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Viral and Innate Immune Factors Controlling Disease Susceptibility During Chikungunya Virus Infection

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by

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ABSTRACT OF THE DISSERTATION

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by

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Chikungunya virus (CHIKV) is a re-emerging pathogen that causes a high fever, painful arthritis and a macular rash. The current outbreak began in Kenya in 2004 and spread throughout the Indian Ocean area and Southeast Asia, infecting millions. During this outbreak, more severe disease manifestations, including lethality, were reported for the first time. To understand one possible reason for this change in severity, we explored the impact of genetic changes in the virus on disease pathogenesis. In addition, we dissected the role of type I interferon (IFN) subtypes in CHIKV infection. From these studies, we have gained important insight into the viral and host factors that contribute to CHIKV pathogenesis.

To test if one factor contributing to the increased disease severity is genetic changes in the viral genome we compared the pathogenesis of two strains: the first from Senegal in 1983 (37997) and the second from La Reunion in 2006 (LR2006 OPY1). Neonatal mice infected with LR2006 OPY1 developed prolonged viremia, elevated viral loads in the muscle, hind limb weakness and massive myonecrosis compared to 37997-infected mice. A detailed analysis revealed that the LR2006 OPY1 strain displayed a unique ability to infect myofibers following intradermal infection. This difference in tropism appeared

to be due to its ability to access the myofiber niche since both infected myofibers and established similar viral loads when given intramuscularly. Thus, LR2006 OPY1 has an enhanced ability to infect myofibers during a natural infection which contributes to severe muscle damage. Studies to identify the genetic differences responsible for the myofiber phenotype are ongoing.

Central to the control of acute CHIKV infection is the type I interferon response which induces a potent anti-viral state and modulates the immune response. To further evaluate the role of IFNs during CHIKV infection, we characterized mice lacking specific IFN subtypes. Mice lacking IFN-β displayed a modest increase in lethality compared to wild-type mice, and increases in viral loads late in infection, particularly in the brain. Surprisingly, IFN-$\kappa^{-/-}$ were resistant to CHIKV-induced lethality, and had similar viral loads and proinflammatory cytokine induction as wild-type mice, suggesting the mechanism for IFN- κ is distinct from other IFN subtypes. Recombinant tools are being developed to further analyze the function of IFN-κ.

Together these data provide important insight into the host factors that control pathogenesis and offer evidence that genetic differences in viral strains that alter tropism can contribute to changes in disease severity. Understanding these factors will aid in the development of future therapeutics that target CHIKV infection.

Introduction

I. Epidemiology

Symptoms

Chikungunya virus was first identified in Tanzania in 1952 and was named in an East African dialect for the painful arthritis it causes (1). In the years following, periodic outbreaks of limited scope were noted throughout Africa and Southeast Asia (2)(3). In 2004, the largest outbreak in record began in Kenya and continues today. The first cases spread from eastern Africa to the islands of the Indian Ocean, India and Southeast Asia, ultimately afflicting millions.

Alphaviruses are classified into two groups, "Old World" and "New World" based on the hemisphere in which they are thought to originate (3). The Old World and New World groups also segregate by symptoms they cause: Old World viruses are known predominately for causing fever, arthritis and rash, while the New World viruses also cause encephalitis (3). CHIKV is also classified by similarity into the Semliki Forest Virus (SFV) antigenic complex group, which also includes O'Nyong-nyong Virus and Ross River Virus (RRV) (3). These viruses are also known to cause similar symptoms as CHIKV. Symptoms typically begin 2-12 days after inoculation by a CHIKV-infected mosquito (4). The first sign of infection is an abrupt-onset fever, that can last for up to two weeks. Shortly after the start of the fever, the majority of infected people develop incapacitating polyarthralgia, which is usually symmetric and effects distal joints, including wrists, elbows, fingers, knees and ankles and is often accompanied with myalgia (5). A variable percentage of patients also develop rash, usually maculopapular on the trunk, palms, soles, or face (4). Additional, but less common symptoms include headache, fatigue, nausea, lymphopenia and conjunctivitis (4).

