and hematogenous dissemination to secondary sites. CHIKV establishes extremely high-level viremia, with some infected individuals experiencing viral loads of greater than 10^9 particles/ml in blood (120). Most blood cells are refractory to CHIKV infection, with the exception of monocyte-derived macrophages (85). Based on *in vitro* studies, endothelial cells also are likely targets of CHIKV in humans (85), but the source of virus responsible for the high levels of viremia observed in infected persons remains undefined.

Because of the arthralgia and myalgia caused by CHIKV, musculoskeletal tissues are the most highly studied site of secondary virus replication. CHIKV infects numerous cells in the synovium and musculature. In the joint, synovial fibroblasts and macrophages are both susceptible to CHIKV infection (32, 39, 121), and macrophages are a potential site of virus persistence (118). Chondrocytes and osteoblasts of the cartilage and bone, respectively, also are proposed targets of CHIKV *in vivo* based on *in vitro* susceptibility (82, 117, 122). Skeletal muscle biopsies have demonstrated that myofibers and satellite cells (muscle progenitors) are major targets of CHIKV in humans (33). Muscle fibroblasts also are likely targets of infection in humans, although this has only been demonstrated in mice where, alongside myofibers, fibroblasts serve as sites of persistence (114).

Infection of secondary sites outside of the musculoskeletal system is less frequently documented and leads to rare manifestations of CHIKV disease. Although CHIKV is not considered to be a neurotropic virus, infection is rarely associated with neurological complications (123). Whether these cases result from CHIKV replication in cells of the central nervous system (CNS) remains unclear, but *in vitro* susceptibility assays indicate that neuroblastoma cells, glial

cells, and microglial cells are potential targets of virus infection (124-127). In infected neonatal mice, CHIKV antigen is detected in astrocytes, ependymal cells, neurons, and oligodendrocytes (39, 128). Ocular disease manifestations also have been reported for CHIKV (18), and immunostaining of corneal grafts from donors on La Reunion Island demonstrated viral antigen in keratocytes, fibroblasts of the connective tissue of the sclera, and in the smooth muscle of the ciliary body (129). Finally, the presence of CHIKV RNA in the spleen, liver, and heart of infected rhesus macaques implicates these organs as secondary target sites (119), although the precise target cells at these sites remain undefined.

Overall, many studies have analyzed CHIKV tropism *in vitro* using both immortalized and primary cells. This information, combined with knowledge of susceptible cells and tissues gained from animal models and CHIKV immunohistochemistry in human musculoskeletal biopsies, has demonstrated the broad tropism of the virus *in vivo*. The broad tropism exhibited by CHIKV has made understanding mechanisms of disease development difficult, whether they be from direct cytopathic effect of viral replication in tissues or from elicitation of damaging immune responses. Dissecting the role of viral tropism in CHIKV pathogenesis is the goal of this dissertation.

1.8 Antiviral and Vaccine Strategies

Treatment of CHIKV disease is mainly palliative, involving pain management and treatment of inflammation using non-steroid anti-inflammatory drugs (NSAIDs). In persons who do not respond to NSAIDs or those suffering from chronic disease, treatment with disease-modifying anti-rheumatic drugs (DMARDs), such as methotrexate, hydroxychloroquine, and sulfasalazine, is favored (130, 131). However, the potential for exacerbation of symptoms due to

immunosuppression following treatment with corticosteroids has limited their use in individuals with acute CHIKF (132).

Attempts to develop CHIKV-specific therapeutics have met with limited success. Many high-throughput screens of previously developed chemical libraries have been conducted to identify antiviral compounds (79, 133-135). In addition, target-based modeling approaches have been used to develop therapeutics impeding the protease activity of the viral nsP2 protein (136, 137). While both of these approaches have identified promising options, no compounds have progressed to clinical trials. An additional method to develop antivirals limiting CHIKV disease has been to repurpose compounds developed to treat other diseases that have antiviral activity, including favipiravir, which is used to treat influenza in Japan, ribavirin, which is used to treat respiratory syncytial virus in infants and chronic hepatitis C, and suramin, which is licensed for treatment of trypanosomiasis (138-140). Of these, only ribavirin has been used to treat CHIKV in humans, although small sample sizes have limited conclusive results about the efficacy of treatment (141).

A potential success in the field of CHIKV therapeutic development has been the isolation and characterization of a number of monoclonal antibodies capable of neutralizing the capacity of the virus to infect cells (74, 142-145). These studies were predicated on the knowledge that antibody responses are critical for control and clearance of CHIKV infection (39, 51, 146, 147). Neutralizing monoclonal antibodies protect immunodeficient mice from lethal challenge with virulent CHIKV (142, 145, 146, 148). Importantly, antibody administration 24 h post-inoculation still provided full protection of immunodeficient mice from lethal challenge, with administration as late as 60 h post-inoculation providing some protection with a subset of mice surviving infection (119). The efficacy of neutralizing monoclonal antibody therapy also has been assessed using

nonhuman primates, in which antibody administration 1 and 3 d post-inoculation led to rapid viral clearance and diminished joint inflammation (149). Perhaps most promising, combination therapy with a neutralizing antibody and abatacept, a biological DMARD that inhibits cytotoxic T-lymphocyte-associated protein 4 (CTLA4) to prevent T cell co-stimulation, significantly diminishes joint inflammation, inflammatory cytokine and chemokine production, and leukocyte infiltration into tissues even when administrated several days post-inoculation (150). One final DMARD, a monoclonal antibody directed against tumor necrosis factor-α (TNFα), has been successfully used to treat six patients with chronic chikungunya arthritis who failed to respond to methotrexate therapy (151). A larger sample size is required to understand whether this therapy is truly effective, especially in light of a study showing administration of anti-TNF antibodies in mice exacerbates disease caused by a Ross River virus (RRV), a closely-related arthritogenic alphavirus (152).

CHIKV vaccine development has advanced more rapidly than development of therapeutics with the characterization of many promising candidates, although none have achieved license for use in humans. The first live-attenuated CHIKV vaccine candidate, strain 181/25, was developed by the United States Army Medical Institute of Infectious Disease (USAMRIID) in 1985. The 181/25 strain was isolated by first passaging a virulent CHIKV strain from Thailand, strain 15561, in green monkey kidney (GMK) cells 11 times (153), followed by 18 passages in MRC-5 human fetal lung fibroblast cells (154). Strain 181/25 is attenuated in cell culture and mouse models and elicits immune responses that protect against challenge with virulent CHIKV strains (154). The vaccine also elicits protective immune responses in humans in Phase II clinical trials. However, the incidence of transient arthralgias experienced by a cohort of vaccinated individuals caused the

cessation of 181/25 development as a CHIKV vaccine (155). Despite this, 181/25 is frequently used to investigate the cell biology of CHIKV infection, as it can be studied at BSL2 conditions.

A number of promising vaccine candidates have been developed since cessation of the 181/25 vaccine program in 2000, including inactivated, subunit, DNA, recombinant, and live-attenuated vaccines. Of these, four have progressed to clinical trials. The furthest along in the clinical trial pipeline is a virus-like particle (VLP) vaccine that was initially developed using the structural proteins of WA CHIKV strain 37997 (156). This vaccine is safe and protective in a non-human primate model, elicits neutralizing antibodies in humans after two inoculations, has recently completed Phase II clinical trials, and is projected for Phase III trials (156-158). A Phase II clinical trial is currently recruiting to evaluate this VLP vaccine in prior recipients of other alphavirus vaccines, and while promising, it will be important to determine the durability and duration of the protective immune responses elicited by this candidate. A second candidate is the same VLP, but instead of administration by intramuscular (i.m.) inoculation, the VLPs are vectored using a live-attenuated strain of measles virus (MV) for production (159). In Phase I clinical trials, the MV-VLP vaccine elicits neutralizing antibodies in a dose-dependent manner, and 100% of individuals seroconverted even at low doses (160). However, the vaccine caused adverse event in 58% of individuals in the high-dose cohort (160). While Phase II clinical trials were completed in April 2018, no results have been made available. Two other vaccines have completed Phase I clinical trials, including a replication-deficient simian adenovirus-vector expressing CHIKV structural proteins and a live-attenuated virus in which replicative capacity is diminished by deletion of portions of the nsP3 protein (161, 162). Results of these vaccine trials are not yet available. While promising candidates exist, barriers to licensing a CHIKV vaccine, including identifying at-risk populations in which to conduct Phase III trials, diminishing costs to allow for deployment of

vaccines in resource-limited populations, and developing a vaccine that elicits a robust and durable protective immune response following a single vaccination, are difficult to overcome, and much work remains to be done.

1.9 Significance of the Research

CHIKV is a global public health concern due to its capacity to cause large epidemics of debilitating musculoskeletal disease following introduction into naïve populations. The threat posed by CHIKV will only grow as the virus continues to emerge due to human population shifts and introduction of mosquito vectors into new areas. Treatment and prevention of CHIKV have been difficult due to an absence of targeted antivirals and vaccines, the development of which has been hindered by gaps in knowledge about mechanisms of CHIKV pathogenesis. One such gap is an incomplete understanding of virus tropism and how this tropism influences pathogenesis.

The overall goal of this research was to dissect the role of CHIKV replication at discrete sites in the mammalian host in development of disease. To this end, CHIKV strains were engineered to diminish replicative capacity in specific cells and tissues. In the work presented here, I discovered that one such strain, a virus incapable of replication in skeletal muscle cells, is attenuated in a mouse model of disease. Through characterization of the immune response elicited by this virus, I describe a new therapeutic target for the treatment of acute CHIKV disease. Additionally, I characterize the capacity of the skeletal muscle-restricted CHIKV to serve as a vaccine candidate both alone and in the context of other attenuating mutations formulated into a single live-attenuated vaccine strain. Future studies will further define the protective capacity of vaccine strains recovered during this research and dissect the role of CHIKV replication in other

cells, including endothelial cells, hematopoietic cells, keratinocytes, and osteoblasts, to pathogenesis. Overall, this research answers key questions about CHIKV pathogenesis and will inform the development of targeted therapeutics and vaccines to limit the global burden of CHIKV disease.

2.0 Recovery of miRNA-Restricted CHIKV Strains

2.1 Introduction

A few methods exist to manipulate virus tropism in order to either enhance or restrict the capacity of a virus to replicate in a specific cell, tissue, or organism. A key determinant of virus tropism is the expression of receptors at the cell surface capable of allowing binding and internalization of virions. Diminishing receptor expression can render susceptible cells refractory to infection. This observation is the premise of a number of genetic screens intended to identify virus receptors (82, 163-166). Additionally, nonsusceptible cells often can be made susceptible to infection by ectopic expression of receptors. For instance, expression of the human poliovirus receptor, CD155, in transgenic mice conferred susceptibility to poliovirus infection, allowing study of the virus in a mouse model (167, 168). The same system was used to develop a transgenic mouse model of MV infection through ectopic expression of the human CD46 receptor (169). In this study, receptor expression conferred susceptibility of lung and kidney cells to MV infection, but CD46 expression in bone marrow-derived macrophages did not allow infection (169). In this example, another cell-intrinsic factor, likely expression of host restriction factors, dictates susceptibility of cells to MV and highlights another important consideration in virus tropism. Expression of restriction factors capable of restricting virus replication can ameliorate the capacity of susceptible cell types to support virus infection. For instance, human immunodeficiency virus type 1 (HIV-1) is potently restricted by rhesus tripartite motif-containing protein 5α (TRIM5α), and expression of this restriction factor in susceptible cells renders them refractory to infection by

HIV-1 (170). Collectively, genetic manipulation of receptors and host restriction factors can assist in dissecting the contribution of virus tropism to pathogenesis.

Identification of a proteinaceous receptor used by CHIKV to gain entry into cells has been difficult. The first bona fide entry receptor for CHIKV, Mxra8, was identified in 2018 (82, 83). Because infection of some cell types appears to be Mxra8-independent, and because CHIKV strains differentially require Mxra8 for infection, it would be difficult to use genetic manipulation of Mxra8 expression to study CHIKV tropism and pathogenesis in a mouse model (82). Additionally, while many host restriction factors are important for control of CHIKV *in vitro*, including IFN-stimulated gene (ISG) 15 (171), viperin (172), and tetherin (173), whether expression of these molecules in tissues is capable of fully restricting CHIKV infection is unknown. Instead of using these traditional techniques to investigate CHIKV tropism, I used a system in which target sequences corresponding to tissue-specific host miRNAs were engineered into the viral genome to specifically restrict replication at discrete sites and evaluate the contribution of replication at those sites to pathogenesis.

2.1.1 miRNA Biogenesis and RNAi

miRNAs are small (~22 nucleotides) RNA molecules that mediate post-transcriptional silencing of messenger RNA (mRNA) molecules. The first miRNAs were described in *Caenorhabditis elegans* in 1993 (174, 175), and significant efforts since that time have promoted an understanding of their biosynthesis, regulation, and mechanism of action. miRNAs are transcribed in the nucleus of the cell most commonly by RNA polymerase II (176). The majority of canonical human miRNAs are produced from intronic regions of noncoding or coding transcripts. However, some miRNAs are produced from exons, and others are intergenic, being

transcribed independently by their own promoters (177, 178). Several miRNAs can be encoded at the same genetic locus, constituting a polycistronic transcription unit called a cluster (179, 180). Regardless of their germline location, miRNAs are transcribed as long (typically over 1 kb) primary miRNA (pri-miRNA) molecules with a characteristic stem-loop structure containing the mature miRNA sequences (180). In the nucleus, the pri-miRNA is cleaved by nuclear RNase III Drosha into a small (~65 nucleotide) hairpin-shaped pre-miRNA (181). Following cleavage, the pre-miRNA is exported into the cytoplasm by the action of exportin 5 (EXP5) in the nuclear pore complex (182, 183). In the cytoplasm, the pre-miRNA is cleaved by a cytoplasmic RNase III, Dicer, into the mature miRNA duplex (184).

Argonaute (AGO) proteins mediate the effector function of the RNA silencing machinery (185). To accomplish this activity, one of the strands of the mature RNA duplex must be loaded onto the AGO protein to form the RNA-induced silencing complex (RISC) (186). While it is possible for both strands of a mature miRNA duplex to be AGO-loaded, one strand, called the guide strand, is often preferentially loaded due to lower thermodynamic stability of the 5' end of the RNA (187). The other strand, called the passenger strand, is unwound from the guide strand following loading (187). The guide RNA then directs the RISC to mRNA molecules due to sequence complementarity between the guide RNA and target sequences in the mRNA (188).These sequences are most often found in the 3' untranslated region (UTR) of the mRNA, although miRNAs exist that guide RISC to target sequences in the 5' UTR, mRNA coding sequences, and gene promoters (188, 189). RISC-mRNA interaction leads to translational silencing of the targeted gene, although the mechanism through which this is accomplished is determined by the degree of complementarity exhibited by the miRNA and mRNA target sequences. If these sequences exhibit perfect complementarity, AGO cleaves the mRNA,

facilitating its degradation by exoribonucleases in the cytoplasm (190-192). Imperfect complementarity promotes sequestration of mRNA to translationally silent sites of mRNA decay called P-bodies (193, 194).

2.1.2 RNAi and Viruses

The RNA silencing machinery is used in many organisms as a form of antiviral defense. Perhaps the most rudimentary form of this strategy is the clustered regularly interspersed short palindromic repeat (CRISPR) system in prokaryotes. CRISPR functions as a bacterial adaptive immune response to infection with phages (195). In this system, foreign phage genetic material that has been cleaved by microbial nucleases is incorporated into CRISPR loci (195-197). Transcription of these loci results in production of CRISPR RNAs (crRNAs) that are loaded onto CRISPR-associated (Cas) nucleases to guide them through complementarity to incoming phage genetic material (196, 197). Stable binding to target DNA adjacent to a protospacer-adjacent motif (PAM) results in introduction of a double-strand break at that site, after which the foreign genetic material can be degraded (198-200).

The RNA silencing machinery also is used as an antiviral defense in plants, nematodes, and arthropods. Instead of using germline encoded miRNAs, however, the small RNAs used in this antiviral defense result from Dicer cleavage of viral genetic material from double-stranded replication intermediates and intramolecular hairpins in viral genomic RNA (201-207). After loading onto AGO proteins, small viral RNAs (viRNAs) are able to potently restrict virus replication, as their perfect complementarity to target sequences in the viral genome mediates direct cleavage and degradation of genomic RNA by AGO (208-210).

Although RNA silencing is deployed successfully as an antiviral defense in some organisms, there is little evidence that the germline-encoded miRNA system employed by vertebrates serves as an antiviral defense. Because host miRNAs are not derived directly from viral genetic material, they do not exhibit the perfect complementarity required to serve as potent suppressors of viral replication. Even if a miRNA could bind through partial complementarity to viral genetic material and cause sequestration to translationally silent regions of the cell, few miRNAs are expressed at high enough copy number to restrict viral replication using this less potent silencing mechanism (211, 212). In spite of this limitation, some miRNAs target viruses to restrict replication. Primate foamy virus type 1 (PFV-1), human T cell leukemia virus type 1 (HTLV-1), HIV-1, influenza A virus (IAV), enterovirus 71 (EV71), porcine reproductive and respiratory syndrome virus (PRRSV), and infectious bursal disease virus (IBDV) are all restricted by vertebrate RNAs (213-224). RNAi also has been coopted by viruses to enhance virulence. For example, binding sites for a hematopoietic-specific miRNA, miR-142, in the 3' UTR of eastern equine encephalitis virus (EEEV) specifically restrict virus replication in myeloid cells, although this restriction actually leads to enhanced virulence, as the virus can escape detection by the host innate immune response (225). Finally, hepatitis C virus (HCV) and bovine viral diarrhea virus (BVDV) both co-opt host miRNAs in complex with AGO proteins to stabilize their own genomes to enhance replication (226-229). While vertebrate RNAi does not serve in the same antiviral capacity as invertebrates and plants, it appears that miRNAs are capable of regulating viral replication in the appropriate contexts.

2.1.3 Harnessing RNAi to Dissect Virus Tropism

A number of studies have used the mammalian RNA silencing machinery in order to manipulate viral tissue and species tropism. This system has been predicated on the engineering of miRNA target sequences exhibiting perfect complementarity to host miRNAs into viral genomes in order to allow for potent restriction by mammalian RNAi. This technique is an attractive strategy for studying tropism because many miRNAs exhibit both species-specific and cell type-specific expression (211). One of the first applications of this approach was for gene therapy in which miRNA target sequences for hematopoietic-specific miR-142 were introduced into a lentiviral vector to prevent immune responses originating in the hematopoietic compartment against the therapeutic lentivirus (230). Since this study, miRNA-mediated restriction of viral replication has been repurposed to understand the pathogenesis of multiple viruses and to attenuate their virulence. For example, incorporation of central nervous system (CNS)-specific miR-124 sequences into the 5' UTR and ORF of the poliovirus genome specifically limits viral replication in the CNS of mice, to attenuating neurovirulence while still eliciting a robust and protective immune response (231). Incorporation of muscle-specific miR-133 and -206 sequences into the 3' UTR of the coxsackievirus A21 (CVA21) genome restricts virus replication in skeletal muscle and prevents lethal myositis observed in wild-type (WT) virus-infected mice (232). This system also has been used to study viral dissemination. For example, restriction of DENV replication in hematopoietic cells through addition of miR-142 target sites in the 3' UTR limits dissemination to sites of secondary virus replication, implicating hematopoietic cells as a critical cell type for hematogenous dissemination (233). Finally, this system has been used to restrict species tropism. Introduction of target sequences for miR-192, which is expressed in the respiratory tract of humans and mice but not ferrets, into the genome of IAV restricts viral replication and disease development

in mice without affecting the course of infection in ferrets (234). One interesting application of introducing species-specific miRNA target sequences into a virus was the integration of both CNS-specific miR-124 and mosquito-specific miR-184 and -275 sequences into the genome of DENV, which limited neurovirulence in mice and restricted virus replication in mosquitoes (235). This study demonstrates that miRNA-target sequences can be engineered into live-attenuated vaccine candidates to limit transmission to vector species.

While usurping the mammalian RNA silencing pathway provides a useful tool for studying the role of viral tropism in pathogenesis, a few caveats should be noted when employing this system. First, near-perfect complementarity between host miRNAs and target sequences engineered into the virus genome is required to restrict virus replication in cells, which leads to a risk that mutations selected in target sites will allow viruses to escape targeting. This risk has been mitigated in studies employing this technique through introduction of multiple target sequences for a specific miRNA into the genome of the virus of interest as well as through engineering target sequences into multiple sites in the viral genome (231-235). Additionally, infecting cells with viruses encoding miRNA target sequences may indirectly affect the transcriptome of the host cell due to competitive hybridization of the host miRNA, leading to inefficient silencing of other host transcripts (236). The potential for saturation of host miRNAs and possible effects on transcriptomics should be studied further, although these effects likely would not be observed in the context of studies of acute disease. With an understanding of the potential for the use of miRNA-targeting to investigate the role of viral tropism in pathogenesis, I sought to apply this technique to studies of CHIKV.

2.2 Results

2.2.1 Design of miRNA-Restricted CHIKV Strains

I chose to engineer miRNA-restricted strains in the background of virulent CHIKV strain SL15649, a virus strain isolated in the serum of a patient during an outbreak of CHIKV disease in Sri Lanka in 2006 (44). This isolate was passaged minimally in cell culture prior to sequencing in order to assemble an infectious cDNA clone (44). To recover miRNA-restricted CHIKV strains, I first had to identify a location in the viral genome capable of accepting large insertions of exogenous genetic material. Initial attempts in our lab to recover these viruses were informed by a previous study using SFV. In this study, SFV neurovirulence was attenuated by insertion of target sequences of CNS-specific miR-124 in-frame in the nonstructural ORF of the viral genome between the nsP3 and nsP4 genes (237). This site is normally cleaved by the nsP2 viral protease, and the protease cleavage site was duplicated to ensure that nsP2 cleaves at the 5' and 3' ends of the miRNA insert cassette. Because the insert was in-frame in the coding sequence of the viral nonstructural polyprotein, the peptide resulting from translation of the insert cassette was cleaved from the mature replicase. We used the same strategy to engineer miRNA-restricted CHIKV strains (Figure 2-1A). To recover these viruses, *in vitro* transcribed RNA was electroporated into baby hamster kidney cells (BHK-21) and harvested when cytopathic effect (CPE) was observed in electroporated cells. Recovery of strains containing miRNA target sequences by this method took much longer than WT SL15649, as CPE was not observed for many days post-electroporation. Analysis of insert maintenance in recovered virus stocks by consensus sequencing showed that strains anticipated to contain miRNA-target sequences had purged the insert cassettes and reverted

to WT sequence. Thus, this strategy for recovering miRNA-restricted CHIKV strains was abandoned.

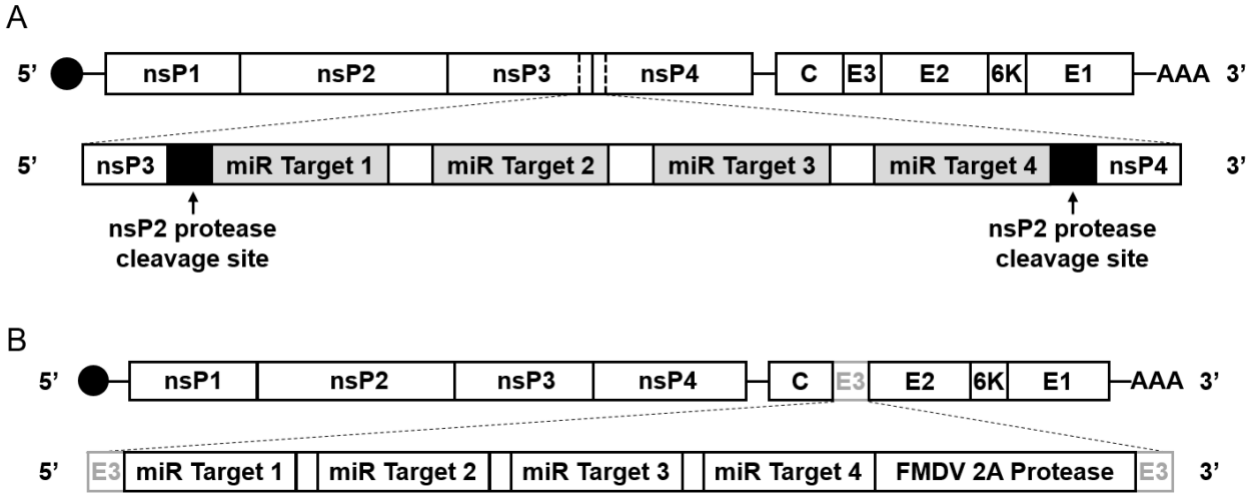

Figure 2-1. Schematic of miRNA-restricted CHIKV strains.

(A) Schematic of strategy in which miR-target sequences were inserted in frame in the nonstructural ORF of CHIKV strain SL15649. Four copies of miRNA seed sequences were appended at the 5' and 3' end by protease cleavage sites recognized by the virus nsP2 protease (black boxes). (B) Schematic of strategy in which miR-target sequences were inserted in frame in the structural ORF of CHIKV strain SL15649 between coding regions of the virus E3 protein. Sequences were appended at the 3' end by coding sequences of the FMDV 2A protease.

Our second attempt to recover miRNA-restricted viruses adapted a cloning strategy employed to recover alphavirus strains expressing reporter proteins to track replication and dissemination in animal models (238). Using this system, I engineered miRNA-target cassettes in-frame in the viral structural ORF between coding regions of the E3 glycoprotein. Insert cassettes were appended at the 3' end with coding sequence of the foot-and-mouth disease virus (FMDV) 2A protease (Figure 2-1B). Because the capsid protein at the 5' end of the insert sequence autocleaves from the structural polyprotein, this strategy ensures that, while a peptide is translated from the inserted sequence, this peptide is not incorporated into the virion. Viruses were readily

recovered following electroporation of *in vitro* transcribed RNA into BHK-21 cells, and analysis of virus stocks by consensus sequence confirmed maintenance of the insert cassettes in all recovered miR-restricted strains. Viruses were engineered with target sequences for endothelial-specific miR-126-3p (239), hematopoietic cell-specific miR-142-3p (211, 225, 233), keratinocyte-specific miR-203-3p (240), osteoblast-specific miR-2861 (241), and skeletal muscle-specific miR-206-3p (232, 242). I termed the recovered viruses ENDO (endothelial-restricted), HEM (hematopoietic-restricted), KER (keratinocyte-restricted), OST (osteoblast-restricted), and SKE (skeletal muscle cell-restricted). Importantly, all miRNAs chosen for this study are identical in humans and mice with the exception of osteoblast-specific miR-2861, which differs by one nucleotide. I also recovered a virus containing target sequences for a mosquito-specific miRNA, miR-275-3p (235), a virus I termed MOS. Because this miRNA is not expressed in mammalian cells, this virus was anticipated to serve as a control in mice and mouse cells for deleterious effects conferred by introducing exogenous genetic material into the E3 coding region of the virus. Nucleotide sequences of miRNA target inserts for all viruses engineered in this study are reported in Table II-1.

Table 2-1. Insert sequences of miRNA-restricted CHIKV strains.

Virus	Targeting miRNA	Insert sequence
Endothelial-restricted	miR-126-3p	GCCATCCGCATTATTACTCACGGTACGACGATCGCATTATTACTCACGGTACGACTCGAGCGCATTATTACTCACGGTACGATCACCGCATTATTACTCACGGTACGAAACTTTGACCTACTTAAGTTGGCGGGAGACGTTGAGTCCAACCCTGGGCCCAGTCTT
Endothelial mismatch	N/A	GCCATCCGGATAATAACCCATGGAACCACGATAGCTTTGTTGCTAACCGTTCGTCTCGAGCGGATAATAACCCATGGAACCATCACGGCTTTGTTGCTAACCGTTCGTAACTTTGACCTACTTAAGTTGGCGGGAGACGTTGAGTCCAACCCTGGGCCCAGTCTT
Hematopoietic-restricted	miR-142-3p	GCCATCATCCATAAAGTAGGAAACACTACAACGATCCATAAAGTAGGAAACACTACACTCGAGATCCATAAAGTAGGAAACACTACATCAATCCATAAAGTAGGAAACACTACAAACTTTGACCTACTTAAGTTGGCGGGAGACGTTGAGTCCAACCCTGGGCCCAGTCTT
Hematopoietic mismatch	N/A	GCCATCATACACAAGGTTGGTAATACAACTACGATACACAAGGTTGGTAATACAACTCTCGAGATACACAAGGTTGGTAATACAACTTCAATACACAAGGTTGGTAATACAACTAACTTTGACCTACTTAAGTTGGCGGGAGACGTTGAGTCCAACCCTGGGCCCAGTCTT
Keratinocyte-restricted	miR-203-3p	GCCATCCTAGTGGTCCTAAACATTTCACCTTCTAGTGGTCCTAAACATTTCACCTCGAGTCTAGTGGTCCTAAACATTTCACACTCTAGTGGTCCTAAACATTTCACCAAAAACTTTGACCTACTTAAGTTGGCGGGAGACGTTGAGTCCAACCCTGGGCCCAGTCTT
Keratinocyte mismatch	N/A	GCCATCCTTGTCGTGCTCAATATATCTCCTTCAAGCGGACCAAAGCACTTTACCTCGAGTCTTGTCGTGCTCAATATATCTCACTCAAGCGGACCAAAGCACTTTACCAAAAACTTTGACCTACTTAAGTTGGCGGGAGACGTTGAGTCCAACCCTGGGCCCAGTCTT
Mosquito-restricted	miR-275-3p	GCCATCGCGCTACTTCAGGTACCTGAACGATGCGCTACTTCAGGTACCTGACTCGAGGCGCTACTTCAGGTACCTGATCACAGCGCTACTTCAGGTACCTGAAACTTTGACCTACTTAAGTTGGCGGGAGACGTTGAGTCCAACCCTGGGCCCAGTCTT
Osteoblast-restricted	miR-2861	GCCATCCCGCCCGCCGCCAGGCCCCCGATCCGCCCGCCGCCAGGCCCCCTCGAGCCGCCCGCCGCCAGGCCCCTCACCCGCCCGCCGCCAGGCCCCAACTTTGACCTACTTAAGTTGGCGGGAGACGTTGAGTCCAACCCTGGGCCCAGTCTT
Skeletal muscle-restricted	miR-206-3p	GCCATCCCACACACTTCCTTACATTCCACGATCCACACACTTCCTTACATTCCACTCGAGCCACACACTTCCTTACATTCCATCACCCACACACTTCCTTACATTCCAAACTTTGACCTACTTAAGTTGGCGGGAGACGTTGAGTCCAACCCTGGGCCCAGTCTT
Skeletal muscle mismatch	N/A	GCCATCCCTCATACATCGTTGCACTCAACGATACATACACTCCCGTATATCCCTCTCGAGCCTCATACATCGTTGCACTCAATCACACATACTCTCCCGTATATCCCTAACTTTGACCTACTTAAGTTGGCGGGAGACGTTGAGTCCAACCCTGGGCCCAGTCTT

E3 sequence; miR-target sequence; FMDV 2A protease sequence

2.2.2 Characterization of miRNA Expression Profiles in Common Cell Lines

In order to validate that miRNA-restricted CHIKV strains replicated with expected kinetics in cell culture, I first identified cells that could be used to assess replication of the engineered strains in restrictive and nonrestrictive conditions. To begin assessing replication in nonrestrictive conditions, I sought to identify cell lines that did not express any of the restrictive miRNAs. Because I used BHK-21 cells to recover virus, African green monkey kidney epithelial cells (Vero81) to titer virus, and human osteosarcoma cells (U-2 OS) to characterize replication kinetics of virus strains, I first defined miRNA expression profiles in each of these cell types. This analysis allowed identification of cells lacking expression of all miRNAs of interest and ensured that artifactual deficits in the replication of any of our miRNA-restricted CHIKV strains were not observed due to endogenous expression of a targeting miRNA. I harvested and purified miRNAs from cells and reverse transcribed and amplified the miRNAs by PCR. Because miRNAs are only ~22 nucleotides long, which is the size of normal PCR primers, they cannot be amplified using conventional RT-PCR. Instead, I used stem-loop RT-PCR (243). In this assay, reverse transcription is achieved using a primer containing a highly stable stem-loop structure that lengthens the target cDNA to ~100 nucleotides that can then be amplified by conventional PCR and detected following electrophoresis on an agarose gel containing ethidium bromide. Using this technique, miRNA expression profiles were determined for BHK-21, Vero81, and U-2 OS cells (Table II-2). Of these, BHK-21 cells did not express any of the miRNAs used to target CHIKV strains engineered for this study. Vero81 cells express endothelial miR-126-3p, indicating all viruses recovered in this study could be titered using these cells, except the endothelial-restricted virus, which could be titered in BHK-21 cells. Finally, U-2 OS cells express both endothelial miR-126-3p and osteoblast miR-2861, indicating that these cells could be used to assess replication of

all viruses in nonrestrictive conditions, except for the endothelial- and osteoblast-restricted viruses, for which BHK-21 cells could be used.

Table 2-2. miRNA expression profiles in common cell lines used to study CHIKV replication.

		Cell line		
		BHK-21	**U-2 OS**	**Vero81**
miRNA	**Endothelial**	Absent	Present	Present
	Hematopoietic	Absent	Absent	Absent
	Keratinocyte	Absent	Absent	Absent
	Osteoblast	Absent	Present	Absent
	Skeletal muscle	Absent	Absent	Absent
	Mosquito	Absent	Absent	Absent

After defining miRNA expression profiles in the cell lines commonly used in the lab to study CHIKV, I next identified cell lines that could be used to define replication restriction of each of the viruses. For this purpose, such cells should express the cognate miRNA for which target sequences were engineered into a miRNA-restricted virus strain. To identify these cells, I turned first to common cell lines immortalized from primary cultures of each of the cell types of interest (Table II-3). To identify a cell line for characterization of replication restriction of the endothelial-restricted virus, I analyzed miRNAs purified from SV40-transformed (SVEC4-10) endothelial cells and human microvascular endothelial cells (HBMECs). Of these, only HBMECs were confirmed to express the endothelial cell-specific miRNA. A monocyte macrophage cell line, J774A.1, was confirmed to express hematopoietic cell-specific miR-142-3p. XB-2 cells, a murine keratinocyte cell line, did not express keratinocyte-specific miR-203-3p, but primary murine keratinocytes isolated from the tail skin of adult mice were confirmed to express this miRNA.

Primary osteoblasts isolated from the skull of mice were confirmed to express osteoblast miR-2861, while murine ST2 cells that were differentiated into osteoblasts through addition of bone morphogenetic protein 2 (BMP-2) did not express this miRNA (244). Finally, a murine stromal cell line (C2C12), which was differentiated into myotubes by culture in 2% horse serum (245, 246), was confirmed to express skeletal muscle-specific miR-206-3p.

Table 2-3. miRNA expression profiles in immortalized and primary cell lines for use in demonstrating miRNA-mediated restriction of CHIKV striains.

Tissue type	Cell line	miRNA	Present/absent
Endothelial	SVEC4-10	miR-126-3p	Absent
	HBMEC	miR-126-3p	Present
Hematopoietic	J774A.1	miR-142-3p	Present
Keratinocyte	XB-2	miR-203-3p	Absent
	Primary murine	miR-203-3p	Present
Osteoblast	Differentiated ST2	miR-2861	Absent
	Primary murine	miR-2861	Present
Skeletal muscle	Differentiated C2C12	miR-206-3p	Present

Following identification of cell lines expressing each of the miRNAs of interest, I next determined whether these cell lines could be used to test restriction of the miRNA-restricted CHIKV strains. To do this, I infected differentiated C2C12 cells at various multiplicities of infection (MOIs) with either OST or SKE. Because these cells express detectable levels of skeletal muscle-specific miR-206-3p, they should specifically restrict replication of SKE but not OST. Unfortunately, both OST and SKE were capable of infecting these cells at the MOIs tested, although SKE infection may have been slightly impaired at higher MOI (Figure 2-2), indicating that these cells could not be used to determine whether SKE is restricted by its cognate miRNA.

While all miRNAs chosen in this study were selected due to high expression in their selected cell types *in vivo*, it is possible that they are expressed at lower levels or by only a small population of cells of a particular type *in vitro*. To ensure that all cells used to assess replication of the putative miR-restricted strains actually expressed the restrictive miRNAs as they would *in vivo*, I instead assessed replication kinetics in cells transfected with siRNAs mimicking the sequence of cell type-specific miRNAs.

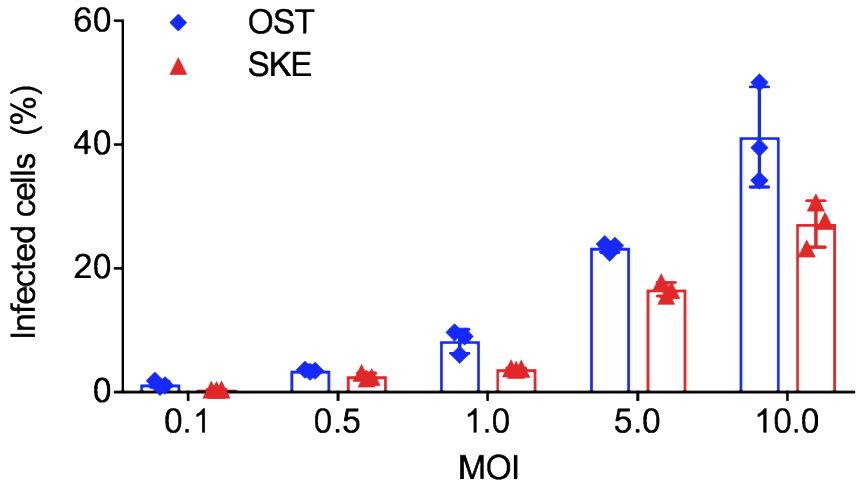

Figure 2-2. Expression of miR-206-3p by differentiated C2C12 cells is insufficient to restrict replication of SKE.

Differentiated C2C12 myotubes were adsorbed with OST or SKE at the MOIs shown at 37$_{o}$C for 1 h. At 6 h post-infection, cells were fixed in cold methanol, and CHIKV infection was assessed by indirect immunofluorescence. Results are expressed as mean percent infected cells from triplicate wells for one independent experiment. Error bars indicate SD.

2.2.3 Recovery and Characterization of miRNA-Restricted CHIKV

2.2.3.1 Mosquito-Restricted CHIKV

I recovered the mosquito-restricted CHIKV strain as I planned to use this virus as a nontargeted control in experiments with each of the mammalian miRNA-restricted strains. To begin characterizing this virus *in vitro*, I analyzed replication kinetics in U-2 OS cells alongside WT SL15649. Both viruses replicated with similar kinetics in these cells, indicating no replicative defects were conferred through insertion of miR-275-3p target sequences in-frame in the structural cassette of MOS (Figure 2-3). Because MOS replicated with the expected kinetics *in vitro*, I next wanted to test whether infection in mice produced a similar progression of disease when compared to infection with WT SL15649. Three-to-four-week-old male C57BL/6J mice were inoculated in the left rear footpad with either MOS or WT SL15649. A major hallmark of CHIKV disease that can be tracked in this model is swelling of the inoculated foot, which can be quantified by measurement with digital calipers. During infection with WT SL15649, mice experience bimodal swelling, with peaks at day 3 and 6 post-inoculation, corresponding to activation of the innate and adaptive immune responses, respectively. Infection with MOS resulted in a similar course of disease (Figure 2-4), indicating MOS-infected mice experience comparable disease to those infected with WT CHIKV.

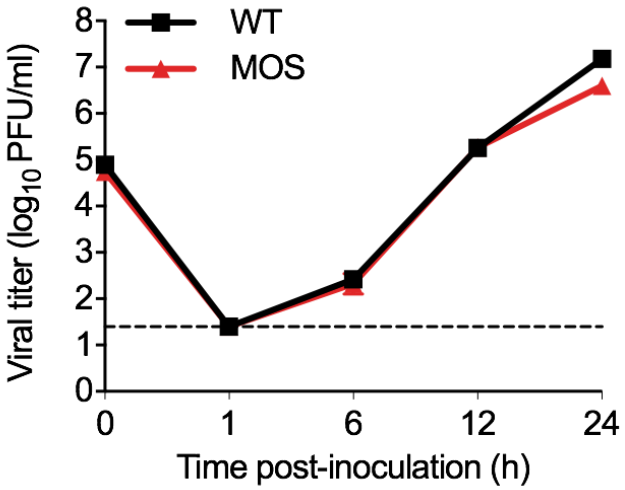

Figure 2-3. Mosquito control virus replicates with kinetics of WT SL15649 *in vitro*.

U-2 OS cells were adsorbed with WT SL15649 or MOS at an MOI of 0.01 PFU/cell. Supernatants were collected at the times shown post-adsorption, and viral titer was quantified by plaque assay. Results are expressed as mean viral titer from duplicate wells of three experimental replicates from one independent experiment. Error bars are present and indicate SD. Dashed line indicates the limit of detection.

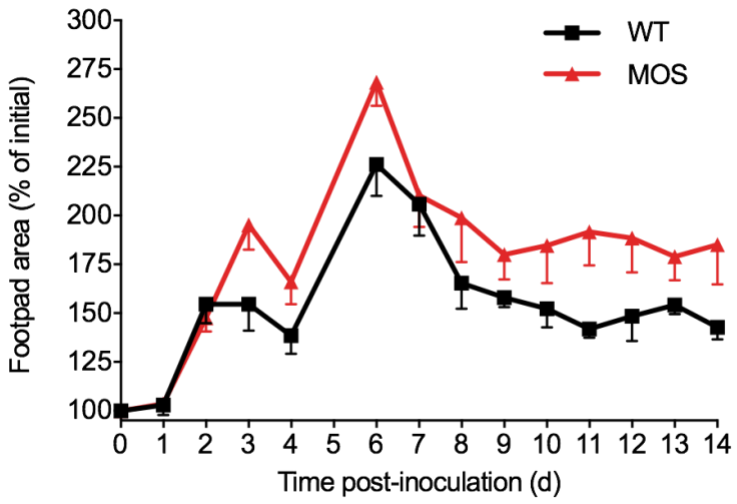

Figure 2-4. Infection with mosquito control virus induces swelling in the inoculated foot comparably to WT SL15649.

Three-to-four-week-old male C57BL/6J mice were inoculated in the left rear footpad with 10^3 PFU of WT SL15649 or MOS. Left rear footpad swelling was quantified using digital calipers on the days shown. Results are normalized to initial footpad area and presented as the mean percent of initial footpad area for 10 mice per group. Error bars indicate SEM.

To test whether CHIKV tropism in mice is altered by insert of exogenous sequence in the viral structural cassette, mice were either mock-infected or inoculated in the left rear footpad with MOS, and left ankle tissue was excised on days 1 and 3 post-inoculation. Musculoskeletal tissue was processed for *in situ* hybridization using a probe specific for CHIKV RNA. No DAB staining corresponding to CHIKV RNA was observed in any section from mock-infected animals. At day 1 post-inoculation, significant staining was only observed in connective tissue of mice infected with MOS (Figure 2-5). This staining also was observed at day 3 post-inoculation, at which time there was significant staining was also observed in myofibers of the interosseous muscle (Figure 2-5). These findings are consistent with previous reports with WT CHIKV (247). Overall, the sites targeted at acute times post-inoculation were comparable to infection with WT strains (247),

indicating that miRNA-restricted viruses do not produce unintended alterations in cell and tissue tropism.

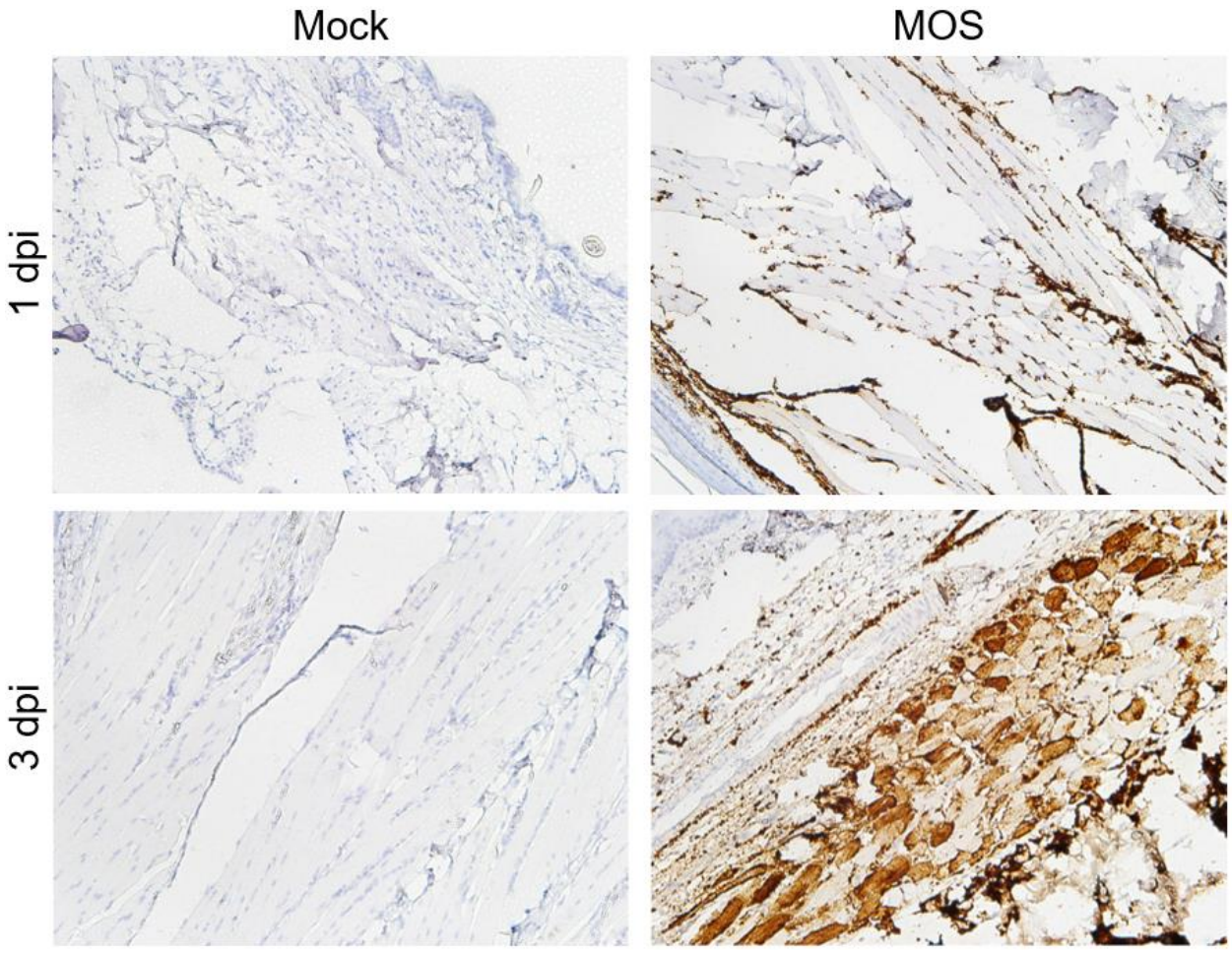

Figure 2-5. Mosquito control virus exhibits expected tropism in musculoskeletal tissue of WT mice.