Persistent Arthralgia

Chikungunya fever generally resolves in two weeks, a subset of patients, however, develop a painful arthritis that can last for months to years. In the first report of CHIKV in 1952, a patient was characterized with persistent arthralgia, however, a large-scale study to determine frequency of persistence before the 2004 outbreak has been lacking (1). In a survey done in South Africa in 1983, most patients had recovered from CHIKV infection 3-5 years earlier, but 12.1% of those surveyed still experienced joint stiffness and pain (6). Due to the ongoing CHIKV epidemic, more extensive studies of chronic symptoms have been performed. A survey of patients from La Réunion 18 months after their infections, fund that 63.8% reported persistent arthralgia attributable to CHIKV infection (7). These patients experienced intermittent or continuous polyarticular arthritis which interfered with their daily activities (7). Similar results were found in another study also done on La Réunion, with 57% of those surveyed experiencing rheumatic symptoms (joint pain, stiffness or swelling) 15 months after infection (8). Like the Borgherini study, the authors found advanced age and severity of the acute infection as risk factors for persistent arthralgia. Even after 27.5 months, 78.6% of those surveyed on the island of Mauritius reported musculoskeletal symptoms (9). Similar affliction rates of persistent arthralgia has also been noted in India and Italy (5) (10). Because of the limited studies performed before the current outbreak, it is unclear whether a higher percentage of patients are developing persistent arthralgia after CHIKV infection. Regardless, many of those infected experience pain for months to years afterward, causing both personal and economic hardship.

Atypical and Severe Manifestations

In the latest outbreak, there have been increased reports of atypical symptoms of CHIKV including meningoencephalitis, acute hepatitis, and pneumonia (11). Further, mortality due to CHIKV was

reported for the first time. Risk factors for disease severity include advanced age, hypertension and pre-existing respiratory and cardiovascular conditions (11). On the island of La Reunion, approximately 260 deaths due to CHIKV occurred during the outbreak, out of 266,000 infected, resulting in a death rate of less than 0.1% (12). Similar disease features have been seen in India, where a study looked at CHIKV infections in hospitals in Ahmedabad and Pune. Of those hospitalized with severe disease. 61.5%, suffered from neurologic symptoms (13). Other severe cases included renal failure, pneumonia, hepatitis, cardiomyopathy and hemorrhagic problems, similar to those reported on La Reunion. Additionally, mortality, particularly in those above 60, was also seen in India (13).

In addition to the elderly, neonates were also found to be highly susceptible to CHIKV infection. While most mothers who contracted CHIKV early in pregnancy had no increase in negative outcomes due to CHIKV, mothers that were viremic at the time of delivery had a 50% rate of transmission to their newborn (14). These infections were not prevented by Cesarean section. Of the newborns that developed an acute infection, all experienced fever, poor feeding and pain, and the majority had joint edema, rash and thrombocytopenia. Ten of the 19 infected neonates had severe disease with encephalopathy or hemorrhagic fever. Four of the children with encephalopathy had lasting neurological deficits 2 years after birth (14). Although data prior to the outbreak is limited, more severe symptoms have reported in this latest outbreak. At particular risk are neonates and the elderly that are more prone to lasting sequelae from CHIKV infection.

Diagnosis

In the setting of an epidemic, a clinical diagnosis can be made based on history or physical. However, for severe cases diagnosis can be made based on PCR detection of virus in the serum or ELISAs for the presence of anti-CHIKV IgM or IgG in the serum (4). CHIKV-specific IgM typically appears 5-7 days

after onset of symptoms and can last for several months (15) (16). The differential diagnosis for CHIKV must also include Dengue Virus, malaria, meningitis, and rheumatic fever (17). Dengue has the same geographical distribution and is spread by the same mosquitoes as CHIKV, but can usually be differentiated by the presence of prominent arthralgias with CHIKV and hemorrhage with Dengue (4). Treatment guidelines from the World Health Organization suggest home care for uncomplicated cases, managing fever and pain with acetaminophen and fluid replenishment. For more severe cases, hospitalization and treatment specific to complications is recommended (17).

Vaccine Development and Anti-virals

There is currently no approved treatment for CHIKV, but several different areas are being pursued, including monoclonal antibody treatment, vaccine development and small molecule anti-viral agents. Chloroquine is a drug that is commonly used to treat malaria, and acts by preventing the acidification of the endosome. Because this step is crucial for CHIKV to infect cells, chloroquine was tested as a treatment for CHIKV infection in a double-blinded placebo-controlled trial (18). However, there was no reduction in viremia or febrile arthralgia duration between chloroquine and placebo-treated groups (18). Further, the chloroquine treated patients were more likely to report arthralgia 200 days after treatment than placebo (18). Inhibitors of furin, the host protein that cleaves the structural viral polyprotein is also a possible drug target that has shown promise in tissue culture studies (19). Sulfasalazine with methotrexate was also used to treat patients suffering from chronic arthritis at least three months after the acute symptoms, and 71.4% of these patients reported an improvement in their symptoms (20).

Human immunoglobulins have also been used to treat viral infections, such as West Nile Virus (21). Treating mice with sera from convalescent CHIKV patients protected mice from lethality and reduced

viral load in serum, muscle and brain, and could be an effective therapy for pregnant women, elderly and the immunocompromised, among others (22). Anti-CHIKV antibodies that can prevent death in infected mice have been developed and characterized and are a promising area for future treatment (23). Interferon α/β Receptor (IFNAR) knockout mice could be treated with pairs of CHIKV-neutralizing antibodies 24 hours post infection to delay death in these highly susceptible mice (23). These mice also did not develop severe arthritis, unlike their counterparts in the control group. Although there is a risk of developing escape mutants for CHIKV, the use of multiple monoclonal antibodies decreased that likelihood (23).