Three-to-four-week-old male C57BL/6J mice were inoculated in the left rear footpad with PBS (mock) or 10^3 PFU of MOS. Left ankle tissue was collected 1 (top) or 3 (bottom) d post-inoculation for RNAscope *in situ* hybridization for CHIKV RNA. Representative images from 3 (mock) or 5 (MOS) mice per group are shown.

Another hallmark of CHIKV disease in mice is infiltration of immune cells into musculoskeletal tissues as a consequence of a robust adaptive immune response. To determine whether this phenotype was also observed following infection with MOS, mice were either mock-infected or inoculated in the left-rear footpad with MOS. Left ankle tissue was harvested at day 7

post-inoculation and processed for histology. Analysis of hematoxylin and eosin (H&E) stained sections of the interosseous muscle at this time point demonstrated significant infiltration by leukocytes, displacing much of the normal musculature and indicative of severe musculoskeletal disease (Figure 2-6). This finding is consistent with previous reports of WT CHIKV disease (44), suggesting that mice experience similar severity of illness following activation of the adaptive immune response.

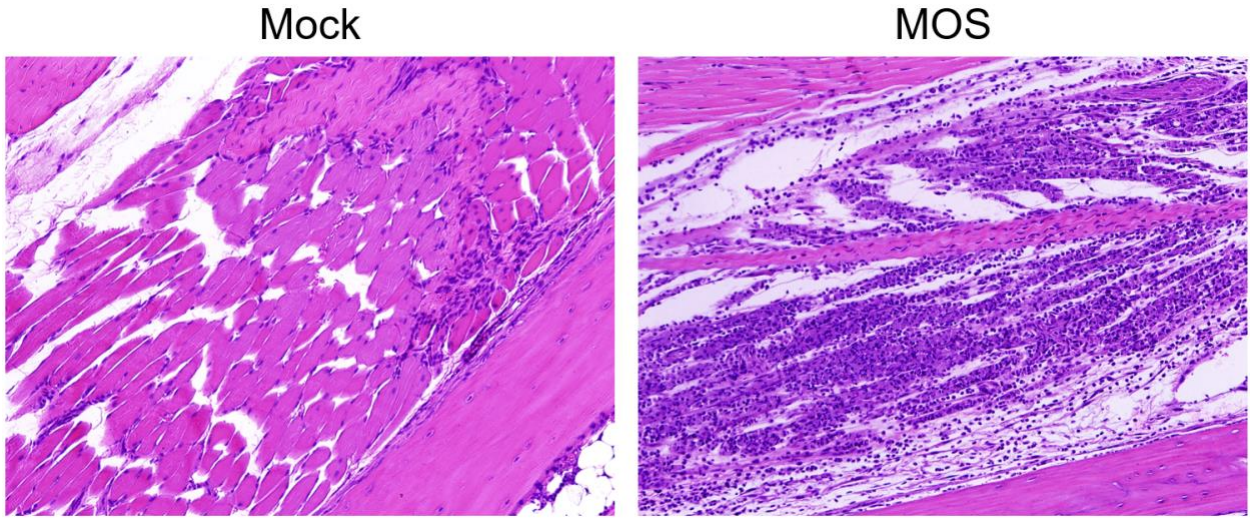

Figure 2-6. Infection with mosquito control virus results in significant infiltration of immune cells during adaptive phase.

Three-to-four-week-old male C57BL/6J mice were inoculated in the left rear footpad with PBS (mock) or 10_3 PFU of MOS. Left ankle tissue was collected 7 d post-inoculation and processed for histology. Representative images of H&E stained sections from 3 (mock) or 5 (MOS) mice per group are shown.

I next wanted to understand how titers in mouse tissues throughout the course of infection with MOS compared with titers in WT-infected mice. Mice were inoculated with WT SL15649 or MOS, and tissues were harvested on day 1 post-inoculation for titer by plaque assay and on days 7 and 28 post-inoculation to determine viral burden by RT-qPCR, as it is difficult to recover infectious virus from mouse tissues at these time points. No differences in titers of MOS and WT

SL15649 were observed in left ankle, left gastrocnemius muscle, or serum of mice at day 1 post-inoculation (Figure 2-7A), indicating that MOS replication and local dissemination are comparable to WT virus. Additionally, titers produced by the two viruses were similar at day 7 post-inoculation in the left and right ankle, left and right gastrocnemius muscle, and left and right quadriceps muscle, indicating that MOS is capable of dissemination to distant sites during infection. Finally, infection with MOS and WT SL15649 resulted in similar levels of CHIKV genome copies in the right ankle at day 28 post-inoculation (Figure 2-7C), indicating that RNA loads at late times post-inoculation are not affected by introduction of miR-target sequences in the structural cassette.

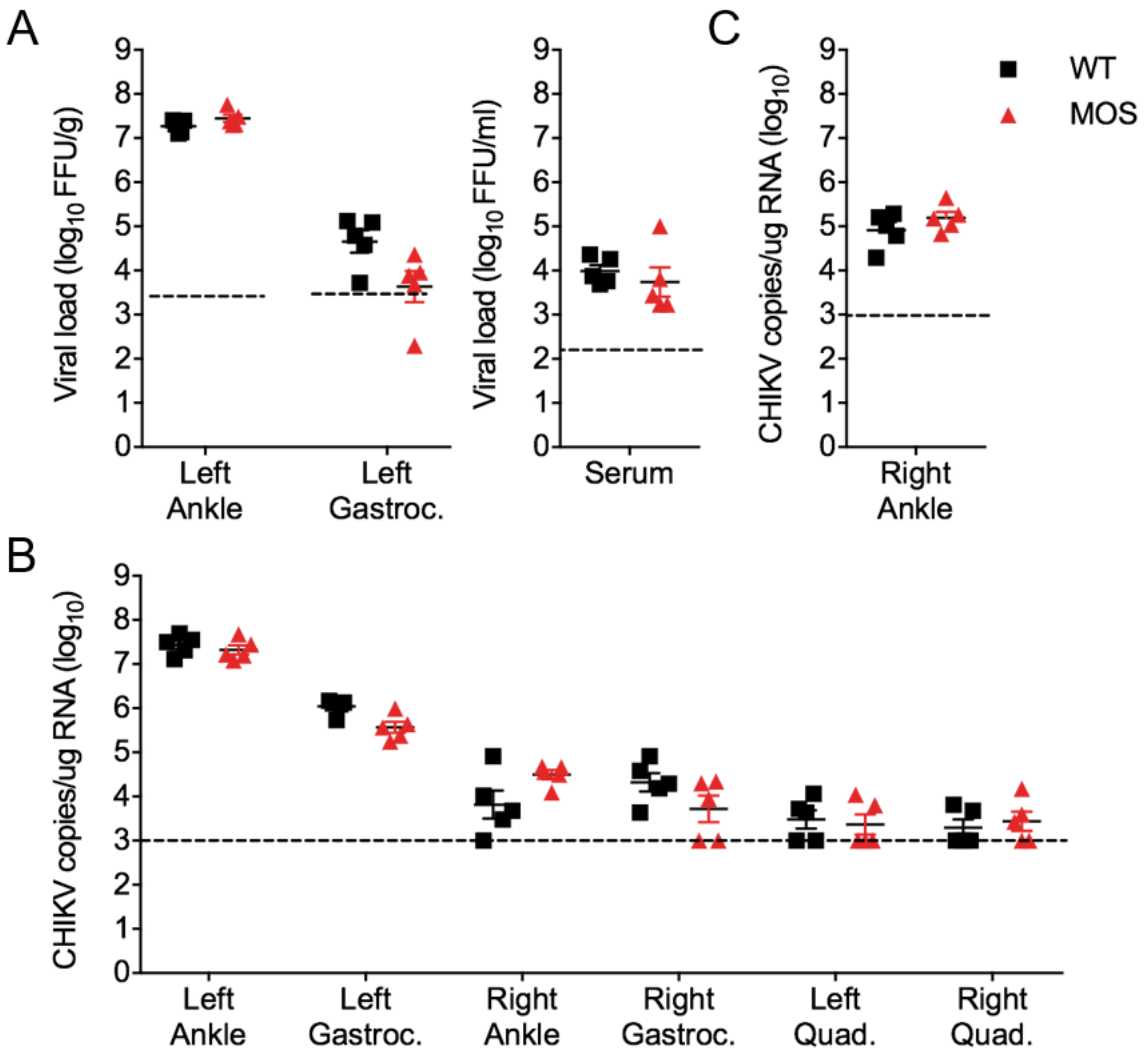

Figure 2-7. Mosquito control virus replicates comparably to WT SL15649 in musculoskeletal tissues.

Three-to-four-week-old male C57BL/6J mice were inoculated in the left rear footpad with 10_3 PFU of WT SL15649 or MOS. At 1, 7, and 28 d post-inoculation, mice were euthanized, ankles, gastrocnemius (gastroc.) muscles, and quadriceps (quad.) muscles were excised, and serum was collected. Viral titers in day 1 (A) tissue homogenates and serum were determined by FFU assay. Horizontal bars indicate mean FFU/g (tissue) or FFU/ml (serum) for 5 mice per group. Viral loads in day 7 (B) and 28 (C) tissue homogenates were determined by RT-qPCR. Horizontal bars indicate mean CHIKV genome copies/mg RNA for 5 mice per group. Error bars indicate SEM. Dashed lines indicate limits of detection.

Finally, while virus stocks of MOS were confirmed to maintain insert cassettes containing miR-275-3p target sites, I wanted to ensure that the miRNA target sequences were maintained following infection in mice. Mice were infected with MOS, and left ankle tissue was harvested at days 3 and 7 post-inoculation. RNA was purified from ankle homogenates, reverse transcribed, and the E3-coding region was amplified by PCR and submitted for consensus sequencing. Analysis of sequencing alongside *in silico*-derived reference sequence demonstrated that the miR-275-3p sequences were quickly altered in such a way as to be completely unrecognizable by day 3 post-inoculation. While exogenous sequence remained at this site, I decided to discontinue use of MOS as a control virus without knowing the basis for selection against the miR-target insert sequences.

2.2.3.2 Design and Recovery of Mismatch-Control Viruses

As an alternative to the mosquito control virus, I engineered mismatched viruses containing silent mutations in miRNA-target sequences to use as controls for each of the miR-restricted CHIKV strains in this study. These mismatch viruses would control for exogenous effects conferred through incorporation of miRNA target sequences in the E3 coding region as well as for any artifact that might arise from peptides translated as a consequence of the miR-target insert sequences. Due to difficulties in early characterization of the osteoblast-restricted viruses (described below), no osteoblast mismatch virus was initially designed. Otherwise, endothelial mismatch (ENDO MM) keratinocyte mismatch (KER MM), hematopoietic mismatch (HEM MM), and skeletal muscle mismatch (SKE MM) control viruses were constructed and recovered following electroporation of *in vitro* transcribed RNA into BHK-21 cells using the same protocol as was used for their cell type-restricted counterparts (Table II-1). All stocks of mismatch control viruses were confirmed to maintain the inserted sequences by consensus sequencing.

2.2.3.3 Endothelial Cell-Restricted CHIKV

The capacity for CHIKV to infect endothelial cells suggests that these cells may be required for dissemination and amplification of the virus during infection. Despite the refractory nature of most blood cells to infection with CHIKV, the virus is capable of reaching very high titers in the blood of infected patients (120), and endothelial cells are a potential source of virus. Additionally, a potential role for endothelial cells in hematogenous dissemination of the virus has yet to be assessed. For these reasons, I engineered a virus that should be restricted in endothelial cells by endothelial cell-specific miR-126-3p. Unfortunately, despite two attempts to recover both ENDO and ENDO MM viruses in BHK-21 cells that do not express miR-126-3p, neither virus could be recovered at titers high enough to assess replication *in vitro* or study *in vivo*.

2.2.3.4 Keratinocyte-Restricted CHIKV

Keratinocytes in the skin represent a potential early target site for CHIKV following deposition by the bite of an infected mosquito, but their role in early dissemination of the virus is unknown. Whether these cells actively support CHIKV replication is a point of contention in the field, with some groups reporting susceptibility of keratinocyte cultures *in vitro* and others reporting failure to infect these cells (82, 115-117). To understand the role of keratinocytes during CHIKV infection, we recovered KER, which should be restricted by keratinocyte-specific miR-203-3p, and mismatch control KER MM. These viruses were both recovered at high titer following electroporation of *in vitro* transcribed RNA into BHK-21 cells.

Before KER and KER MM were used to infect mice, replication of these viruses was assessed in cell culture. BHK-21 cells were adsorbed with 0.01 PFU/cell of either WT SL15649, KER, or KER MM, and production of viral progeny in cell supernatants was quantified at various times post-inoculation by focus-forming unit (FFU) assay. Both KER and KER MM produced

lower titers than WT SL15649 in BHK-21 cells, which should represent nonrestrictive conditions (Figure 2-8). Additionally, these viruses exhibited a small plaque phenotype in Vero81 cells. These findings suggest a replicative defect was conferred by the introduction of sequences containing four target sites for miR-203-3p in the structural cassette of CHIKV.

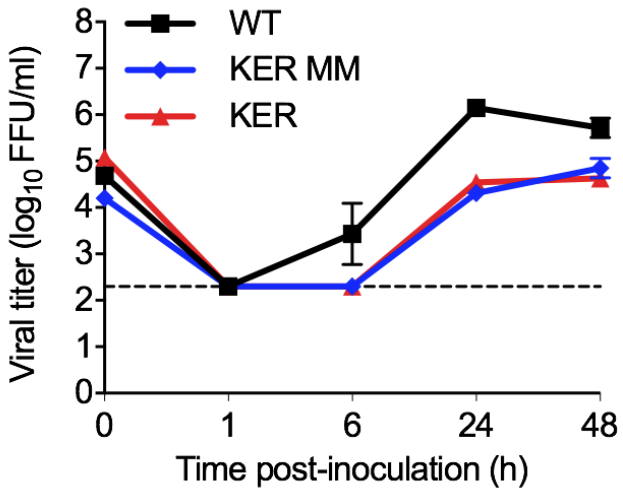

Figure 2-8. Keratinocyte-restricted and mismatch control viruses exhibit diminished replicative capacity *in vitro*.

BHK-21 cells were adsorbed with WT SL15649, KER MM, or KER at an MOI of 0.01 PFU/cell. Supernatants were collected at the times shown post-adsorption, and viral titer was quantified by FFU assay. Results are expressed as mean viral titer from duplicate wells of three experimental replicates from one independent experiment. Error bars indicate SD. Dashed line indicates the limit of detection.

2.2.3.5 Osteoblast-Restricted CHIKV

Infection-mediated death of osteoblasts has been thought to play a role in bone resorption observed in CHIKV-infected individuals based on studies conducted *in vitro* (122). Additionally, disruption of the receptor activator of nuclear factor-κB ligand/osteoprotegerin (RANKL/OPG) ratio, which promotes osteoclastogenesis, has been observed in the joints of infected mice as well as in serum of infected humans, and infection of osteoblasts is thought to contribute to this

phenotype (43, 122). To understand how infection in osteoblasts influences the course of CHIKV disease in mice, I recovered OST, a CHIKV strain that should be incapable of replication in osteoblasts by incorporation of target sequences of osteoblast-specific miR-2861.

I attempted to assess replication kinetics of OST in cell culture before defining the miRNA expression profiles of cell lines used for this characterization. Due to this fact, I analyzed OST replication in U-2 OS cells, which I later confirmed to express detectable levels of miR-2861. In this experiment, U-2 OS cells were absorbed with 0.01 PFU/cell of either WT SL15649 or OST, and titer in culture supernatants was quantified at various times post-adsorption by plaque assay. OST produces lower peak titers in U-2 OS cells compared with WT SL15649 (Figure 2-9A). This replicative defect was potentially exacerbated following transfection of U-2 OS cells with 10 nM miR-2861-mimic siRNA. Following transfection, OST replication in U-2 OS cells was reduced by an additional 4-to-5-fold, although differences in the inoculum may have been responsible for this observation (Figure 2-9B). These data indicate that OST is likely restricted by its cognate miRNA, but additional characterization in cell culture is required to confirm this hypothesis.

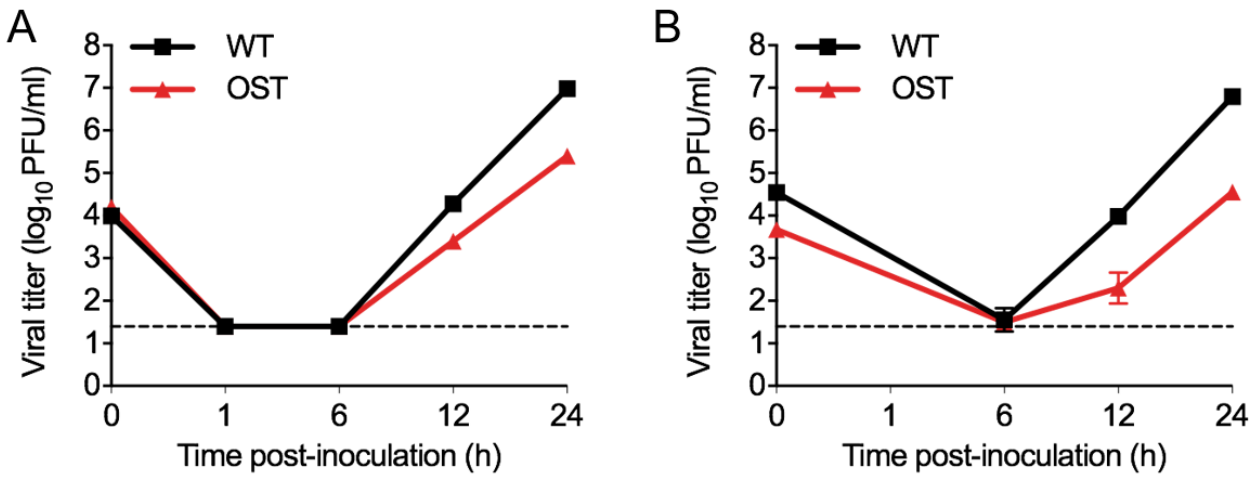

Figure 2-9. Osteoblast-restricted CHIKV exhibits diminished replicative capacity in U-2 OS cells that is exacerbated following transfection of cells with cognate miR-2861.

U-2 OS cells were either untransfected (A) or transfected with osteoblast-specific miR-2861-mimic siRNA (B) prior to adsorption with WT SL15649 or OST at an MOI of 0.01 PFU/cell. Supernatants were collected at the times shown post-adsorption, and viral titer was quantified by plaque assay. Results are expressed as mean viral titer from duplicate wells of one (A) or three (B) experimental replicates from one independent experiment. Error bars indicate SD. Dashed lines indicate the limits of detection.

2.2.3.6 Hematopoietic-Cell-Restricted CHIKV

The second-to-last virus I recovered to dissect the role of CHIKV tropism in pathogenesis (and the last discussed in this chapter as characterization of SKE is the subject of Chapter III) is HEM, a virus exhibiting diminished replicative capacity in hematopoietic cells by insertion of target sequences for hematopoietic-specific miR-142-3p. Although CHIKV seems capable of replicating only in monocyte-derived macrophages from the hematopoietic lineage, this tropism may influence disease. First, it is possible that replication in monocytes can contribute to the high serum viremia observed during CHIKV infection. Second, another alphavirus, EEEV, specifically co-opts miR-142 to limit replication in myeloid cells and prevent detection by the innate immune system (225). Third, macrophages are a reservoir of CHIKV antigen and RNA during the chronic

stage of disease (118). The role of these cells in the detection of CHIKV, the resulting immune response, and whether maintenance in macrophages is responsible for chronic disease phenotypes have not been defined.

To define the role of CHIKV replication in hematopoietic cells in disease development, I recovered HEM alongside its mismatch control, HEM MM, at high titer following electroporation into BHK-21 cells. To quantify replication in nonrestrictive conditions, Vero81 cells were adsorbed with HEM or HEM MM at an MOI of 0.01 PFU/cell, and virus titers in culture supernatants at various times post-adsorption were assessed by FFU assay. HEM and HEM MM replicated with similar kinetics to WT SL15649 in these cells (Figure 2-10). To assess restriction of HEM specifically by miR-142, viral replication was examined in Vero81 cells transfected with various siRNAs. HEM and HEM MM replicated with similar kinetics in cells transfected with a nontargeting siRNA directed against luciferase (Figure 2-10). However, in cells transfected with miR-142-mimic siRNA, HEM was restricted, replicating to titers ~270-fold lower than HEM MM in the same conditions. Collectively, these data indicate that HEM replication is not compromised due to incorporation of miR-142 target sequences except in the presence of its cognate miRNA.

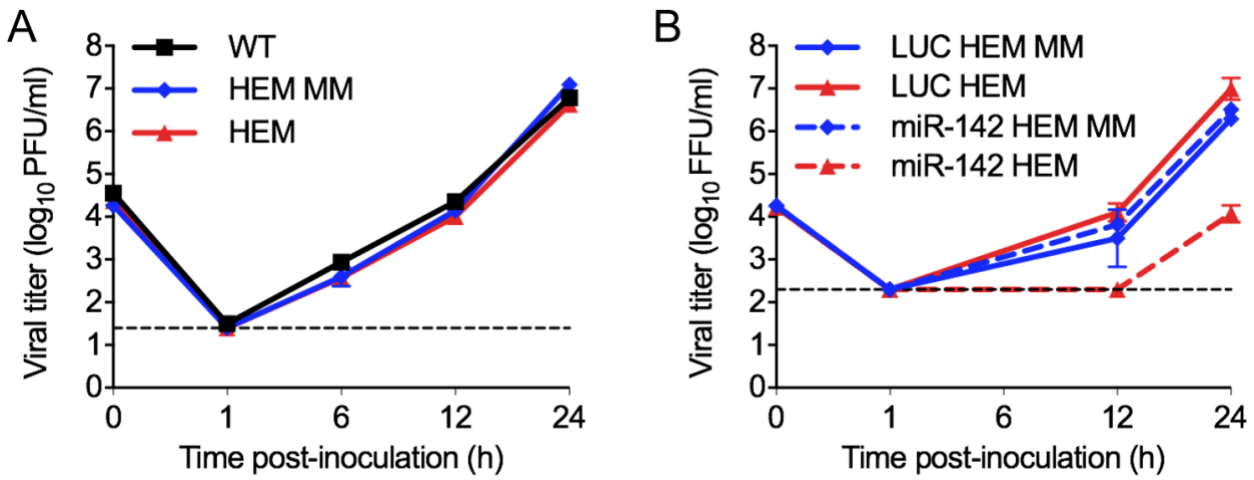

Figure 2-10. Hematopoietic cell-restricted virus replicates with kinetics of WT SL15649 in nonrestrictive conditions and is restricted following transfection of cells with cognate miR-142.

U-2 OS cells were either untransfected (A) or transfected with either a nontargeting siRNA directed against luciferase (LUC) or hematopoietic cell-specific miR-142-mimic siRNA (B) prior to adsorption with HEM or HEM MM at an MOI of 0.01 PFU/cell. Supernatants were collected at the times shown post-adsorption, and viral titer was quantified by plaque (A) or FFU (B) assay. Results are expressed as mean viral titer from duplicate wells of three experimental replicates from one independent experiment. Error bars indicate SD. Dashed lines indicate the limits of detection.

To begin assessing the contribution of replication in hematopoietic cells to disease development, mice were inoculated with HEM or HEM MM, and swelling of the inoculated foot was quantified using digital calipers. In a small preliminary experiment, mice infected with HEM experienced enhanced when compared with HEM MM-infected counterparts during the initial phase of swelling, corresponding to activation of the innate immune response (Figure 2-11). However, swelling experienced by HEM MM-infected mice was slightly greater during the second phase at day 6 post-inoculation (Figure 2-11). These data suggest that replication and detection of HEM in macrophages limits disease early in infection, although it may potentially exacerbate disease following activation of the adaptive immune response.

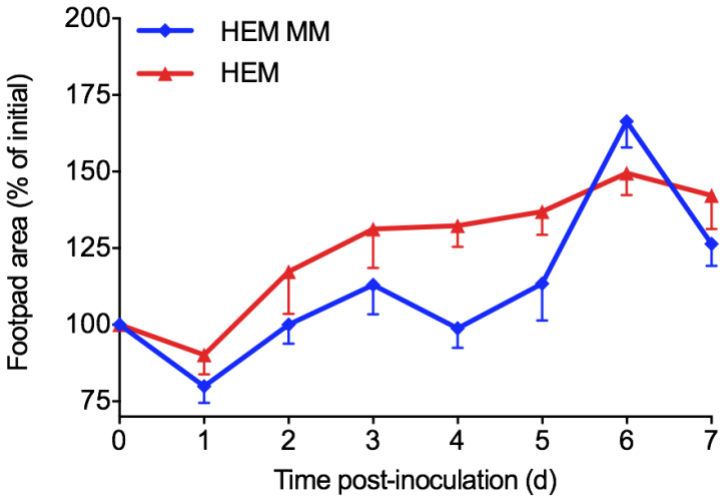

Figure 2-11. Infection with HEM causes enhanced virulence during early phase of swelling in mice.

Three-to-four-week-old male C57BL/6J mice were inoculated in the left rear footpad with 10^3 PFU of HEM MM or HEM. Left rear footpad swelling was quantified using digital calipers on the days shown. Results are normalized to the initial footpad area and presented as the mean percent of initial footpad area for 4 mice per group. Error bars indicate SEM.

I last sought to determine the role of replication in macrophages in persistence of viral RNA at late times post-inoculation. Mice were inoculated with HEM or HEM MM, and tissues were excised at day 28 post-inoculation. RNA was purified from tissue homogenates, and CHIKV genome copies per μg RNA were quantified by RT-qPCR. In this assay, HEM and HEM MM produced comparable genome copies per μg RNA in the left and right ankle and spleen of infected mice (Figure 2-12). These data suggest that while RNA can be detected in macrophages during chronic disease, it likely is not the major reservoir of viral RNA at late time points.

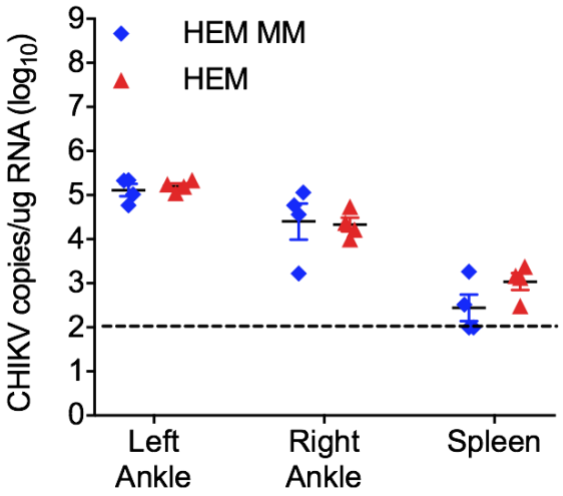

Figure 2-12. HEM and HEM MM are maintained to a similar degree at sites of persistence in mice during chronic phase of disease.

Three-to-four-week-old male C57BL/6J mice were inoculated in the left rear footpad with 10^3 PFU of HEM MM or HEM. At 28 d post-inoculation, mice were euthanized, and ankles and spleens were excised. Viral loads in day 28 tissue homogenates were determined by RT-qPCR. Horizontal bars indicate mean CHIKV genome copies/mg RNA for 4 mice per group. Error bars indicate SEM. Dashed lines indicate limits of detection.

2.3 Discussion

In the experiments described in this chapter, I recovered and preliminarily characterized viruses exhibiting diminished replicative capacity following targeting by tissue-specific miRNAs. I found that a virus strain engineered to contain target sequences for a mosquito-specific miRNA that is not expressed in mammalian cells replicates with anticipated kinetics in cultured cells and causes acute disease in mice comparable to WT CHIKV. However, target sequences for mosquito-specific miR-275-3p are rapidly altered following infection in mice. Additionally, I found that two miRNA-restricted CHIKV strains, ENDO and KER, have impaired replicative capacity in the

absence of their respective restrictive miRNAs. Two additional strains, OST and HEM, replicate with anticipated kinetics *in vitro*. In preliminary experiments in mice, HEM produces enhanced disease during the phase of innate immune activation and persists to a degree similar to its mismatch control virus at late time points. This study will serve as a foundation for future studies designed to better understand the role of CHIKV replication in these cell types in development of disease.

Using target sequences for endothelial cell-specific miR-126-3p, I could not recover virus at sufficiently high titer for use in experiments, despite multiple attempts. Inability to recover virus could be for a variety of reasons. It is possible that introduction of miR-126 target sequences results in formation of deleterious secondary structure in the CHIKV genome, which is an important consideration as RNA secondary structures essential in the replication of alphavirus RNA (248, 249). Additionally, it is possible that, although BHK-21 cells do not express restrictive miR-126, some other miRNA or factor in these cells restricts replication of CHIKV containing target sequences for this miRNA. Attempts could be made to recover this virus in another cell type, either Vero81 cells or mosquito C6/36 cells, which allow production of high-titer viral stocks following electroporation of viral RNA. Additionally, target sequences corresponding to other miRNAs that are highly expressed in endothelial cells could be used to recover a different endothelial cell-restricted strain. Both the miR-23-27-24 and miR-17-92 clusters are highly expressed in endothelial cells, where they govern angiogenesis and vascularity (250-254).

Two other viruses engineered in this study, KER and KER MM, exhibited unexpected replication. Although recovered stocks of these viruses were at sufficient titer, both viruses displayed a replicative defect in BHK-21 cells and produced small plaques in Vero81 cells. Whether the defect in replication occurred in BHK-21 cells over the course of the replication

experiment or was due to restriction in Vero81 cells during the FFU assay remains unclear. This experiment raises an important consideration when using multiple cell lines to assess the replicative fitness of a miRNA-restricted CHIKV strain: if defects in replication are observed, it is difficult to determine whether the defects are due to a virus-intrinsic replicative problem that would be observable regardless of cell line or due only to restriction in a single cell type. Future studies should quantify viral titers by RT-qPCR in parallel with conventional plaque or FFU assays and study viruses using a variety of cell lines. Because I have not completely assessed replication kinetics of KER and KER MM in various cell types, I cannot say with certainty that these viruses are less fit than WT SL15649. Additional experiments should be conducted before these viruses are deemed unusable in a mouse model. If these experiments lead to the determination that KER and KER MM cannot be used in mice, target sequences for other miRNAs could be inserted to yield a keratinocyte-restricted CHIKV strain. In particular, miR-9, -95, -146a, -223, and -508-3p are all strongly induced upon terminal differentiation of human keratinocytes (255).

Another important consideration in studying a virus such as the keratinocyte-restricted virus used to dissect primary replication of CHIKV following deposition of virus in the skin is to ensure that a proper model is used to study primary replication and dissemination. The mouse model employed by the lab to study CHIKV pathogenesis assesses disease development following subcutaneous inoculation of virus in the footpad of mice. However, because the skin of mice is thin, these inoculations are subdermal, thus bypassing cell types, including keratinocytes, that may be the first cells that the virus would encounter following the bite of an infected mosquito. To study the role of cells involved in primary replication and early dissemination, including keratinocytes, hematopoietic cells, and fibroblasts, virus could be inoculated by a mosquito bite as opposed to needle inoculation (256). This inoculation procedure is difficult, however, as it requires

the use of a BSL3 insectary, and the inoculum from a mosquito bite cannot be controlled. An additional option would be using a bifurcated needle to introduce the virus into the epidermal and dermal layers of mouse skin. This is the method of inoculation of the smallpox vaccine (257) and would allow analysis of sites of primary replication required for dissemination without requiring the use of an insectary.

The osteoblast-restricted CHIKV strain engineered in this study may provide an interesting virus to study the contribution of infection of these cells to focal erosion of bone and disruptions in the RANKL/OPG ratio observed during CHIKV infection. I initially halted characterization of this virus due to its diminished replication in U-2 OS cells compared with replication of the parental SL15649 strain. However, characterization of the miRNA profile of U-2 OS cells confirmed that these cells express detectable levels of this miRNA. Preliminary experiments suggest that endogenous levels of this miRNA were capable of restricting virus replication ~38-fold (Figure 2-9A). Transfection of miR-2861 enhanced restriction to ~177-fold. These findings should be confirmed by analysis of replication kinetics in either BHK-21 or Vero81 cells that lack expression of miR-2861. Additionally, an osteoblast mismatch CHIKV strain should be designed and recovered as a control to accurately define the role of osteoblasts in CHIKV-induced musculoskeletal disease. If *in vitro* characterization in cell types lacking expression of miR-2861 reveals a replication defect that would preclude use of OST in animal models, target sequences for another miRNA that promotes osteoblast differentiation, miR-433-3p, could be used to recover a virus incapable of replication in these cells (258).

Additional characterization of HEM in mice is required to determine whether this virus is more virulent during the first phase of swelling observed in three-to-four-week-old mice. If this finding is confirmed, it will be important to understand the mechanism of enhanced virulence. It

is possible that, similar to EEEV, diminished replication in myeloid cells by incorporation of target sequences for miR-142 allows the virus to escape immune detection (225). If this is the case, it would be interesting to investigate whether enhanced virulence occurs by increased virus replication and resulting cytotoxicity, alterations in tropism achieved by delayed induction of an immune response, or an aberrant immune response mounted against the virus following detection at other sites. Characterization of the cytokine/chemokine response early in infection as well as identification of the infiltrating immune cell populations may help understand how a virus incapable of replication in hematopoietic cells causes disease.

Finally, to summarize these attempts to engineer miR-restricted CHIKV strains exhibiting cell-type specific restriction, I would like to provide a few lessons learned during the design of these viruses. First, perhaps the most important step is choosing appropriate tissue-specific miRNAs. These miRNAs should exhibit as close to tissue-specific expression as possible. Some expression at other sites may still allow the characterization of disease contributions of replication in a specific cell type, but only in cases in which the miRNA exhibits very limited expression at other sites and high expression at the site of interest. Second, the location in the viral genome into which the target sequences are engineered must be carefully chosen. Like CHIKV, many viruses form secondary RNA structures that serve regulatory functions in replication and translation, and disruption of these sites should be avoided. If a site inside a viral ORF is chosen, the miRNA target sequences must not shift the reading frame of downstream sequence, and stop codons should not be present in the inserted sequence. Because the inserted sequence will give rise to a peptide product, contributions of this peptide to pathogenesis should be controlled by recovery of a mismatch control. Third, multiple target sequences or incorporation of target sequences at multiple sites in the virus genome should be used to limit reversion. Finally, characterization of recovered

viruses should involve one or two cell lines in which the miRNA expression profiles are known. I defined expression profiles of cell lines by resolving products of stem-loop RT-PCR in an agarose gel, but characterizing these profiles by RT-qPCR would have provided more sensitive and comprehensive information. Even with cell lines that are confirmed to lack expression of restrictive miRNAs, it may be important to study replication using cell-based assays such as FFU or plaque assays alongside RT-qPCR. Overall, given proper attention to these considerations, the use of RNAi to manipulate viral tropism provides a powerful approach to study mechanisms of viral pathogenesis.

3.0 Skeletal Muscle-Restricted CHIKV is Attenuated in Mice

3.1 Introduction

3.1.1 Infectious Myositis

Healthy muscle is generally resistant to infection (259). Infectious myositis results when pathogens invade and replicate in skeletal muscle and is accompanied by myalgia, swelling, tenderness, or weakness. Cases of bacterial and fungal myositis are rare. The most common type of bacterial myositis is pyomyositis that results from hematogenous dissemination to muscle tissue. Even *Staphylococcus aureus* infection, the most common cause of pyomyositis (260, 261), only results in this disease manifestation in 0.5% of bacteremic individuals (262). Less frequently, bacterial myositis results from infection of contiguous sites or penetrating trauma that allows access to muscle tissue. Features of fungal myositis, which is most commonly the result of infection with *Candida* species (259), are similar to bacterial myositis. Both bacterial and fungal myositis occur most commonly and cause more severe disease in immunocompromised individuals (260, 263).

Parasitic myositis results from the formation of cysts in the musculature. While uncommon in the United States, the three most common global causes of parasitic disease in skeletal muscle are *Trichinella spiralis*, *Taenia solium*, and *Toxoplasma gondii* (259). Trichinosis and toxoplasmosis are caused by ingesting undercooked meat, usually pork or lamb, containing *T. spiralis* and *T. gondii* cysts, respectively (264, 265). In trichinosis, parasites encyst in human muscle, causing weakness and myalgias with disease severity dependent on parasite load (264). *T.*

gondii-associated polymyositis also occurs following the formation of cysts in skeletal muscle but is associated with musculoskeletal disease often only in immunocompromised individuals (266). Unlike *T. spiralis* and *T. gondii*, cysticercosis from *T. solium* infection results from ingestion of eggs excreted by humans who are already carrying the parasite, which results in the formation of cysts at a variety of sites in humans, including the musculature (267). Musculoskeletal disease associated with these organisms is most frequently subclinical (259), although more severe disease manifestations occur following penetration of parasites into other sites such as the heart and CNS (264, 265, 267).

Many viral infections also result in myositis, with the majority of cases attributed to infection with IAV, influenza B, or enteroviruses (268). Influenza-associated myositis primarily involves the gastrocnemius and soleus muscles of children, potentially due to enhanced susceptibility of differentiating myoblasts (269). Isolation of influenza virus from homogenates of muscle biopsies taken from patients experiencing myositis indicates virus is capable of replicating in these cells (270, 271). Viral RNA from enteroviruses, including coxsackieviruses and echoviruses, also has been detected in biopsies of muscle tissue from individuals diagnosed with myositis (272-275). In all cases, it has been difficult to assert whether damage to muscle is a direct result of accumulation of cytotoxic viral components or by a damaging immune response.

Regardless of the cause of infectious myositis, understanding molecular mechanisms of disease has been difficult. Many infections are self-limiting and do not require biopsy for diagnosis, limiting the use of patient samples to study determinants of disease development. Symptoms of viral myositis often resolve following palliative treatment, which has likely prevented diagnosis and limited attempts to understand mechanisms of pathogenesis, as specific therapeutics are not always required to manage disease. However, emergence of new causes of

viral myositis have necessitated an understanding of how infection in skeletal muscle can result in disease.

3.1.2 CHIKV Infection in Skeletal Muscle

While CHIKV has traditionally been studied in the context of virus-induced polyarthritis and polyarthralgia, mounting evidence implicates skeletal muscle as an important site in CHIKV disease development. CHIKV infection in humans results in myalgia in most patients (37, 276-279). Biopsies taken from the quadriceps muscle of patients during both acute and chronic stages of disease show atrophy and necrosis of muscle fibers with detection of virus antigen in muscle progenitor cells (33). Myositis and rhabdomyolysis, a syndrome in which death of muscle fibers results in release of their contents into the blood stream and resultant renal failure, have both been documented in cases of severe CHIKV disease (280-284). Understanding the molecular underpinnings of these disease manifestations has been difficult using patient samples, requiring the use of animal models.

A limitation of nonhuman primate models of CHIKV pathogenesis is failure to recapitulate musculoskeletal disease observed in infected humans. In a cohort of 13 cynomolgus macaques inoculated with low (10 PFU), intermediate (10^2-10^6 PFU), or high ($\geq 10^7$ PFU) doses of CHIKV, muscle necrosis and mononuclear cell infiltration were observed only in one intermediate- and one high-dose animal (118). Additionally, inflammation and mononuclear infiltration into synovial tissue was observed only in one macaque receiving high-dose inoculum (118). Thus, while the macaque model recapitulates viremia, fever, rash, and abnormalities of the liver, spleen, and lymph nodes observed during infection in humans (118, 119), CHIKV-infected macaques rarely develop musculoskeletal disease.

CHIKV disease in muscle has been more successfully studied in mouse models. Infection of mice recapitulates arthritis, tenosynovitis, myositis, and persistence that are all characteristic of severe disease in humans (44, 114). CHIKV strains capable of replicating in skeletal muscle cells in mice cause more severe disease than strains incapable of replication at that site (285), implicating myotropism as an important determinant of disease severity. Unfortunately, the strains chosen for this study, an ECSA and WA strain, are too divergent to allow for a mechanistic analysis of the contributions of muscle infection to disease. Mouse myofibers also survive infection and harbor viral RNA during the chronic phase of disease, implicating this tissue in chronic disease phenotypes (114). Understanding the pathogenic contribution of CHIKV replication in skeletal muscle cells is important for discerning mechanisms of virus pathogenesis and will inform development of targeted therapeutics to limit musculoskeletal disease experienced by CHIKV-infected individuals.

3.2 Results

3.2.1 Recovery of Skeletal Muscle-Restricted CHIKV

CHIKV infection in humans results in a syndrome characterized by severe arthralgia and myalgia (286-288). Replication in skeletal muscle has been associated with development of severe disease (247, 285, 289), but the pathologic outcome of virus replication in muscle cells has not been well defined. To understand the contribution of viral replication in skeletal muscle cells to CHIKV disease, we engineered a CHIKV strain exhibiting diminished replication at this site. To achieve this goal, we incorporated into the CHIKV genome target sequences with perfect

complementarity to a skeletal muscle cell-specific microRNA, miR-206 (290, 291). miR-206 is expressed at detectable levels in skeletal muscle progenitor satellite cells, strongly induced upon differentiation, and then stably expressed at high levels throughout the life of the muscle fiber (292). Target sequences for miR-206 have been employed with other viruses, including coxsackieviruses A21 (232) and B3 (293), to specifically limit replication in myofibers. To ensure stability of the inserted sequences and limit reversion, four copies of the miR-206 target sequence were placed in tandem and in-frame into the structural open reading frame (ORF) of virulent CHIKV strain SL15649 within coding sequences of the viral E3 glycoprotein (Figure 3-1A-B). This site was chosen because it can accommodate insertions of exogenous sequences without compromising replication capacity (238, 294). A mismatch control virus was engineered containing silent mutations at synonymous nucleotide positions in miRNA target sequences to alleviate restriction by miR-206 (Figure 3-1C). WT SL15649 containing no miRNA target insertion, SKE, and SKE MM CHIKV strains were recovered following electroporation of *in vitro* transcribed viral RNA into BHK-21 cells. Consensus sequencing of SKE and SKE MM stocks harvested at 48 h post-electroporation confirmed maintenance of inserted sequences.

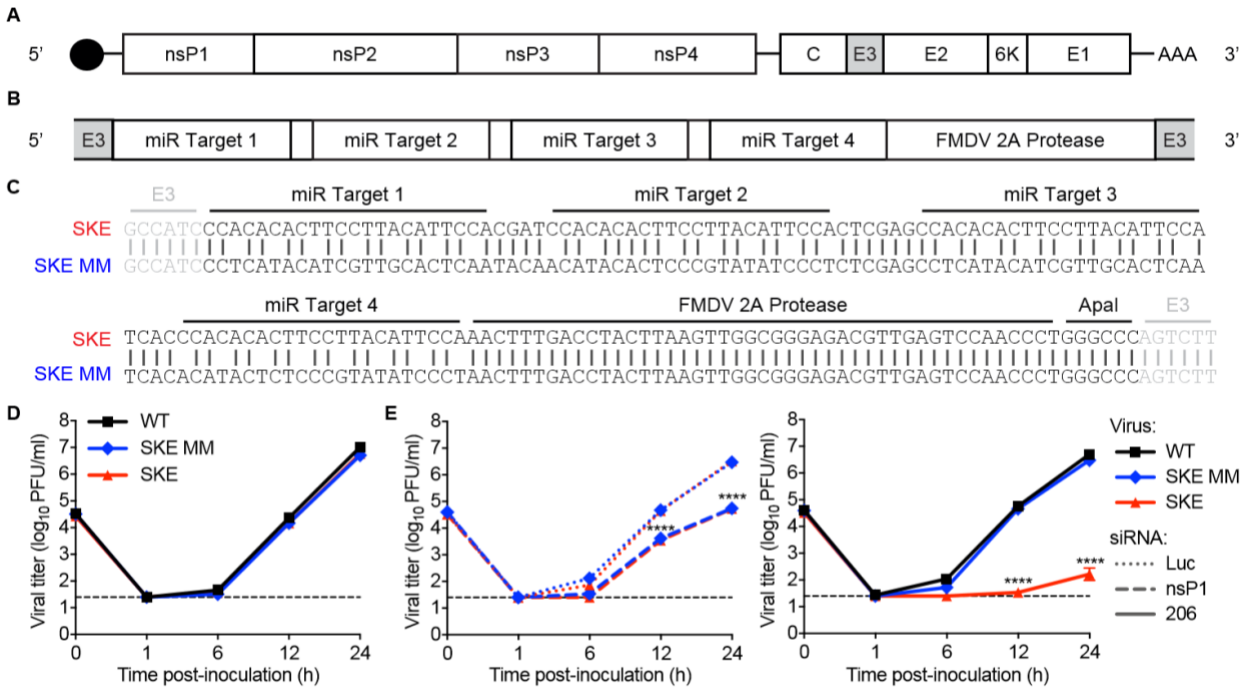

Figure 3-1. CHIKV engineered to contain target sequences for skeletal muscle-specific miR-206 is specifically restricted by its cognate miRNA.

(A) Schematic of the CHIKV genome. Sequences were engineered in-frame within the E3 protein-coding region (shown in grey). (B) Insert cassettes consist of four target elements in tandem appended at the 3' end by sequences of the foot-and-mouth disease virus (FMDV) 2A protease. (C) Nucleotide sequence of insert cassettes. Four target sequences exhibiting perfect complementarity to skeletal muscle-specific miR-206 were introduced into SKE. Silent mutations were engineered into target sequences to produce SKE mismatch (MM). (D) U-2 OS cells were adsorbed with WT SL15649, SKE MM, or SKE at an MOI of 0.01 PFU/cell. Supernatants were collected at the times shown post-adsorption, and viral titer was quantified by plaque assay. (E) U-2 OS cells were transfected with siRNA directed against luciferase (Luc; dotted lines), CHIKV nsP1 (dashed lines), or muscle-specific miR-206-mimic siRNA (solid lines) and adsorbed with WT SL15649 (black), SKE MM (blue), or SKE (red) at an MOI of 0.01 PFU/cell. Supernatants were collected at the times shown post-adsorption, and viral titer was determined by plaque assay. (D) and (E) Results are expressed as the mean viral titer from duplicate wells of three independent experiments. Error bars indicate SEM. Dashed lines indicate the limit of detection. *P* values were determined at 12 and 24 hpi by ANOVA followed by Tukey's *post hoc* test. The following comparisons were statistically significant (*, $P < 0.05$; ****, $P <$

0.0001): SKE MM-Luc vs. SKE MM-nsP1 and SKE-Luc vs. SKE-nsP1 (E, left panel); WT-206 vs. SKE-206 and SKE MM-206 vs. SKE-206 (E, right panel).