Currently, there is no vaccine for CHIKV prevention. A live-attenuated vaccine was developed in 1986 by the U.S. Army, and was tested in healthy volunteers (24) (25). Most participants developed neutralizing antibody response after one month, and 85% were still positive after one year (25). Transient arthralgia was reported in 8% of patients, but the vaccine was otherwise well tolerated (25). Other CHIKV vaccines are in development, including virions that express only CHIKV capsid and envelope glycoproteins, a live attenuated CHIKV/IRES vaccine, as well as a DNA vaccine expressing the CHIKV nsp2 protein (26) (27) (28). A virus-like particle (VLP), consisting lentiviral-vector expressing the envelope glycoproteins was tested in mice and rhesus monkeys for neutralizing antibody responses (29). Both mice and monkeys produced high titer neutralzing antibodies in response to treatment with the VLP, which is likely to have fewer side-effects than the attenuated viruses because the VLPs are unable to replicate in the host (29). Although these additional strategies have not yet been tested for safety and efficacy in humans, they show promise in several animal models.

II. Life cycle and Pathogenesis

Mosquito Vectors

Chikungunya is spread by mosquitoes, particularly those of the genus Aedes, most commonly the species *Ae. aegypti* and *Ae. albopictus*, but also including *Ae. furcifer*, *Ae. taylori*, and *Ae. luteocephalus* (2). In Africa, CHIKV is maintained through a sylvatic cycle, transferring between mosquitoes and undetermined mammalian host reservoirs, possibly primates. In this case, human outbreaks are relatively small and sporadic as human infections are incidental. In Asia and densely populated areas in Africa, an urban cycle is implicated, spreading between mosquitoes and humans directly without an animal intermediate.

Strains from early in the Indian Ocean outbreak were probably spread by the original mosquito vector, *Ae. aegypti* (30). However, a residue change in the E1 protein from an alanine to a valine at position 226 allows for increased infectivity of a new vector, *Ae. albopictus*, the Asian tiger mosquito. (31). A mutation in a similar position for another alphavirus, the Semliki Forest Virus (SFV), is thought to decrease the need for cholesterol in the membranes of mosquito cells, thus allowing for infection of *Ae. albopictus* (32). The E1 A226V mutation results in increased infectivity of *Ae. albopictus*, increased dissemination within the mosquito, and more efficient infection of suckling mice, but slightly decreased viral growth in *Ae. aegypti* (31). In India, where *Ae. Aegypti* predominates, early in the epidemic most strains were 226A, but later 226V was more common (30). In areas where *Ae. albopictus* dominates, the lower titer necessary for infection of *Ae. albopictus* could facilitate spread from humans to mosquitoes and back to humans, resulting in the large scale epidemic we see today.

Importantly, *Ae. Albopictus* is present in areas naive to CHIKV, such as North and South America and Europe. In Northeastern Italy, a CHIKV outbreak was traced to a visitor from India who became febrile two days after arrival (33). Subsequently, 205 people in two neighboring towns became infected, and CHIKV-positive *Ae. albopictus* mosquitoes were found (33). Fortunately, aggressive mosquito control prevented further spread (33). In France, an infected individual returning from Asia contributed to

spread to two others living nearby (34). In the United States, as of 2009, there have been 109 cases from travelers returning to the US from Africa and Southeast Asia (16). Although some of these travelers were viremic and lived in areas with CHIKV vectors, there have not been any reports of spread (16).

Chikungunya Virus Lifecycle

CHikungunya virus is an enveloped alphavirus in the Togaviridae family with a positive strand RNA genome. The single-stranded genome is approximately 11.8 kb in length, and has a 5' untranslated region, followed by the non-structural genes, and another subgenomic promoter followed by the structural genes and finally a 3' untranslated region. A summary of CHIKV replication is presented in Figure 1.1.

Although a cellular receptor is not yet known, other alphaviruses bind via the E2 glycoprotein present on the virion surface. Because CHIKV must be able to infect both mammalian and insect cells, the receptor may either be conserved across species, or there may be multiple receptors that vary with the host (35). For Sindbis, a related alphavirus, the receptor was found to be natural resistance-associated macrophage protein (NRAMP) in both mammalian and insect cells (36). However, this receptor is not responsible for CHIKV binding. Once bound, CHIKV is internalized in a clatharin-dependent manner and requires a low-pH endosome for proper infection (37). The change in pH causes a conformational change in the E1-E2 dimer, exposing the E1 fusion spike. This spike inserts into the membrane of the endosome, juxtaposing the vesicle membrane and virion membrane and allow for fusion of the two membranes. The uncoated nucleosome is ejected into the cytoplasm, where it disassembles, removing the capsid proteins from the genomic RNA.