3.2.2 SKE Is Restricted by Cognate miR-206 *In Vitro*

Replication kinetics of SKE and SKE MM were first assessed using nonrestrictive conditions to ensure that no defects in replication were conferred by incorporation of exogenous sequences into the structural ORF of the viral genome. U-2 OS cells, which do not naturally express miR-206, were infected with WT CHIKV, SKE, or SKE MM at an MOI of 0.01 PFU/cell, and supernatants were harvested at various intervals post-adsorption to quantify viral progeny production by plaque assay. SKE and SKE MM replicated with kinetics comparable to WT CHIKV and reached similar peak titers in U-2 OS cells (Figure 3-1D). These data indicate that sequences inserted into the E3 coding region do not compromise CHIKV replication in U-2 OS cells.

To determine susceptibility of SKE and SKE MM to miRNA-mediated restriction, a multi-step replication experiment was conducted using U-2 OS cells transfected with various siRNAs. Both SKE and SKE MM replicated with kinetics comparable to WT CHIKV in cells transfected with a nontargeting siRNA directed against luciferase (Figure 3-1E). Cells transfected with an siRNA targeting the viral nsP1 gene allowed diminished but equivalent replication of SKE and SKE MM, indicating that both strains are equally susceptible to nsP1 siRNA-mediated restriction (Figure 3-1E). In cells transfected with an siRNA mimicking the sequence of miR-206, replication of SKE was restricted, reaching peak titers significantly lower than those produced by SKE MM and WT CHIKV, which replicated with similar kinetics and to equivalent peak titers (Figure 3-

1E). These data demonstrate that SKE is specifically restricted by its cognate miRNA in cell culture and that mismatch mutations present in SKE MM are sufficient to alleviate this restriction.

3.2.3 SKE Displays Diminished Replication in Murine Skeletal Muscle

To assess whether SKE is restricted in skeletal muscle tissue in vivo, three-to-four-week-old C57BL/6J mice were inoculated in the left rear footpad with 10^3 PFU of either SKE or SKE MM. At day 3 post-inoculation, mice were euthanized, and the left rear limb was processed for histology. Myofibers were identified in H&E-stained sections as striated, multinucleated cells containing nuclei at the cell periphery (Figure 3-2). CHIKV replication in skeletal muscle was assessed by in situ hybridization of serial tissue sections using a probe specific for CHIKV RNA. In mice infected with SKE MM, abundant staining was observed in the interosseous muscles of the foot (Figure 3-2), which is consistent with prior studies with WT CHIKV (247). This staining was significantly reduced in mice infected with SKE (Figure 3-2C), demonstrating that SKE replication is restricted in skeletal muscle cells in mice. Importantly, intense staining was observed in connective tissue of mice infected with either SKE or SKE MM (Figure 3-2B), indicating that introduction of target sequences for miR-206 into SKE specifically restricts replication in skeletal muscle cells while still allowing replication at other sites.

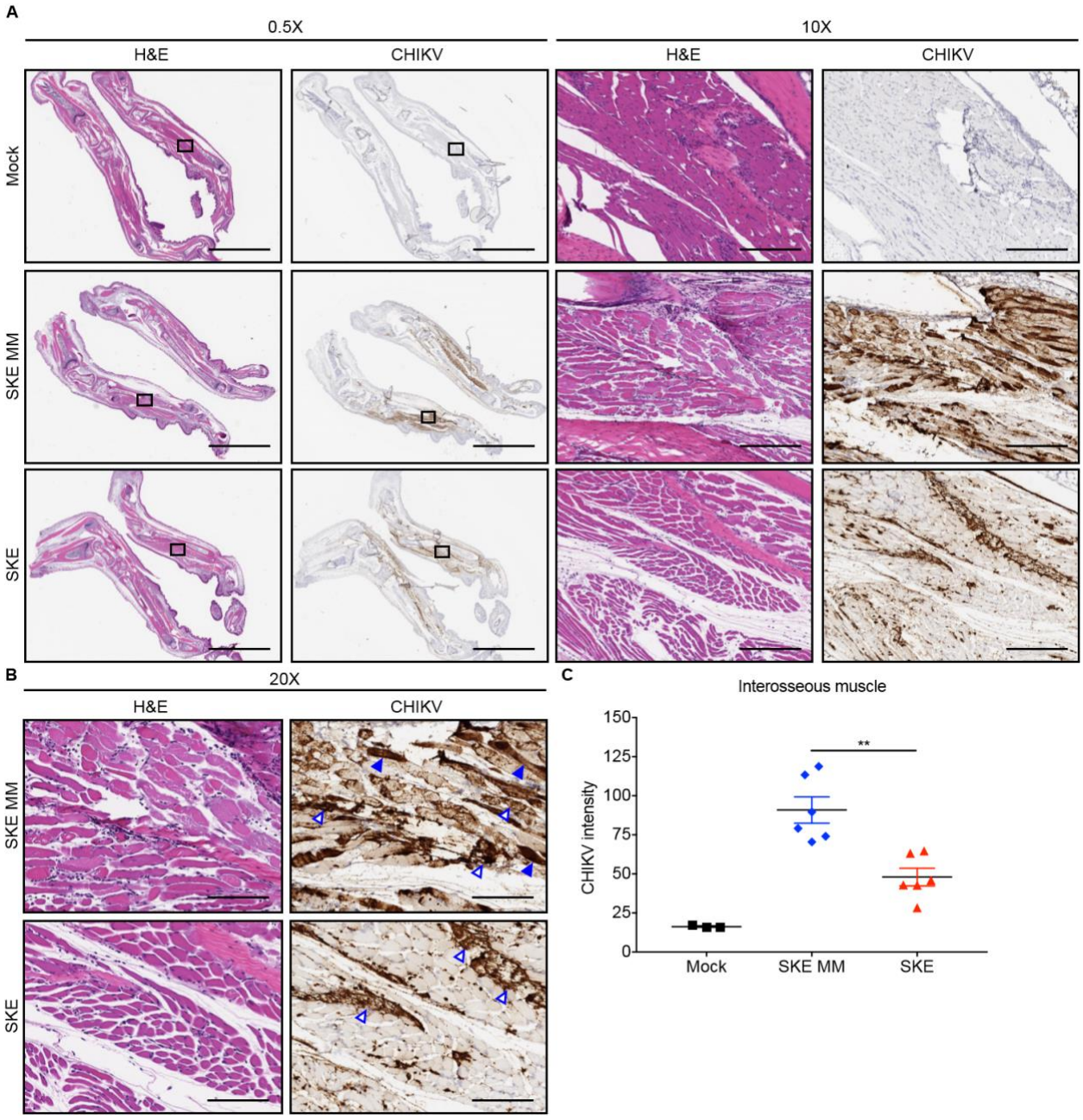

Figure 3-2. Replication of CHIKV containing muscle-specific miRNA target sequences is restricted in skeletal muscle.

Three-to-four-week-old male C57BL/6J mice were inoculated in the left rear footpad with PBS (mock) or 10_3 PFU of SKE MM or SKE. Left ankle tissue was collected 3 d post-inoculation and processed for either H&E staining or RNAscope in situ hybridization for CHIKV RNA. (A) Regions corresponding to high-magnification insets (10X) of the interosseous muscle are indicated in the overview micrographs (0.5X) by black boxes. Representative images of 3 (mock) or 6 (SKE MM and SKE) mice per group are shown. Scale bars, 0.5X = 6 mm; 10X = 300 μm. (B) 20X

magnification insets with indicated CHIKV staining in myofibers (closed arrows) and connective tissue (open arrows). Scale bars, 20X = 150 μm. Images were acquired using an Aperio ScanScope XT slide scanner and processed with Aperio ImageScope software. (C) DAB signal corresponding to CHIKV staining in interosseous muscle was quantified using ImageJ software. Horizontal bars indicate mean CHIKV intensity. Error bars indicate SEM. *P* values were determined comparing SKE and SKE MM by two-tailed Student's *t* test (*, $P < 0.05$; **, $P < 0.01$).

3.2.4 SKE and SKE MM Produce Comparable Titers in Musculoskeletal Tissue of the Inoculated Foot in Mice

Because SKE replication is restricted in skeletal muscle cells, we assessed whether replication in these cells contributes to overall viral titers in foot tissue and viral dissemination during acute infection. Mice were inoculated with WT CHIKV, SKE, or SKE MM, and loads of infectious virus in various tissues both proximal and distal to the site of inoculation were quantified at days 1, 3, and 7 post-inoculation. Because the level of infectious virus at day 7 post-inoculation is often at or below the limit of detection in our infectivity assays, we used an FFU assay to quantify infectious virus at days 1 and 3 post-inoculation and RT-qPCR to quantify viral genome copies at day 7 post-inoculation. Tissue burdens of SKE MM and SKE did not differ significantly in the left ankle near the site of inoculation or at sites of dissemination, including the left gastrocnemius muscle, right ankle, and spleen, at days 1 (Figure 3-3A), 3 (Figure 3-3B), or 7 (Figure 3-3D) post-inoculation. At all time points examined, both viruses produced titers comparable to those produced by WT CHIKV. CHIKV RNA in the interosseous muscle of the left rear foot was significantly reduced at day 3 post-inoculation in our in situ hybridization analysis (Figure 3-2C). Because total viral titers in the ankle did not differ between mice infected with SKE and SKE MM at this time point by FFU assay (Figure 3-2B), we also analyzed total ankle homogenates by RT-qPCR. This analysis confirmed that, while SKE replication is specifically restricted in interosseous

muscle, total viral titers in the ankle produced by SKE and SKE MM at this time point are comparable (Figure 3-3C). These data indicate that replication in skeletal muscle does not contribute significantly to overall CHIKV titers in tissues or dissemination during acute infection and that replication in other cell types, likely connective tissue fibroblasts that are highly susceptible for CHIKV infection, is responsible for high viral titers observed in musculoskeletal tissues at these time points. Additionally, titers of SKE and SKE MM reached similar levels in the serum at day 1 post-inoculation (Figure 3-3A) and were cleared by day 3 post-inoculation (Figure 3-3B), indicating that restriction of CHIKV replication in skeletal muscle cells does not affect establishment or clearance of viremia.

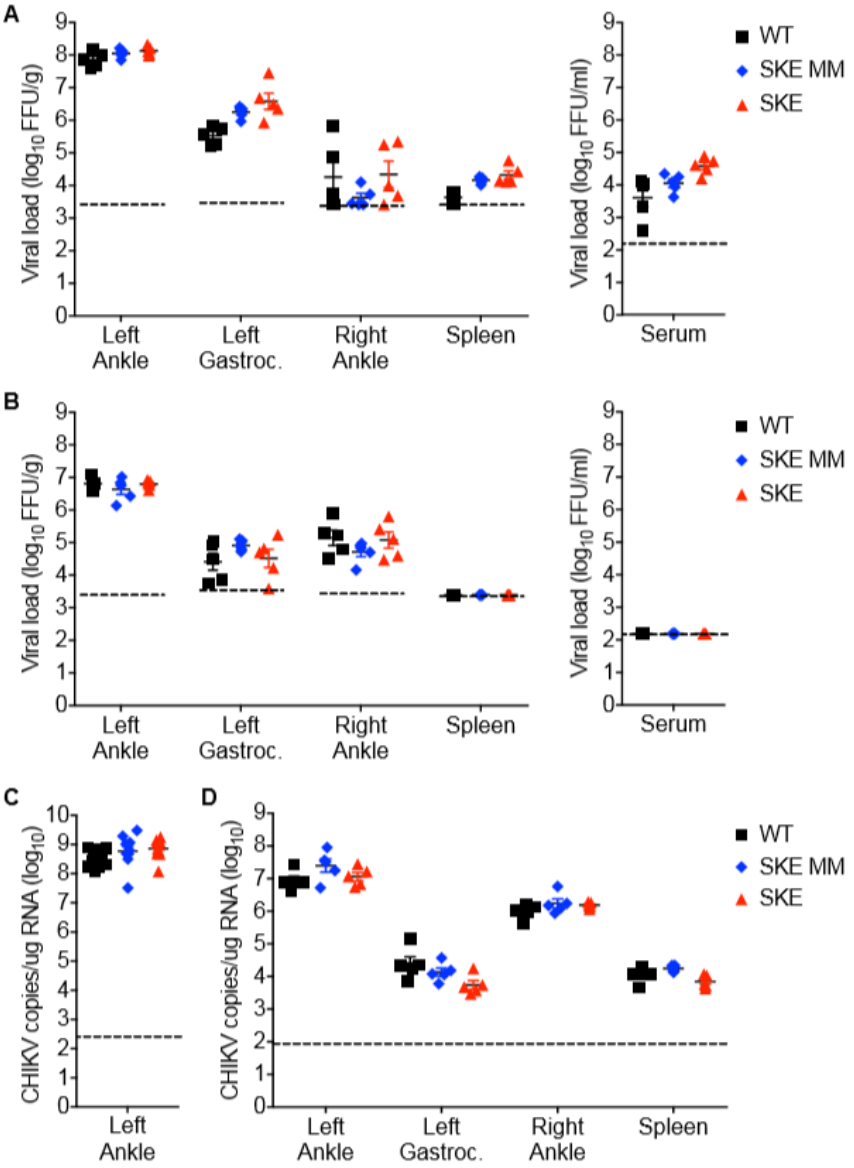

Figure 3-3. CHIKV replication in skeletal muscle does not contribute significantly to viral titer in tissue during acute infection.

Three-to-four-week-old male C57BL/6J mice were inoculated in the left rear footpad with 10^3 PFU of WT SL15649, SKE MM, or SKE. At 1, 3, and 7 d post-inoculation, mice were euthanized, ankles, gastrocnemius (gastroc.) muscles, and spleens were excised, and serum was collected. Viral titers in day 1 (A) and 3 (B) tissue homogenates and serum were determined by FFU assay. Horizontal bars indicate mean FFU/g (tissue) or FFU/ml (serum) for 5 mice per group. Viral loads in day 3 (C) and 7 (D) tissue homogenates were determined by RT-qPCR. Horizontal bars indicate mean CHIKV genome copies/mg RNA for 5 (7 dpi) or 10 (3 dpi) mice per group. Error bars indicate SEM. Dashed lines

indicate the limits of detection. *P* values were determined by comparing SKE and SKE MM by ANOVA followed by Tukey's *post hoc* test (*, $P < 0.05$).

3.2.5 Restriction of CHIKV Replication in Skeletal Muscle Cells Attenuates Viral Virulence

To define the contribution of viral replication in myofibers to pathogenesis, mice were inoculated with WT CHIKV, SKE, or SKE MM, and swelling of the inoculated foot, a hallmark of CHIKV-mediated disease in mice (44), was measured using digital calipers. Following inoculation with either WT CHIKV or SKE MM, mice exhibited a bimodal pattern of swelling, with swelling peaking at days 3 and 6 post-inoculation (Figure 3-4), which is consistent with prior studies (41, 49). Mice infected with SKE displayed significantly diminished swelling during both phases, with the second phase peaking later at day 7 post-inoculation (Figure 3-4). These data implicate skeletal muscle cells as a site of viral replication essential to the induction of both phases of swelling following CHIKV infection in mice.

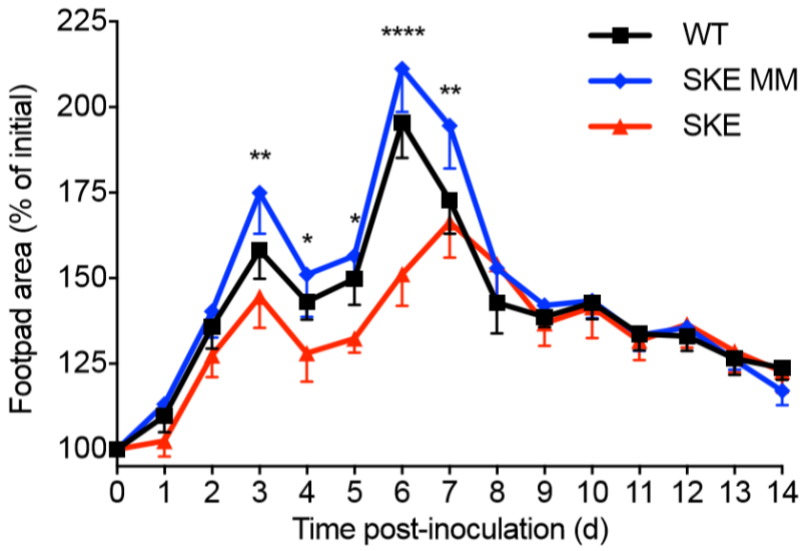

Figure 3-4. Restriction of CHIKV replication in muscle diminishes footpad inflammation.

Three-to-four-week-old male C57BL/6J mice were inoculated in the left rear footpad with PBS (mock) or 10_3 PFU of WT SL15649, SKE MM, or SKE. Left rear footpad swelling was quantified using digital calipers on the days shown. Results are normalized to initial footpad area and presented as the mean percent of initial footpad area for 10 mice per group. Error bars indicate SEM. *P* values were determined by comparing SKE and SKE MM by ANOVA followed by Tukey's *post hoc* test (*, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$).

To understand how CHIKV replication in skeletal muscle cells influences tissue injury, mice were inoculated with SKE or SKE MM, euthanized at day 7 post-inoculation, and the left rear limb was processed for histology. H&E-stained tissue sections were scored by a veterinary pathologist blinded to the conditions of the experiment to compare severity of synovitis, inflammation and necrosis in the interosseous muscle, and tenosynovitis. SKE MM-infected mice displayed significant myositis and necrosis of the interosseous muscle, with most mice displaying greater than 40% displacement of muscle tissue with infiltrating leukocytes (Figure 3-5A). This finding is consistent with prior studies with WT CHIKV (44). Synovitis was reduced in SKE-infected mice compared with those infected with SKE MM, although scores did not differ significantly (Figure 3-5B). In contrast, mice infected with SKE exhibited significantly diminished

necrosis and inflammation in the interosseous muscle as well as diminished tenosynovitis in the tendon sheath compared with SKE MM-infected mice (Figure 3-5B). These findings suggest that while replication in skeletal muscle cells does not contribute significantly to tissue viral burden, these cells are an important site of replication for development of muscle inflammation and necrosis.

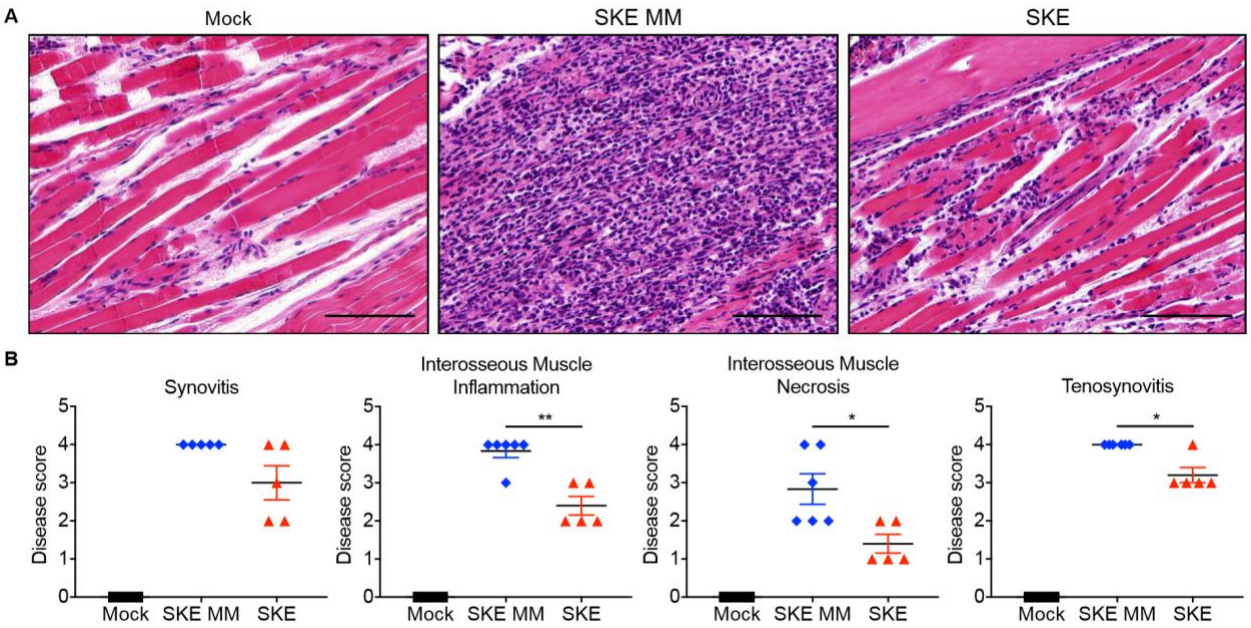

Figure 3-5. SKE infection causes diminished interosseous muscle inflammation and necrosis.

Three-to-four-week-old male C57BL/6J mice inoculated in the left rear footpad with PBS (mock) or 10_3 PFU of WT SL15649, SKE MM, or SKE. (A) H&E staining of left rear interosseous muscle of mock-, SKE MM-, or SKE-infected mice 7 d post-inoculation. Data are representative of two independent experiments with 3 mice per group in each experiment. Scale bars, 100 mm. (B) H&E-stained sections were scored for histological evidence of synovitis, inflammation and necrosis in the interosseous muscle, and tenosynovitis. Results are expressed as disease score of tissues from individual animals for 5-6 mice per group. Horizontal bars indicate mean disease score. Scores were assigned based on the following scale: 0, no lesions; 1, mild, < 5 areas of small clusters of leukocytes; 2, moderate, leukocytes forming larger clusters to thin tracts throughout the tissue, multiple sites/tissues affected; 3, severe, clusters and tracts of leukocytes coalescing into at least one large area that displaces/replaces tissue; 4, markedly severe, leukocytes in aggregates sufficient to replace > 40% of normal tissue. Error bars indicate SEM. *P* values were determined by comparing SKE and SKE MM by Mann Whitney test (*, $P < 0.05$; **, $P < 0.01$).

3.2.6 Diminished CHIKV Replication in Skeletal Muscle Cells Results in Decreased Infiltration of T Cells into Interosseous Muscle

$CD4^+$ T cells are important mediators of inflammation and disease following CHIKV infection in mice (49). To understand how CHIKV replication in skeletal muscle cells affects recruitment of T cells into musculoskeletal tissues, mice were inoculated with SKE or SKE MM and euthanized at day 7 post-inoculation. The left rear limb was processed for immunohistochemistry using an antibody directed against CD3. Mice infected with SKE exhibited significantly diminished infiltration of $CD3^+$ cells into the interosseous muscle relative to mice infected with SKE MM (Figure 3-6A,C). This reduction in infiltrating $CD3^+$ T cells appears to be specific for the interosseous muscle, as the calcaneal tendon, a representative connective tissue, of both SKE- and SKE MM-infected mice was heavily infiltrated with these cells compared with mock-infected mice. These results were reproduced when immunohistochemistry was conducted using an antibody to CD4 (Figure 3-6D), although staining was less intense. Overall, these data indicate that replication in skeletal muscle cells is a required precursor to T cell infiltration into this site.

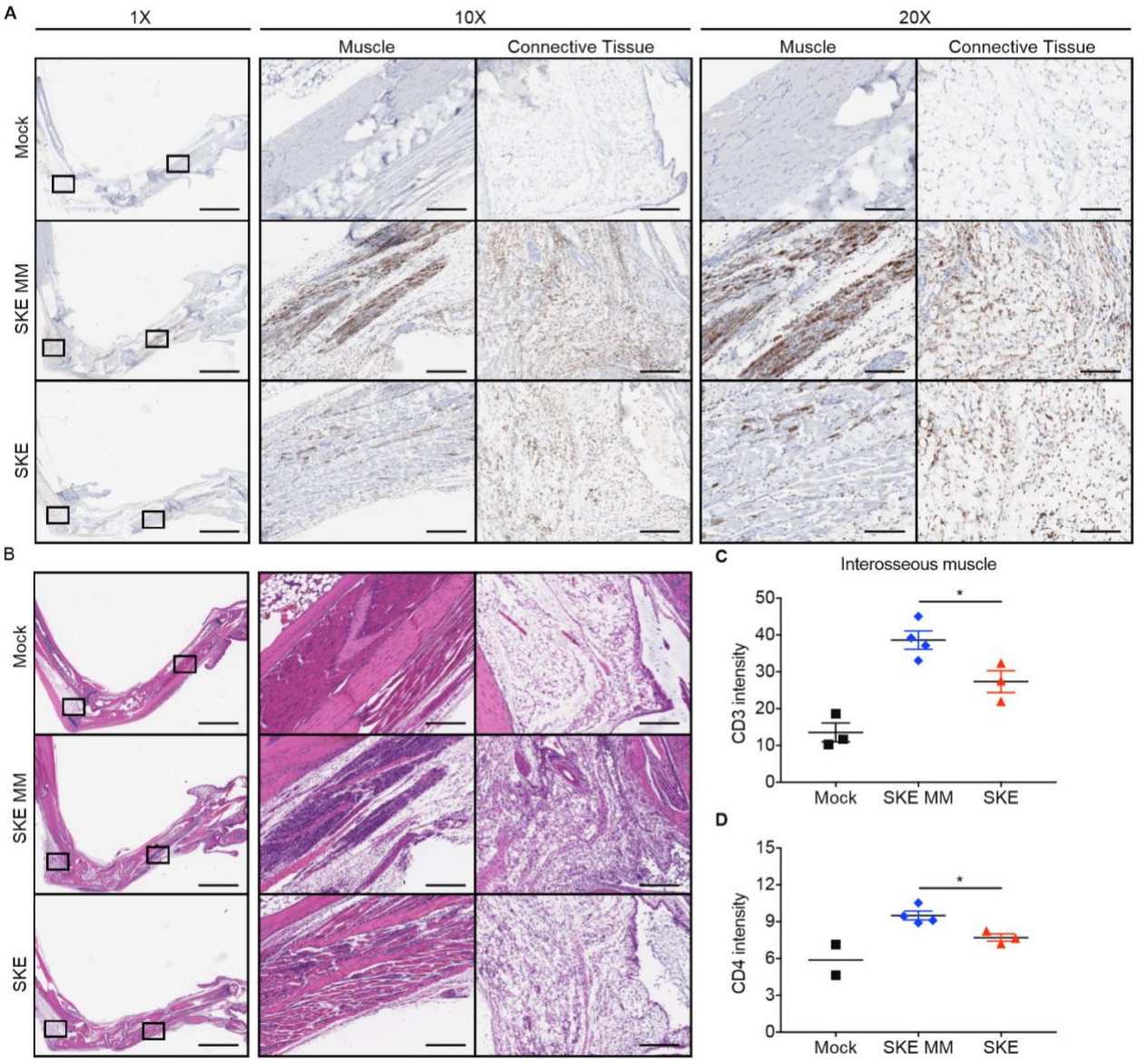

Figure 3-6. CHIKV replication in myofibers promotes T cell infiltration into interosseous muscle.

Three-to-four-week-old male C57BL/6J mice were inoculated in the left rear footpad with PBS (mock) or 10^3 PFU of either SKE MM or SKE. Left ankle tissue was collected 7 d post-inoculation and processed for either CD3 immunohistochemistry (A) or H&E staining (B). Regions corresponding to high-magnification insets (10X) of the interosseous muscle and calcaneal tendon (connective tissue) are indicated in the overview micrographs (1X) by black boxes. Representative images of 3 (mock and SKE) or 4 (SKE MM) mice per group are shown. Scale bars, 1X = 3 mm; 10X = 300 μm; 20X = 150 μm. Images were acquired using an Aperio ScanScope XT slide scanner and processed with Aperio ImageScope software. DAB signal corresponding to CD3 (C) or CD4 (D) staining in interosseous muscle

was quantified using ImageJ software. Horizontal bars indicate mean intensity. Error bars indicate SEM. *P* values were determined comparing SKE and SKE MM by two-tailed Student's *t* test (*, $P < 0.05$).

3.2.7 CHIKV Replication in Skeletal Muscle Cells Is Important for Production of Proinflammatory Mediators

We next tested whether diminished foot swelling and pathology following infection with SKE is attributable to altered immune responses elicited by virus incapable of replicating in skeletal muscle cells. Severe CHIKV disease in humans is associated with the production of IL-6, RANTES, and TNFα, with levels of IFNγ, IL-1β, IP-10, MCP-1, and MIP-1α also increasing during infection (35, 37, 295). To understand how replication in myofibers influences production of these proinflammatory mediators, mice were infected with SKE or SKE MM, and proinflammatory mediator induction in the left rear foot at day 3 post-inoculation was quantified by RT-qPCR. Relative to mice infected with SKE MM, mice infected with SKE exhibited a significant reduction in IL-1β, IL-6, IP-10, and TNFα transcripts in the left rear foot (Figure 3-7A). Importantly, this reduction was not due to a global decrease in the transcript levels of inflammatory molecules, as mRNA levels of IFN-γ, MCP-1, Mip-1α, and RANTES did not differ significantly in mice infected with SKE compared with SKE MM (Figure 3-7A). Additionally, while levels of IL-1β, IL-6, IP-10, and TNFα transcripts trended to be higher following infection with SKE MM, induction of these proinflammatory mediators did not differ significantly in the contralateral foot (Figure 3-7B), which does not swell following infection with either SKE or SKE MM (data not shown). These data suggest that local production of specific proinflammatory mediators drives swelling of the inoculated foot during CHIKV infection in mice.

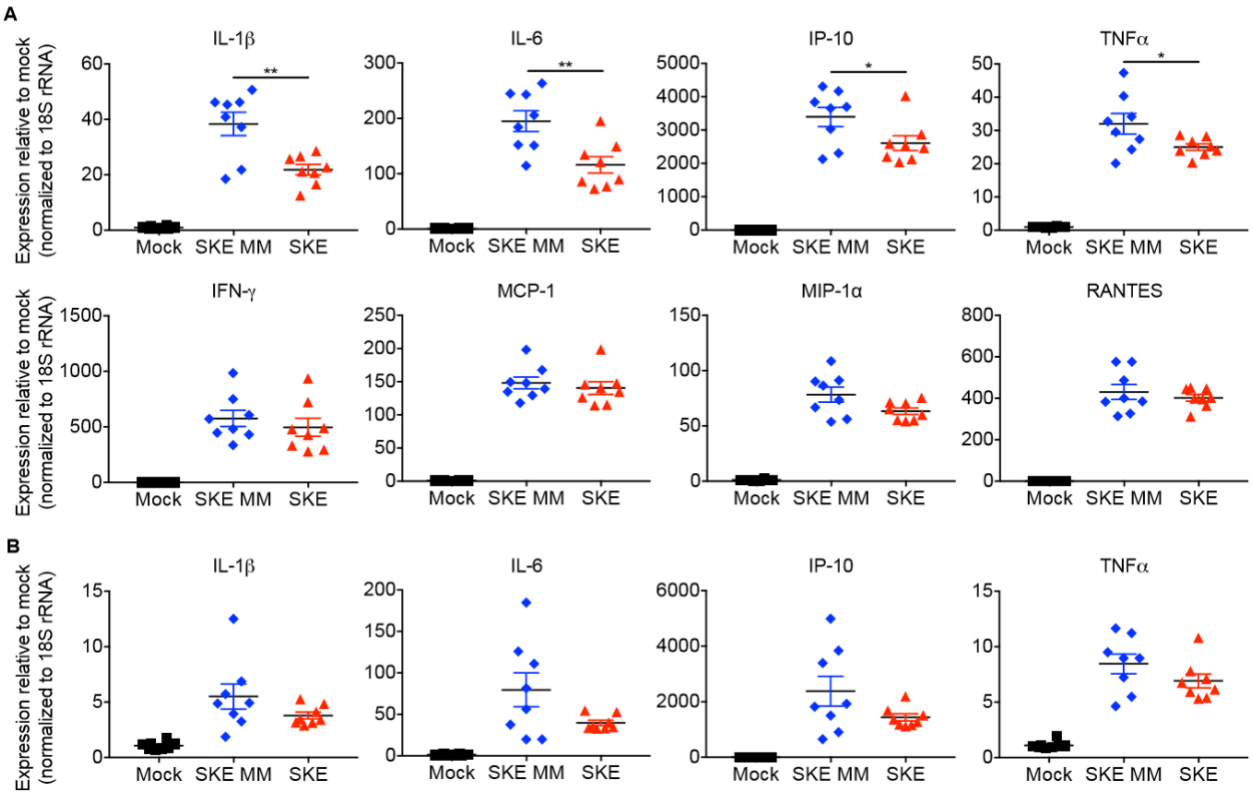

Figure 3-7. CHIKV replication in skeletal muscle is required for induction of IL-1b, IL-6, IP-10, and TNFa.

Three-to-four-week-old male C57BL/6J mice were inoculated in the left rear footpad with PBS (mock) or 10_3 PFU of either SKE MM or SKE. At 3 d post-inoculation, mice were euthanized, and left (A) and right (B) ankles were excised. RNA was extracted and reverse transcribed, and expression of the genes shown was quantified by qPCR. Data are normalized to 18S rRNA levels and expressed as the relative expression (*n*-fold increase) over that in mock-infected tissue. Horizontal bars indicate means of two independent experiments with 4 mice per group in each experiment. Error bars indicate SEM. *P* values were determined by comparing SKE and SKE MM by two-tailed Student's *t* test (*, $P < 0.05$; **, $P < 0.01$).

3.2.8 IL-6 Produced Following Viral Replication in Skeletal Muscle Mediates CHIKV-Induced Inflammation

Because increased IL-6 production is associated with severe CHIKV disease and production of IL-6 is dependent on virus replication in skeletal muscle cells (Figure 3-7), we next

assessed the contribution of IL-6 to CHIKV pathogenesis. Mice were inoculated intraperitoneally with 200 μg of either an IL-6 receptor blocking antibody or an IgG2b isotype control at 0, 3, and 5 days post-inoculation with either SKE or SKE MM. Swelling of the inoculated foot was quantified using digital calipers. As expected, SKE MM-infected mice treated with the isotype control exhibited significant swelling that peaked 6 days post-inoculation (Figure 3-8A). In contrast, swelling in SKE MM-infected mice treated with the IL-6 receptor blockade antibody was significantly reduced 5, 6, and 7 days post-inoculation, with levels more comparable to the swelling induced in SKE-infected mice treated with the isotype control antibody (Figure 3-8A). This phenotype was not due to differences in virus replication, as viral loads in the left and right ankles of SKE MM-infected mice treated with either the IL-6 receptor antibody or isotype control were comparable at day 7 post-inoculation (Figure 3-8B). Thus, IL-6 released following CHIKV infection in skeletal muscle cells is a critical mediator of CHIKV disease in mice.

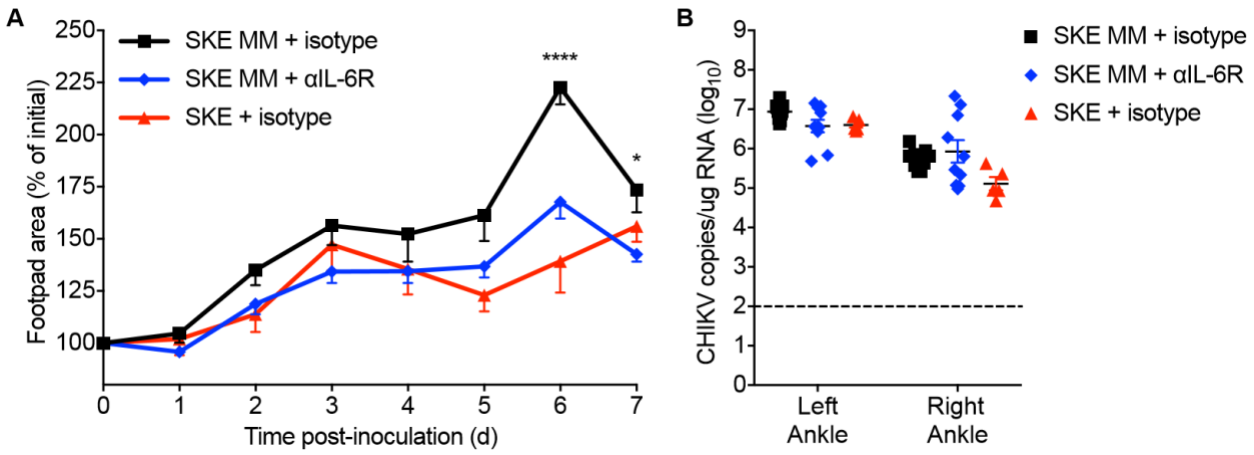

Figure 3-8. IL-6 mediates CHIKV-induced inflammation.

Three-to-four-week-old male C57BL/6J mice were inoculated intraperitoneally with 200 µg of either a monoclonal anti-IL-6 receptor antibody (clone 15A7) or an IgG2b isotype control antibody (clone LTF-2) on days 0, 3, and 5 post-inoculation with 10_3 PFU of either SKE MM or SKE. (A) Swelling of the left rear footpad was quantified using digital calipers on the days shown. Results are normalized to initial footpad area and presented as the mean percent of initial footpad area for 10 mice per group (SKE MM + isotype and SKE MM + αIL-6R) or 5 mice per group (SKE + isotype). Error bars indicate SEM. *P* values were determined by comparing SKE MM-infected animals receiving IL-6R antibody or isotype control by ANOVA followed by Tukey's *post hoc* test (*, $P < 0.05$; ****, $P < 0.0001$). (B) Viral loads in day 7 tissue homogenates were determined by RT-qPCR. Horizontal bars indicate mean CHIKV genome copies/mg RNA of 10 mice per group (SKE MM + isotype and SKE MM + αIL-6R) or 5 mice per group (SKE + isotype). Error bars indicate SEM. Dashed line indicates limit of detection. *P* values were determined by comparing SKE MM-infected animals receiving IL-6R antibody and SKE MM-infected animals receiving isotype control by ANOVA followed by Tukey's *post hoc* test (*, $P < 0.05$).

3.3 Discussion

The broad tropism of CHIKV has made it challenging to define determinants of CHIKV pathogenesis. In this study, we exploited the host RNAi machinery to assess how CHIKV

replication in skeletal muscle cells influences disease development. Target sequences for a skeletal muscle cell-specific miRNA, miR-206, were engineered into the structural ORF of the CHIKV genome. The presence of these target sequences was sufficient to specifically restrict CHIKV replication in a miR-206-dependent manner in vitro. Additionally, viral replication was diminished in the interosseous muscles of infected three-to-four-week-old mice following footpad inoculation with CHIKV, as assessed by in situ hybridization for CHIKV RNA. Interestingly, restriction of replication in skeletal muscle cells did not significantly affect CHIKV titers in tissues, implicating other cell types, likely connective tissue fibroblasts (39), as the major contributor to viral loads in tissues. Restriction of CHIKV replication in skeletal muscle cells led to significantly reduced inflammation and necrosis in the interosseous muscles of the left foot, with a concomitant decrease in T cell infiltration and transcript levels of select proinflammatory mediators including IL-1β, IL-6, IP-10, and TNFα. Finally, we found that IL-6 released following CHIKV replication in skeletal muscle cells is an important mediator of inflammation. Accordingly, treatment with an IL-6 receptor antibody significantly diminished swelling in the inoculated foot of mice infected with a control virus capable of replication in skeletal muscle cells to levels similar to those produced by virus restricted at this site.

The capacity to replicate in skeletal muscle is common among arthritogenic alphaviruses and other viruses that cause myalgia and myositis (296, 297). Whether these viruses cause disease in skeletal muscle using mechanisms similar to CHIKV is unknown. Importantly, it is unclear even for the leading causes of viral myositis, including enteroviruses and influenza virus, whether virus replication in muscle cells directly damages muscle tissue or if disease results from immunologic processes induced by viral infection (268, 269, 298). While CHIKV has primarily been studied in the context of disease manifestations in the joints, CHIKV-patient biopsies demonstrate that virus

also elicits significant infection and damage in muscle tissue (33), a facet of disease that was previously underappreciated. Additionally, intradermal inoculation of newborn mice with a virulent strain of CHIKV isolated in Senegal in 1983 results in diminished hind limb weakness and viral titer in muscle compared with inoculation of a more contemporary strain isolated in La Reunion in 2006, implicating viral replication in skeletal muscle as an important, and perhaps strain-specific, mediator of pathogenesis (285). Unfortunately, the divergent nature of these strains has precluded a precise determination of the exact mechanism of attenuation. Understanding how CHIKV causes damage to skeletal muscle is important both to gain a broader understanding of mechanisms of CHIKV pathogenesis and to inform studies defining mechanisms by which other viruses cause muscle pathology.

Restriction of CHIKV replication in skeletal muscle resulted in diminished induction of IL-1β, IL-6, IP-10, and TNFα in mice. Of these, IL-1β and IL-6 are biomarkers of severe CHIKV disease in humans (35), although IP-10 and TNFα also are significantly induced following CHIKV infection (37, 295). The cellular source of these cytokines during infection is unknown. Although produced by a variety of cells including activated macrophages and T cells, IL-1β, IL-6, IP-10, and TNFα could conceivably be directly elaborated by skeletal muscle cells following infection. Skeletal muscle is capable of producing IL-6 and IP-10 following contraction of muscle fibers, which is important in muscle regeneration and homeostasis (299-303). Additionally, muscle injury results in elaboration of TNFα by muscle fibers (304). In the context of inflammation, IL-1β and TNFα are potent inducers of IL-6 production by skeletal muscle (302). The precise mechanism through which these molecules are elaborated following CHIKV infection of skeletal muscle remains undefined.

The three-to-four-week-old mice used in these studies recapitulate many hallmarks of human disease, including muscle necrosis, myositis, and tenosynovitis (44). However, the inflammatory response to CHIKV in mice is not well understood. Although molecular mediators of biphasic swelling in the inoculated foot are not known, $CD4^+$ T cells are required for the second phase of swelling (49). We discovered that restriction of CHIKV replication in skeletal muscle results in diminished infiltration of $CD4^+$ T cells into the interosseous muscle at 7 dpi. Additionally, blockade of the IL-6 receptor significantly diminishes swelling during this phase, indicating that IL-6 receptor signaling is important for this disease manifestation. How IL-6 receptor signaling and $CD4^+$ T cells synergize to mediate this phenotype is unknown. IL-6 receptor expression is limited in mice, reaching detectable levels mainly on hepatocytes and some leukocytes (305). IL-6 induces chemotaxis of T cells in vitro, indicating a potential function in T cell homing to sites of CHIKV infection (306). Additionally, stimulation of naïve T cells with IL-1β and IL-6 induces naïve T cells to differentiate into TH17 cells (307). IL-17 elaborated by these cells is a mediator of inflammatory myopathy (308, 309). Stimulation of muscle cells with a combination of IL-1β, IL-17, and TNFα results in upregulation of MHC class I, which is directly cytotoxic to muscle cells (310). It is also possible that IL-6 functions in the activation of $CD4^+$ T cells. IL-6 is a costimulatory molecule involved in T cell activation, expansion, and survival (311, 312). Additionally, costimulation of $CD4^+$ T cells with IL-6 and TNFα is sufficient to activate these cells in an antigen-independent manner (313). Finally, while $CD4^+$ T cells have a pathogenic role during CHIKV infection, it is also possible that T cells are required for viral clearance and that diminished infiltration by these cells caused by restriction of CHIKV replication in skeletal muscle could enhance persistence in musculoskeletal tissues at late times post-inoculation.

While our studies have demonstrated that skeletal muscle cells are a critical infection site for development of severe musculoskeletal disease, there are many other important questions related to CHIKV pathogenesis that can be answered by coopting the host RNAi machinery. Cell-culture-based susceptibility assays have implicated keratinocytes, fibroblasts, and skin-resident macrophages as potential primary sites of virus replication following deposition by the bite of an infected mosquito (82, 85, 115). The function of these cells in the amplification or dissemination of CHIKV following primary infection is unknown. Additionally, CHIKV is one of few arboviruses capable of achieving sufficiently high levels of viremia in humans to infect a naïve mosquito following a blood meal (314). Because CHIKV is incapable of appreciable replication in leukocytes, serum viremia is likely the result of virus released from another cell type into the bloodstream (85). These gaps in knowledge could be addressed by engineering CHIKV strains restricted in other cell types through miRNA targeting.

Our study contributes to a better understanding of cellular and molecular determinants of disease development during CHIKV infection. These data also may enhance both the development of CHIKV vaccines and targeted antivirals. To date, no CHIKV vaccines have achieved licensure, though a number of vaccine candidates are currently in clinical trials (160, 315, 316). Live-attenuated vaccines are often preferable, due to their capacity to elicit robust humoral and cellular immune responses and long-lived protection (317). One challenge in the development of live-attenuated vaccine candidates is to ensure an appropriate degree of attenuation to eliminate disease while allowing sufficient replication to induce a protective immune response. Addition of skeletal muscle target sequences is an attractive method of attenuating CHIKV disease without affecting viral loads in tissues. Skeletal muscle cell-specific miRNA target sequences could potentially be incorporated into existing candidates to achieve ideal attenuation. Additionally, our studies

identify IL-6 receptor signaling as a critical mediator of CHIKV disease. Importantly, an IL-6 receptor antibody, tocilizumab, is approved for use in the treatment of rheumatoid arthritis (318). Our data indicate that this therapeutic could be repurposed in the treatment of CHIKV disease. Overall, findings reported here establish the foundation to understanding the contributions of discrete CHIKV-infected cell types to pathogenesis and potentially inform the development of effective vaccines and antivirals to limit the global burden of CHIKV disease.

4.0 SKE Vaccination Protects Against Challenge With Virulent CHIKV

4.1 Introduction

4.1.1 Live-Attenuated Virus (LAV) Vaccines

The capacity of a vaccine to promote durable, robust immunity to subsequent viral infection is reliant on multiple factors. Effective vaccines should provide enough antigen in the right location of the body to elicit a protective response while at the same time avoiding vaccine-associated disease. Inactivated and subunit vaccines frequently meet the latter criteria, as they are incapable of infection and replication characteristic of their live, attenuated counterparts. However, because they cannot replicate and disseminate from sites of inoculation, they often require multiple doses to promote a long-lasting, protective response. LAV vaccines are often capable of eliciting desired immune responses following a single dose, although adverse events are more frequently associated with this vaccine type. Live-attenuated candidates can theoretically serve as potent vaccines if a proper balance is achieved in attenuating the virus to a degree that limits disease without compromising replication to the extent that the vaccine is no longer capable of evoking a protective immune response. Striking this balance has been a major hurdle in the development of live-attenuate vaccine candidates.

Many different methods have been employed to recover LAV vaccine candidates. Initial attempts at developing LAVs involved passage of virulent virus strains in cell culture until they were empirically attenuated, exhibiting a small plaque phenotype *in vitro* and diminished virulence in animal models. This is the method of attenuation of the live-attenuated Sabin poliovirus vaccine

(319), the use of which has led to the near-eradication of poliovirus and illustrates the potential of this approach. The major caveat to LAVs developed through this method is that, because viruses are passaged until attenuation is observed in cell culture, little information is known about mechanisms of attenuation. Reversion of LAV candidates to WT sequence can result in vaccine-associated disease. This occurs following administration of live-attenuated poliovirus, which is associated with reversion and vaccine-associated paralytic poliomyelitis in ~1 in 500,000 first-time vaccinees (320-322). This caveat has promoted attempts to recover LAV strains for which the method of attenuation is known.

One approach to deliberately attenuate a virus is by deletion or targeted mutation of genes essential for replication. The first attempt to develop an LAV using this approach occurred when researchers recovered HSV strains lacking either ICP8 or ICP27, two proteins essential for virus replication (323). These strains are tolerated in mice even at doses that would constitute a lethal infection with virulent virus and elicit a protective cellular immune response after a single inoculation (323). Because these viruses are attenuated on the basis of removal of a gene required for replication, they must be recovered in cell lines complemented with the essential gene. Another important consideration is that, because viruses recovered using this method are often replication-incompetent, they may not disseminate and replicate at sites other than the site of inoculation, limiting one of the major benefits in using LAVs. For the HSV strains lacking ICP8 or ICP27, this potential problem is not significant, as the immune response to the replication-deficient LAVs is equal in magnitude and duration to that elicited by the replication-competent parental virus (324). Likely, the protective immune response to replication-competent HSV is evoked during early replication at the site of inoculation, and it is not likely that this same effect would be observed for viruses that have to disseminate to secondary sites to elicit a protective immune response.

For RNA viruses, manipulation of the fidelity of the virus-encoded RdRp represents another method by which LAVs can be engineered. Compared to other polymerases, virus-encoded RdRps exhibit low replication fidelity that results in the incorporation of 0.1-10 mutations per replication cycle (325). This lack of replication fidelity is important in escape from host immune responses and adaptation to selective pressures. Enhancing replication fidelity of viral RdRps is attenuating due to decreased capacity of the virus to evolve under selective pressures encountered in the host (326). The reverse, diminishing replication fidelity, permits incorporation of too many mutations during each replication cycle and is also attenuating (326). One attempt to harness this method to develop an LAV was the recovery of poliovirus strains encoding a mutation, either G64S, G64A, or G64L, in the RdRp that leads to increased replication fidelity (327). These mutants are attenuated in mice and stimulate a protective immune response that is greater by an order of magnitude than vaccination with the Sabin strain (327). Additionally, due to enhanced replication fidelity, no evidence of reversion is observed following 20 passages in HeLa cells or 5 mouse-to-mouse passages (327).

One final technique employed to engineer LAV strains uses codon deoptimization to recover strains that display diminished translation efficiency in cells. This approach relies on the observation that the genetic code is degenerate, and many codons can specify the same amino acid (328). Organisms can exhibit a codon bias depending on expression levels of transfer RNAs (tRNAs) that mediate incorporation of encoded amino acids during translation (329, 330). To recover a codon-deoptimized virus, many synonymous mutations are engineered into a viral genome such that the least favored codon for each amino acid is encoded at each position. Poliovirus strains recovered using this method exhibit diminished translation efficiency in mammalian cells compared with WT virus (331). Additionally, three i.p. inoculations in mice

provide protection against lethal challenge with WT poliovirus (332). This approach represents an interesting method for LAV recovery as, because vaccine candidates are antigenically identical to their WT counterparts, only the amount of antigen present due to diminished replication differs from WT infection. Additionally, because of the sheer number of mutations required to generate this type of vaccine, the risk of reversion is minimal, although additional studies are required to define safety and immunogenicity of this type of vaccine in humans.

4.1.2 Attempts to Recover Live-Attenuated CHIKV Vaccines

Various live-attenuated CHIKV strains have been described as potential vaccines. The first candidate, strain 181/25, is described in detail in Chapter I. In brief, this strain was derived following plaque-to-plaque passage of virulent strain AF15561 until attenuation was empirically achieved based on a small-plaque phenotype in cell culture and attenuation in a suckling mouse model (154). Clinical trials were halted following the development of transient arthralgias in a small cohort of vaccinees (155). It was later discovered that 181/25 is attenuated due only to two point mutations in the E2 glycoprotein, with the majority of attenuation attributed to mutation G82R (79, 80, 333). While 181/25 is still commonly used as a BSL2 strain to study the cell biology of CHIKV infection, the ease with which the virus reverts to WT sequence has precluded its use as a vaccine.

The G82R mutation present in 181/25 enhances the capacity of the virus to bind glycosaminoglycans, which is attenuating in mice and humans. Another mutation affecting glycosaminoglycan binding, E79K, was identified following passage of CHIKV in Chinese hamster ovary (CHO) K1 fibroblasts and C6/36 *Aedes albopictus* cells (40). This mutation enhances attenuation in mice over that observed following infection with E2 G82R mutants

without compromising protection upon virulent virus challenge (40). Viruses encoding this mutation alone still have a high likelihood of reversion, but it could potentially be used in combination with other attenuating mutations to enhance the safety of a vaccine candidate.

Four LAV vaccine candidates for CHIKV have been described in which targeted mutations in the virus result in diminished replicative capacity. The first candidate, CHIKV-IRES, was recovered following replacement of the subgenomic promoter with an internal ribosomal entry sequence (IRES) (334). This virus is safe in IFN-deficient mice and protects against virulent challenge following a single dose in both mice and nonhuman primates (316, 334, 335). Because reversion would require the virus to mutate the IRES back to subgenomic promoter sequence, this candidate exhibits highly stable attenuation. The virus also is incapable of replicating in mosquitos because insect ribosomes do not recognize mammalian IRES sequences. One caveat to this LAV candidate is that the virus exhibits a severe defect in replication *in vivo*, as no infectious virus is detected in any mouse tissue throughout the course of acute infection (334). It is possible that more than one dose will be required to elicit a protective immune response in humans.

Additional LAV candidates exhibiting diminished replication by virtue of targeted mutations were recovered following deletion of either the transmembrane domain of E2 (336), 183 base pairs of the viral nsP3 protein (162), or the N-terminal nucleolar localization signal of the capsid protein (337), termed TM17-2, Δ5nsP3, and CHIKV-NoLS, respectively. Each of these candidates is attenuated and protective in mice against virulent challenge following a single dose of vaccine (162, 336, 337). Of these, only Δ5nsP3 has entered clinical trials, although no results have been reported. Whether any of these LAV vaccine candidates is associated with adverse events in humans is unknown.

One final mutant LAV is a high-fidelity variant of CHIKV. This variant was identified following 20 serial passages of CHIKV in the presence or absence of ribavirin or 5-fluorouracil until a population was recovered with resistance to these mutagens. Sequencing of this population identified a mutation in the viral nsP4 RdRp, C483Y, capable of limiting mutation frequency and genetic diversity of viral progeny during replication in mammalian cells (338). This mutation is also attenuating in both mice and mosquitoes, resulting in diminished titers in various tissues post-inoculation (338). Introduction of the C483Y mutation in concert with other attenuating mutations could yield an LAV candidate with limited potential for reversion due to enhanced replication fidelity.

Overall, a number of live-attenuated variants of CHIKV have been described and are currently in development as vaccines. While these candidates are promising, clinical trials with 181/25 have demonstrated that live-attenuated CHIKV vaccines can cause disease, albeit with diminished virulence. Therefore, it is important to identify additional attenuating mutations and begin combining methods of attenuation in the attempt to recover a vaccine capable of achieving licensure for use in humans.

4.2 Results

SKE represents an interesting vaccine candidate, as it is markedly attenuated in mice without compromising replication in most tissues during acute infection, which should elicit a protective immune response. To understand whether infection with SKE can protect against challenge with virulent CHIKV, mice were inoculated subcutaneously in the left rear footpad with PBS or 10^3 PFU of either SKE or WT SL15649. At day 30 post-inoculation, mice were challenged

in the same foot with 10^3 PFU of WT SL15649, and swelling of the inoculated foot was assessed with digital calipers. PBS-vaccinated mice exhibited bimodal swelling of the left rear footpad following virulent challenge, which is characteristic of WT CHIKV infection (Figure 4-1). In contrast, mice vaccinated with either SKE or WT SL15649 were protected against disease post-challenge (Figure 4-1). These data suggest that SKE vaccination is capable of eliciting a protective immune response in mice.

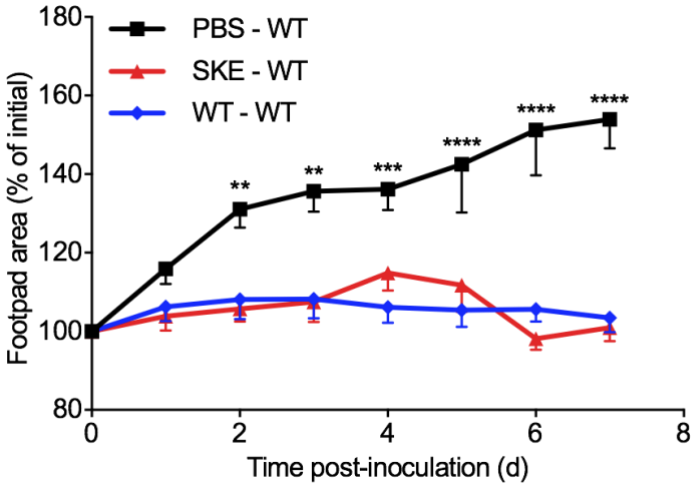

Figure 4-1. Vaccination with SKE is protective upon virulent virus challenge.

Three-to-four-week-old male C57BL/6J mice were inoculated in the left rear footpad with PBS (mock) or 10^3 PFU of either WT SL15649 or SKE. At d 30 post-inoculation, mice were challenged in the left rear footpad with 10^3 PFU of WT SL15649. Swelling in the left rear footpad was quantified using digital calipers on the days shown. Results are normalized to initial footpad area and presented as the mean percent of initial footpad area for 5 mice per group. Error bars indicate SEM. *P* values were determined by comparing SKE-vaccinated animals with mock-vaccinated animals by ANOVA followed by Tukey's *post-hoc* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

Because SKE is capable of serving as a vaccine candidate, I next wanted to determine whether a combination of SKE with other attenuating mutations would be safe and protective in mice. In particular, I wanted to use an attenuating mutation that, while protective, was still too

reactogenic in humans. The G82R mutation described as attenuating in 181/25 represented an attractive option because it is potently attenuating in immunodeficient and immunocompetent mice, but vaccination results in transient arthralgias in humans. To test whether combining G82R with the target sequences for skeletal muscle-specific miR-206 present in SKE could enhance the safety of a vaccine candidate without diminishing robustness of protection, I recovered strain SKE G82R (Figure 4-2) using site-directed mutagenesis of SKE. To control for contributions of G82R alone, I also recovered SL15649 containing the G82R mutation (Figure 4-2). Along with WT SL15649 and SKE, these viruses will be used to determine whether a combination of SKE with G82R limits reversion and reactogenicity without compromising protective capacity in mice.

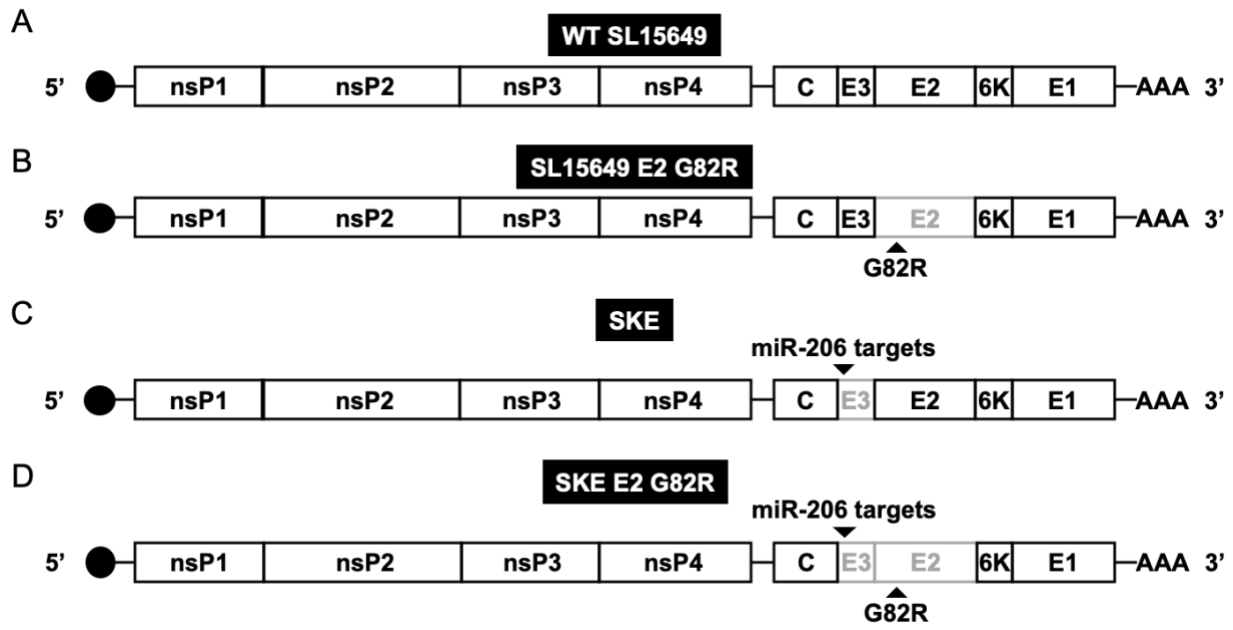

Figure 4-2. Schematic of vaccine candidate CHIKV strains.

Schematic for engineering of vaccine strains in background of WT SL15649 (A). Vaccine strains were recovered by mutation of E2 G82R (B), incorporation of target sequences for skeletal muscle-specific miR-206-3p in the E3 coding region (C), or both (D).

4.3 Discussion

Data presented in this chapter demonstrate that SKE vaccination protects mice from disease following virulent CHIKV challenge. However, additional experimentation is necessary to evaluate the capacity of SKE to serve as a vaccine either alone or in concert with other attenuating mutations. Firstly, the degree of attenuation of SL15649 G82R and SKE G82R should be evaluated in mice. This includes measurement of swelling in the inoculated footpad in immunocompetent mice as well as determination of attenuation in mice deficient in type I IFN responses. These virus

strains also should be evaluated for protection of immunocompetent mice from disease following challenge with WT CHIKV.

For other live-attenuated CHIKV vaccines, the humoral immune response has been the protective component, and passive transfer of serum from CHIKV-infected mice to naïve mice limits disease (51, 339). To test whether this is also the case for SKE and SKE G82R, serum from immunocompetent mice infected with CHIKV can be transferred to immunodeficient mice to determine protective capacity. Alternatively, μMT mice, which lack mature B cells could be inoculated with vaccine strains and then challenged with WT virus to determine whether vaccination is protective in the absence of antibody responses. Taken together, these experiments will serve as proof-of-concept that SKE is a viable vaccine candidate and that multiple attenuating mutations can be incorporated into the same vaccine strain to improve safety without compromising immunogenicity and protection.

This study focuses on a combination of SKE and G82R, although I described a variety of different attenuating mutations earlier in this chapter that comprise a CHIKV vaccine toolkit. Conceivably, a combination of any two or more of these mutations could lead to an ideal vaccine. In particular, the high-fidelity mutation, nsP4 C483Y, in combination with mutations like SKE and those that affect glycosaminoglycan utilization such as E79K and G82R, presents an interesting combination in developing a vaccine candidate with optimal safety and immunogenicity profiles. Viruses encoding SKE, E79K, and G82R mutations still replicate to some extent in tissues of infected animals (40, 79), and the resultant amplification of antigen at sites targeted by WT virus is the major benefit of LAVs. The major concern in developing these vaccines is in reversion, but this potential problem would be largely ameliorated by incorporation

of multiple mutations in the context of the high-fidelity variant. Such considerations may prove critical in achieving licensure of a vaccine to limit the global burden of CHIKV disease.

5.0 Summary and Future Directions

5.1 Thesis Summary

Infection by viruses can elicit dramatically different responses depending on the type of cell infected, context of surrounding tissue, and organism in which the cell resides. The capacity of a virus to infect a cell, which is reliant on expression of receptors capable of supporting internalization and absence of restriction factors, is a critical determinant of disease progression in the host. Elucidating the role of viral tropism in pathogenesis promotes an understanding of the molecular basis of disease progression and informs development of targeted antivirals and vaccines. However, defining the role of tropism in disease development has been difficult for viruses exhibiting a wide tropism, especially when the receptors and other host factors supporting infection are unknown.

CHIKV gained global awareness following epidemics in 2004 and 2013 in which emergence of the virus in naïve populations resulted in millions of cases arthritic disease. While the clinical course of CHIKF has been well-documented in infected individuals, we do not fully understand the molecular underpinnings of illness. Such an understanding has been made difficult by the broad tropism displayed by CHIKV for various cells and tissues in mammalian hosts. This broad tropism, along with an incomplete understanding of the host factors required for CHIKV infection of mammalian cells, has made elucidating the pathogenic contributions of replication at discrete sites difficult. To begin to answer this important question, I engineered a panel of viruses exhibiting cell type-restricted replication by incorporation of tissue-specific miRNA target sequences. In Chapter II, I described important considerations in the recovery of miRNA-restricted

strains. Viruses containing miR-target inserts in the coding sequence of the E3 protein were recovered at high titer, and the inserted sequences were stable by consensus sequencing. Insertion of exogenous sequence at this site did not affect replication in cultured cells or the course of disease in mice in the absence of restrictive miRNAs, validating the use of this method to dissect the pathologic contribution of CHIKV replication in various cells in the mammalian host.

In Chapter III, I provided evidence that viral replication in skeletal muscle cells is an important precursor to disease development. A CHIKV strain incapable of replication in skeletal muscle cells was significantly attenuated, eliciting diminished swelling in the inoculated foot in mice compared with WT-infected counterparts. Restriction of viral replication in skeletal muscle also limited muscle inflammation and necrosis, which are characteristic of CHIKV disease in mice. Infection of muscle fibers promoted infiltration of T cells and release of IL-6. Treatment of mice with an antibody directed against the IL-6 receptor protected mice from severe CHIKV-induced musculoskeletal disease. Finally, in Chapter IV, I demonstrated that vaccination with the skeletal muscle-restricted CHIKV was protective upon challenge with virulent virus and may serve as a vaccine candidate either alone or in combination with other attenuating mutations.

5.2 Future Directions

5.2.1 Determine the Role of CHIKV Replication at Other Sites in Disease Development

In the discussion of Chapter II, I describe future directions for the panel of miRNA-restricted viruses I initially recovered for this study. In addition to experiments proposed in that chapter, there are other CHIKV-targeted sites in the mammalian host that express highly specific

miRNAs. In particular, chondrocytes, which specifically express miR-140 (340), and hepatocytes, which specifically express miR-122 (341), are both infected by CHIKV. CHIKV infection causes focal necrosis in cartilage tissue in some mouse models (45, 342, 343), and destruction of cartilage may contribute to rheumatic disease in humans. A pathogenic role for CHIKV replication in chondrocytes is supported by studies with RRV, which show that virus infection of cartilage cells stimulates release of heparanase (344), a molecule associated with joint pathology and cytokine signaling (345-347). Detection of CHIKV antigen in chondrocytes indicates that the virus is also capable of replicating in these cells (343), although additional studies are necessary to determine whether infection of chondrocytes has a pathogenic outcome.

Hepatitis is a rare manifestation of CHIKV infection in humans (19), although CHIKV RNA was isolated from a patient's liver in a fatal case of CHIKV disease (348). Infectious virus and viral RNA also have been isolated from the liver of infected nonhuman primates (118, 119). Additionally, because the liver filters blood, it is possible that cells of the liver contribute to high serum viremia observed during CHIKV infection. The precise target cells of CHIKV infection in the human liver remain unclear, although virus infection in macaques causes hepatocyte necrosis (118). A virus incorporating target sequences for miR-122 could be recovered to test whether replication in hepatocytes contributes to viremia and causes necrosis observed during infection.

5.2.2 Assess Sites of Replication Required for Dissemination in Mosquitoes

While my studies focused primarily on the mammalian host, RNAi also can be coopted to elucidate the role of various mosquito cell types in viral dissemination. Following a bloodmeal, CHIKV first enters the midgut of a mosquito. The virus then infects epithelial cells, and progeny virions accumulate in the surrounding basal lamina (349). The virus must penetrate the basal

lamina to disseminate to secondary sites, and the mechanism by which CHIKV breaches the basal lamina is unknown. Subsequently, virus must infect a secondary site for amplification following escape of the midgut, but it is not known how the virus disseminates back to the salivary glands for deposition into a new host.

Many mosquito miRNAs are expressed in a tissue-specific manner. In particular, tissue-specific miRNAs have been described for the head, fat body, ovary, and midgut of the mosquito under resting conditions and during digestion following bloodmeal (350). All of these are target sites of CHIKV in mosquitoes (351). Conceivably, viruses engineered to contain target sequences could be used to infect mosquitoes to determine how CHIKV disseminates in vector species. Additionally, CHIKV can be passed to mosquito progeny through vertical transmission, a process that is thought to result from virus replication in mosquito ovary cells (352, 353). Viruses engineered to contain ovary-specific miRNA-target sequences could be used to test this hypothesis. Ovary-specific miRNA-target sequences also could be used alongside other mosquito-specific miR targets to ensure that vaccine candidates do not propagate in mosquito vectors.

5.2.3 Define Pathogenic Contribution of Skeletal Muscle Replication for Other Alphaviruses

In experiments described in Chapter III, I assessed the role of CHIKV replication in skeletal muscle cells in the development of myositis. Infection with other arthritogenic alphaviruses also causes myositis in mouse models, including Mayaro virus (MAYV) (354), o'nyong-nyong virus (ONNV) (355), and RRV (296). Of these, RRV is capable of replicating in skeletal muscle cells (296). *In situ* hybridization analysis of MAYV and ONNV is required to determine whether these viruses exhibit the same myotropism, but I suspect that they do. For viruses capable of replicating

in skeletal muscle cells, the same strategy I used to manipulate CHIKV replication can be employed to restrict replication in myofibers. Analysis of virulence following infection of mice with these strains would promote an understanding of whether these viruses induce myositis in a similar mechanism to CHIKV, thus allowing my findings to be generalized to other arthritogenic alphaviruses.

5.2.4 Assess Importance of Encephalitic Alphavirus Replication in Various Cell Types for Dissemination to and Pathogenesis in the Central Nervous System (CNS)

While my studies with CHIKV infection in skeletal muscle demonstrated a mechanism by which arthritogenic alphaviruses cause rheumatic disease, the use of miRNA-restricted viruses also can be used to study the pathogenesis of encephalitic alphaviruses. These viruses, including EEEV, Western equine encephalitis virus (WEEV), and Venezuelan equine encephalitis virus (VEEV), are capable of invading the CNS in humans to elicit neurological disease. These viruses also are capable of infecting cells in the brain in various animal models (356-361). How these viruses are capable of penetrating the blood-brain barrier to establish infection of cells of the CNS and promote encephalitis remains undefined. Endothelial-specific miRNA target sequences, such as those discussed in Chapter II, could be engineered into these viruses to determine whether invasion of the brain is accomplished following infection of cells lining the vasculature, such as brain microvascular endothelial cells. Additionally, neuron-specific miRNAs, such as miR-9 or miR-124 (362), could be used to assess the role of virus replication in neurons to invasion and pathogenesis in the CNS. Viruses found to be attenuated through miRNA restriction could potentially serve as vaccines or be used to improve tolerability of other live-attenuated vaccine candidates.

5.2.5 Development of Nonhuman Primate Models for Study of CHIKV Musculoskeletal Disease

One hurdle to studies of CHIKV myositis is an absence of nonhuman primate models that faithfully recapitulate the CHIKV-induced musculoskeletal disease observed in humans. While rhesus macaques recapitulate some aspects of CHIKV disease not observed in mice, such as fever and rash, joint and muscle manifestations have not been described in this model. Clues from studies of CHIKV infection in mouse and human skeletal muscle cells may inform development of a better nonhuman primate model for study of these disease manifestations. Firstly, Mxra8 is highly expressed on human synovial fibroblasts and skeletal muscle, and expression of Mxra8 is required for infection in these tissues, as antibody blockade significantly diminishes the number of cells supporting infection (82). Mxra8 expression in rhesus macaque synovial fibroblasts and skeletal muscle cells has not been reported, but it is possible that low expression of this receptor in macaques explains the absence of disease in the joints and muscle following CHIKV infection. To develop a better animal model, Mxra8 expression in these tissues could be assessed in a variety of nonhuman primate models. Severity of musculoskeletal disease following CHIKV infection can then be correlated with Mxra8 expression profiles to define the role of this receptor in musculoskeletal disease progression in nonhuman primates and potentially identify a better model system.

It also is possible that study of musculoskeletal disease in the current rhesus macaque model could be improved by use of genetically modified CHIKV strains or immunomodulation of infected macaques. A mutation in the viral E2 glycoprotein, K200R, enhances musculoskeletal disease in mouse models (289). Infection of mice with CHIKV strains encoding the K200R mutation results in enhanced viremia and dissemination to musculoskeletal tissues distal to the site

of inoculation (289). Infection of macaques with E2 K200R mutant CHIKV strains may enhance musculoskeletal disease. In mice, IRF1 expression restricts CHIKV infection in skeletal muscle cells (247). IRF1 is a transcription factor mediating the expression of ISGs following ligation of the IFN-γ receptor (363). Blocking this signaling, by antibodies specific for IFN-γ or its receptor, may exacerbate musculoskeletal disease in macaques, allowing for study of the molecular basis of disease in joints and muscle.

5.2.6 Evaluate Efficacy of Anti-IL-6R Antibody Treatment in Alleviating CHIKV Disease

In Chapter III, I describe how blockade of IL-6R signaling prevents severe CHIKV disease development in mice. A similar antibody derived against human IL-6R, tocilizumab, is licensed for treatment of rheumatoid arthritis in humans. To date, no studies have reported efficacy of this therapeutic to treat CHIKV arthritis. To determine the therapeutic potential of IL-6R blockade, treatment with these antibodies should be evaluated further in mouse and nonhuman primate models. In particular, it would be important to assess the required dosing and therapeutic window in which IL-6R antibody administration is capable of protecting against CHIKV-induced musculoskeletal disease. In our studies, we administered 200 μg antibody on days 0, 3, and 5 post-inoculation with CHIKV. Whether less antibody could be used and whether three doses are required for protection is unclear. Additionally, analysis of protection following antibody administration on different days will help to understand whether this therapeutic must be administered prior to CHIKV infection or could be used as a therapeutic option for those with CHIKV-induced arthritis. Finally, as rhesus macaques experience rash and fever following CHIKV infection, this model could be used to evaluate whether these manifestations of CHIKV infection are affected by IL-6R blockade.

5.3 Conclusions

The work described in this thesis elucidates the importance of replication in skeletal muscle cells in disease development. Additionally, studies presented here build a foundation for future studies designed to define the pathogenic contributions of infection of other cell types. Collectively, this work enhances an understanding of CHIKV pathogenesis and identifies a live-attenuated vaccine candidate and therapeutic target to limit the global threat posed by CHIKV.

6.0 Materials and Methods

6.1 Cells

Human osteosarcoma cells (U-2 OS; ATCC HTB-96) were maintained in McCoy's 5A medium (Gibco) supplemented to contain 10% fetal bovine serum (FBS; VWR). Baby hamster kidney cells (BHK-21; ATCC CCL-10) were maintained in Alpha minimal essential medium (αMEM; Gibco) supplemented to contain 10% FBS and 10% tryptose phosphate. Vero81 cells (ATCC CCL-81) were maintained in αMEM supplemented to contain 5% FBS. HBMECs (provided by Kwang Sik Kim, Johns Hopkins University) were maintained in RPMI1640 (Gibco) supplemented to contain 10% FBS, 10% NuSerum (BD Biosciences), nonessential amino acids (Sigma), 1 mM sodium pyruvate, and MEM vitamins (Mediatech). ST2 cells (provided by Julie A. Sterling, Vanderbilt University) were maintained in RPMI1640 with 25 mM HEPES supplemented to contain 10% FBS. C2C12 cells (ATCC CRL-1772) were maintained in DMEM supplemented to contain 10% FBS. To differentiate C2C12 cells, confluent cultures were maintained in DMEM supplemented to contain 2% horse serum (Sigma) replaced daily for two weeks. J774A.1 (ATCC TIB-67), SVEC4-10 (ATCC CRL-2181), and XB-2 (ATCC CL-177) cells were maintained in DMEM supplemented to contain 10% FBS. All cell maintenance medium was supplemented to contain 2 mM L-glutamine (Gibco).

6.2 Isolation of Primary Murine Keratinocytes

Adult (≥6 weeks of age) C57BL/6 mice were euthanized by isoflurane overdose followed by cervical dislocation. The tail was excised and ~2 mm of the tip was removed to create a visible hole. Using a sharp blade, the skin of the tail was cut from base to tip, one pair of forceps was used to grasp the exposed vertebra of the tail and another pair was used to grasp the skin at the opposite end, and the skin was gently removed by pulling apart the forceps. The skin was cut into pieces perpendicularly to the mid-line such that each piece was ~2 cm long. Skin tissue was placed in a 10-cm Petri dish, washed with sterile PBS$^{-/-}$ (Gibco), transferred to a tube containing ice cold dispase digestion buffer (4 mg/ml dispase in keratinocyte basal media; Lonza CC-3103), and rotated at 4^{o}C overnight. After 12-18 h in dispase, forceps were used to transfer skin from dispase solution to a Petri dish containing cold PBS$^{-/-}$ to wash sections. Skin was transferred to a new Petri dish with epidermal side down and dermal side up and stretched to full extension on a Petri dish. Using forceps, the dermis was removed from each section of skin and discarded. EDTA-free trypsin (Thermo, 15050) was added to a fresh Petri dish (500 μl per section of skin). Isolated epidermal tissue was transferred to a trypsin solution and floated on top with the basal layer facing down. Epidermal tissue was incubated over trypsin with gentle shaking at RT for 20 min. Following incubation, keratinocyte growth medium was added to the Petri dish. Epidermal tissue was transferred to growth medium. Edges of epidermal tissue were grasped using forceps, and basal portions of tissue were rubbed together to release trypsinized keratinocytes. The cell suspension was collected in a new tube, and the process of rubbing epidermal sheets and collecting released cells was repeated twice more, adding cell suspensions to the same tube. Cells were triturated by pipetting to break up clumps and passed through a 100 μm filter to a fresh tube. Cells

were centrifuged at 180*g* to pellet, the supernatant was aspirated, and the cells were resuspended in keratinocyte growth medium. Cells were counted and seeded at a density of $5x10^4$ cells/cm^2 in fresh growth medium.

6.3 Isolation of Primary Murine Osteoblasts

Three-to-four-week-old C57BL/6J mice were euthanized by isoflurane overdose followed by decapitation. Skin covering the calvaria was removed, and the bone of the skull cap was resected using Rongeurs. Bone was cut into small pieces (~1-2 mm^2) and placed into sterile tubes containing 0.1% collagenase I (Sigma C0130) in Hanks' balanced salt solution (HBSS) with no calcium and no magnesium (Thermo). Tubes were vortexed to submerge bone pieces and secured on their sides in an orbital incubator shaking at 125 rpm at 37^{o}C for 15 min. Bone pieces were vortexed and placed back into the orbital incubator shaking at 125 rpm at 37^{o}C for an additional 15 min. Following incubation, tubes were centrifuged at 1000*g* at 4^{o}C for 5 min. Bone pieces were resuspended in DMEM supplemented to contain 10% FBS, placed in a T75 flask, and incubated at 37^{o}C. Cultures were checked daily until a sufficient number of cells had extravasated from bone tissue to subculture.

6.4 Stem-Loop RT-qPCR of miRNAs

miRNAs were isolated from $1x10^6$ cells using the *mir*Vana miRNA isolation kit (Thermo). Isolated miRNAs were reverse transcribed using the SuperScript III First-Strand Synthesis system

(Thermo) with a stem-loop reverse transcription primer specific to each miRNA of interest (Table IV-1). cDNAs were amplified by PCR using KOD polymerase (EMD Millipore) with a forward primer specific for each miRNA and a reverse primer specific to a conserved region of the stem-loop primers (allowing one reverse primer to be used for detection of all miRNAs) (Table VI-1). Reaction products were resolved by electrophoresis in 1% agarose gels.

Table 6-1. List of primers used for detection of miRNAs by stem-loop RT-qPCR.

Primer	Sequence
miR-126 Stem-Loop RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACCGCATT
miR-126 PCR For	GCGCTCGTACCGTGAGTAATAAT
miR-142 Stem-Loop RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACTCCATA
miR-142 PCR For	GGCGGCGTGTAGTGTTTCC
miR-203 Stem-Loop RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACCTAGTGGTC
miR-203 PCR For	GCGGCGGCGTGAAATGTTTA
miR-206 Stem-Loop RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACCCACAC
miR-206 PCR For	GGCCGGGGTGGAATGTAAGG
miR-275 Stem-Loop RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACGCGCTA
miR-275 PCR For	GCGGCGTCAGGTACCTGA
miR-2861 Stem-Loop RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGG ATACGACCCGCCC
miR-2861 PCR For	TATATAGGGGCCTGGCGGCC
Universal PCR Rev	CCAGTGCAGGGTCCGAGGTA

6.5 Cloning of miRNA-Targeted Virus Infectious Clone Plasmids

The wild-type (WT) CHIKV strain SL15649 infectious clone (pMH56) as well as a variant SL15649 infectious clone in which eGFP was introduced into the virus structural ORF (pMH75) were provided by Dr. Mark Heise (University of North Carolina at Chapel Hill). Skeletal muscle-restricted (SKE) and mismatch control (SKE MM) SL15649 strains were engineered to contain inserts designed in silico containing either four target sequences for tissue specific miRNAs or four mismatch target sequences. These sequences were appended to a nucleotide sequence encoding 15 amino acids of the 2A protease of foot-and-mouth disease virus (FMDV). Insert cassettes were synthesized by Genscript containing BssHII and ApaI restriction sites at the 5' and 3' termini, respectively. The pMH75 plasmid was digested with BssHII to release a 331 bp fragment. The vector lacking this fragment was religated to produce a plasmid containing a single BssHII site (pMH75.1), which was then digested with BssHII and ApaI to remove the eGFP insert from the structural ORF. Inserts containing miR-target and mismatch sequences were digested from Genscript constructs using BssHII and ApaI and ligated into pMH75.1. The BssHII-BssHII fragment was then reinserted and screened for orientation by consensus sequencing.

Virus was recovered by linearization and in vitro amplification of infectious clone plasmids using the mMessage mMachine SP6 transcription kit (Ambion). BHK-21 cells were electroporated with in vitro transcribed viral RNA using a Gene Pulser Xcell electroporator (Bio-Rad) and incubated at 37$_{o}$C for 48 h. Supernatants containing progeny virions were harvested, clarified by centrifugation at 1,500*g* at 4$_{o}$C for 10 min to remove cell debris, and stored at -80$_{o}$C. Titers of virus stocks were determined by plaque assay. All experiments using SL15649 and variant clones were conducted using biosafety level 3 conditions.

6.6 Virus Stock Sequencing

RNA from virus stocks was isolated using the PureLink RNA minikit (Thermo). Isolated RNAs were reverse transcribed using gene-specific primers (Table VI-2) and PCR amplified using KOD polymerase. PCR products were purified using the QIAquick PCR purification kit.

Table 6-2. List of primers used for sequencing CHIKV strain SL15649.

Genome Region (Approximate)	Forward Primer	Reverse Primer
1-809	GTG AGA CAC ACG TAG CCT ACC	TTC CGT CAG GTC TGT TGA AC
455-1300	AGA CAG AGA GCA GAC GTC GC	CTT ACT GAA GGC TTG GGC G
1032-1900	ATG ACC GGC ATC CTT GCT AC	ACG TCT TCA CTT GCT CCG CT
1539-2518	ACT GCC CAA CTA ACA GAC CAC GTC G	CGG TGC TGA TTT CTT GGC AGT TTT C
2239-3104	GCG GAA AGA AAG AAA ACT GC	TCC ACC TCC CAC TCC TTA AT
2827-3700	TCA GAG CAC GTC AAC GTA CT	TTA GTA GGC AGT GCA AGG TT
3442-4307	CAC ACT CAT TAG TGG CCG AA	TTT GCG GTT CCT ACT GGT GT
4028-4956	GTT ACC GGG TGA CGG TGT T	TGC ATC ATC CAC CGG GCA TT
4655-5545	ACA GAG GCC AAT GAG CAA GT	GTA CTC GGT GGT GCC TGA AG
5247-6100	AAC CTG ACT GTG ACA TGT GAC	ACT CAT TAC ATG CTG CCA CT
5840-6760	CGG ACT ACA TAT CCG GCG	CCC ACA TAG GTA TGC TGT CG
6424-7300	CAG GAT GTA CCA ATG GAT AGG	CAA GCT GTT CCT GTC ACA GT
7094-7921	TGG ATG AAC ATG GAA GTG AAG	CGA TTT TCA TGC ACA TCC TC
7656-8530	CGG TAC CCC AAC AGA AGC CA	CGT AGG GTT TCC TCC GGT TC
8280-9136	GGG CCG AAG AGT GGA GTC TT	AAT GTG CGA TCA GGG GTG TC
8894-9720	CAC CCA TTT CAC CAC GAC CC	CCA TAC CCA CCA TCG ACA GG
9452-10320	GAG GTC ACG TGG GGC AAC AA	ATG GGT AGA CGC CGG TGA AG
10093-10920	TCG CTT GAT TAC ATC ACG TG	CGA CAT GTC CGT TAA AGA GG
10601-11520	TGG CTA AAA GAA CGC GGG GC	GGT TGC GTA GCC CTT TGA TC
11239-12039	AAT TAA GTA TGA AGG TAT ATG TG	GCG CGC TTT TTT TTT TTT TTT TTT TTT TTG

6.7 Viral Plaque Assays

Serial 10-fold dilutions of samples in virus dilution buffer (VDB; RPMI medium with 25 mM HEPES [Gibco] supplemented to contain 1% FBS) were adsorbed to Vero81 cells at 37^{o}C for 1 h. Monolayers were overlaid with 0.5% immunodiffusion agarose (VWR) in completed αMEM and incubated at 37^{o}C for 40 to 42 h. Plaques were visualized following staining with neutral red (Sigma). Plaques were enumerated in duplicate and averaged to calculate PFU.

6.8 FFU Assays

Serial 10-fold dilutions of samples in DMEM/F12 medium supplemented to contain 2% FBS were adsorbed to Vero81 cells at 37^{o}C for 2 h. Monolayers were overlaid with 0.5% methylcellulose (Sigma) in medium and incubated at 37^{o}C for 16 to 18 h. Cells were fixed with 1% paraformaldehyde (PFA) in PBS at room temperature (RT) for 1 h, washed three times with PBS, and permeabilized with perm/wash buffer (PBS supplemented to contain 0.1% saponin and 0.1% bovine serum albumin) at RT for 5 min. Cells were incubated with CHIKV-specific monoclonal antibody CHK-11 (142) diluted to 500 ng/ml in perm/wash buffer at RT for 2 h, washed three times with perm/wash buffer, incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (SouthernBiotech, 1030-05, 1:2,000 dilution) at RT for 1 h, and washed three times with perm/wash buffer. Foci were visualized following incubation with TrueBlue Substrate (Fisher) at RT for 10 min and enumerated using a CTL Biospot analyzer and Biospot software (Cellular Technology) to calculate FFU.

6.9 Assessment of CHIKV Replication Kinetics

U-2 OS cells were adsorbed with CHIKV strains diluted in VDB at an MOI of 0.01 PFU/cell at 37^{o}C for 1 h. The viral inoculum was removed, cells were washed twice with PBS, and complete medium was added. Following incubation at 37^{o}C for various intervals, 10% of the cell supernatant was collected and replaced with fresh complete medium. Viral titers in culture supernatants were determined by plaque assay.

6.10 Transfection of miRNA-Mimic siRNAs

U-2 OS cells were transfected with 10 nM of nonspecific siRNA (Luc), siRNA directed against the viral nsP1 gene, or tissue-specific miRNA-mimic siRNAs using Lipofectamine RNAiMAX (Invitrogen) diluted in serum-free OPTI-MEM according to the manufacturer's instructions. Cells were incubated at 37^{o}C for 12 h and then adsorbed with CHIKV strains diluted in VDB at an MOI of 0.01 PFU/cell at 37^{o}C for 1 h. The viral inoculum was removed, cells were washed twice with PBS, and complete medium was added. After incubation at 37^{o}C for various intervals, 10% of the cell supernatant was collected and replaced with fresh medium. Viral titers in culture supernatants were determined by plaque assay.

6.11 Mouse Infections

C57BL/6J mice were obtained from The Jackson Laboratory. All mouse infection studies were conducted in an animal biosafety level 3 laboratory. Three-to-four-week-old male mice were used for all studies. Mice were inoculated in the left rear footpad with 10 μl containing either 10^3 PFU of virus in diluent (PBS supplemented with 1% bovine calf serum [BCS]) or diluent alone (mock). Mice were weighed at 24-hour intervals and monitored for signs of disease. The area of the left rear footpad was determined prior to infection by measurement of footpad width and thickness with digital calipers and then at 24-hour intervals thereafter for either 7 or 14 days. For IL-6 receptor blockade studies, mice were inoculated intraperitoneally (i.p.) with 200 μg of either αIL-6R antibody (BioXCell, BE0047) or an IgG2b isotype control antibody (BioXCell, BE0090) diluted in PBS on days 0, 3, and 5 post-inoculation with CHIKV. For experimental endpoints, mice were euthanized by exposure to isoflurane followed by cervical dislocation. Blood was collected, and mice were perfused by intracardiac injection of PBS or 4% PFA in PBS, depending on the experiment. PBS-perfused tissues were resected and homogenized using a MagNA Lyser (Roche) in either TRIzol reagent (Life Technologies) for RNA isolation or PBS supplemented to contain 1% BCS for viral titer determination by FFU assay.

6.12 RT-qPCR

RNA was isolated using a PureLink RNA minikit (Life Technologies), and 1 μg RNA was reverse transcribed to cDNA using random primers (Thermo, 48190011) and the SuperScript IV first strand kit (Invitrogen). CHIKV sequence-specific forward primer (5′-

TTTGCGTGCCACTCTGG-3′), reverse primer (5′-CGGGTCACCACAAAGTACAA-3′), and an internal TaqMan probe (5′-ACTTGCTTTGATCGCCTTGGTGAGA-3′) were used to amplify and detect a region of the viral nsP2 gene. A standard curve was established from known samples containing 10 to 10^8 genome copies of in vitro transcribed CHIKV RNA. Purified RNA from BHK-21 cells was added to bring each standard sample to 1 μg total RNA prior to reverse transcription in an identical manner to that used for experimental samples. Experimental samples were amplified concurrently with standards to quantify CHIKV genome copies/μg RNA. Controls without template were processed in parallel. To determine host gene expression, cDNAs were subjected to qPCR analysis using TaqMan primer/probe sets specific for murine 18S rRNA, IFN-γ, IL-1β, IL-6, IP-10, MCP-1, MIP-1α, RANTES, and TNFα (ThermoFisher). Murine gene expression was normalized to 18S rRNA values to control for differences in cDNA input. The relative fold induction of amplified mRNA relative to samples from mock-infected mice was determined using the Ct method (364).

6.13 Histopathological Analysis

At defined times post-inoculation, mice were euthanized, and PFA-perfused tissues were resected and fixed in 4% PFA in PBS at 4°C for at least 72 h. Fixed tissue was embedded in paraffin, sectioned (5-μm thick), stained with hematoxylin and eosin (H&E), and visualized by light microscopy to assess histopathologic changes. Tissues resected at day 7 post-inoculation were scored by a board-certified veterinary pathologist blinded to the conditions of the experiment for the presence, distribution, and severity of histopathological damage. For all tissue changes, the following scoring system was used: 0, no lesions; 1, mild, < 5 areas of small clusters of leukocytes;

2, moderate, leukocytes forming larger clusters to thin tracts throughout the tissue, multiple sites/tissues affected; 3, severe, clusters and tracts of leukocytes coalescing into at least one large area that displaces/replaces tissue; 4, markedly severe, leukocytes in aggregates sufficient to replace > 40% of normal tissue. Immunohistochemical staining was conducted with tissue sections obtained from mice on day 7 post-inoculation by incubation with antibodies directed against CD3 (Dako, A0452, 1:200 dilution) or CD4 (Abcam, ab183685, 1:1000 dilution) followed by incubation with a horseradish peroxidase-conjugated OmniMap anti-rabbit IgG secondary antibody (Ventana, 760-4311, ready to use). CD3 and CD4 signal in the interosseous muscle was quantified using ImageJ. Regions of interest containing interosseous muscle were defined from H&E-stained serial sections of each foot. CD3- and CD4-stained images were separated into hematoxylin and DAB channels. DAB staining was quantified in the DAB channel for each interest region, and the intensity of CD3 and CD4 signal was calculated as the average of the staining intensity in each region weighted by its relative area.

6.14 *In Situ* Hybridization

At defined times post-inoculation, mice were euthanized, and PBS-perfused tissues were resected and fixed in 4% PFA in PBS at 4$_{o}$C for at least 72 h. Tissue was washed three times in PBS at RT for 15 min, then three times in deionized water at RT for 15 min. Tissue was decalcified in 14% EDTA and placed on an orbital shaker at RT. EDTA was replaced after 24 h and at 72-h intervals thereafter for 10-14 days until tissue was fully decalcified. Tissue was dehydrated, embedded in paraffin, and sectioned (5-μm thick). Viral RNA in situ hybridization was conducted using RNAscope 2.5 (Advanced Cell Diagnostics) according to the manufacturer's instructions.

Tissue sections were incubated twice in xylene at RT for 5 min to remove paraffin, twice in 100% ethanol at RT for 1 min, once in hydrogen peroxide at RT for 10 min, and boiled in RNAscope Target Retrieval reagent (Advanced Cell Diagnostics) for 15 min. Slides were cooled to RT in deionized water and treated with RNAscope Protease Plus (Advanced Cell Diagnostics) at 40$_{o}$C for 30 min before incubation with the hybridization probe. CHIKV RNA was detected using a probe (V-CHIKV-sp, 479501) designed by Advanced Cell Diagnostics. Tissues were counterstained with Gill's hematoxylin (Sigma) and visualized by light microscopy.

6.15 Site-Directed Mutagenesis

The G82R substitution was engineered by site-directed mutagenesis into the SKE or WT SL15649 infectious clone plasmid using KOD polymerase. cDNA from each clone was sequenced to verify that only the desired mutation was introduced.

6.16 Statistics

All statistical tests were conducted using GraphPad Prism 7 software. Significant differences were detected using two-tailed Student's *t* test, Mann-Whitney test, or ANOVA with Tukey's post hoc test to correct for multiple comparisons. *P* values of less than 0.05 were considered to be statistically significant. Descriptions of the specific statistical tests used for each experiment are provided in figure legends.

6.17 Study Approval

All animal work reported here conforms to Public Health Service policy and was approved by the Institutional Animal Care and Use Committees at the University of Pittsburgh and University of Colorado School of Medicine.

Appendix A Copyright Permissions

Figures in Chapters 1 and 2 and text from Chapter 2 were previously published and presented herein in a modified form with permission of the copyright holder.

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