The replication of CHIKV proceeds in the cytoplasm, associated with membranous organelles (3). The non-structural genes are translated as a polyprotein, referred to as p1234. This polyprotein is cleaved by the internal protease, nsp2. The nsp1 protein acts as a guanine-7-methyltransferase and guanyltransferase to cap newly synthesized viral RNAs. In addition to its protease activity, the nsp2 protein also functions as a helicase to unwind RNA for replication and transcription. The role of nsp3 has not been well defined, though nsp3 has been shown to be required for RNA synthesis, and have ADP-ribose 1-phosphate phosphatase and RNA binding activity. The nsp4 protein is the RNA-dependent RNA polymerase that transcribes the RNA (35).

First, minus-strand synthesis occurs, which serves as a template for synthesizing positive-strands. The positive-strands are then used both to translate structural and non-structural polyproteins, as well as to package in virions. The structural polyprotein is translated from a subgenomic promoter located in the junction after the non-structural proteins. The capsid protein autocleaves itself from the polyprotein, exposing an ER localization signal. Once in the ER, the remaining polyprotein is processed by the host protein signalase, separating the 6k protein from E1 and pE2 (E2 and E3). The pE2 and E1 heterodimer then continues to the golgi, where E2 and E3 are cleaved by the host protein furin. Also during this time E1 and E2 are glycosylated, and E2 is also thought to be palmitoylated. The viral proteins are shuttled to the plasma membrane, where the E1 and E2 heterodimers interact to form trimers that will be present on the surface of the virion. The capsid protein, free in the cytoplasm, auto-oligmerizes on the positive-stranded RNA that is abundant in the cytoplasm. The E3 protein assists in envelope glycoprotein spike formation. The 6k protein is thought to be involved in budding and virus formation (35). Interactions of the capsid proteins with the portion of the E2 on the cytoplasmic side of the cell surface allow for maturation of the virion, and budding from the host cell.

Mouse Model for Chikungunya Virus

A mouse model was developed to study CHIKV *in vivo* (38) (39). Wild-type adult mice are resistant to CHIKV infection, and do not show any signs of illness and the virus is rapidly cleared without spread from the injection site. Neonatal mice are highly susceptible, although the susceptibility decreases with increasing age. Six day old pups have 100% lethality to CHIKV-21, a clinical isolate from La Reunion, while 12 day old pups display 0% lethality. Nine day old pups have intermediate survival, with virus disseminating from the injection site to primarily muscle, joint and skin tissue (38). Similar muscle damage has also been observed in neonatal mice compared to human biopsy samples (38, 40). Further, many of the same cytokines, sucha as IL-6, RANTES and type I interferons, which have been implicated in CHIKV disease in humans are also upregulated in the mouse model (41, 42). This mouse model is reflective of the increased susceptibility of human neonates to CHIKV infection.

A mouse model of CHIKV-induced arthritis has also been developed. Fourteen day-old mice were inoculated in the foot pad with 100 pfu of CHIKV strain SL15649 (43). These mice exhibited decreased weight gain and swelling of the ankle joint compared to mice injected with PBS. Histological analysis of inflamed ankle joints exhibited tenosynovitis and myositis, with the inflammatory cell infiltrate consisting primarily of NK cells, neutrophils, monocytes, macrophages and lymphocytes. Similar results were found with two Asian clinical isolates of CHIKV (44). CHIKV RNA was still detectable in the injected ankle 21 days post infection, suggesting this strain may be useful in investigating the pathogenesis of chronic arthritis (43).

Cellular Tropism and Pathogenesis

The symptoms of CHIKV reflect the tropism of the virus for primarily connective tissue fibroblasts in the skin, muscle and joint. In skeletal muscle, fibroblasts and satellite cells, but not muscle fibers stained for CHIKV antigen after infection in IFNAR and WT pups (38). Staining for CHIKV was also seen in fibroblasts in the joint as well as in the dermis. This distribution has been confirmed in human biopsy samples (38, 40). CHIKV does not appear to infect neurons *in vivo*, but can maybe infect neurons and astroglia *in vitro* (45). In IFNAR$^{-/-}$ and WT pups, staining in the brain was limited to the choroid plexus and lepto-meingeal cells (38).

The mechanism of transmission to neonates in the womb is not yet known. When pregnant IFNAR mice are infected, high viremia is seen in the mother and some viral load is seen in the placenta, but not in fetal tissue (38). In vitro, syncytiotrophoblasts a cell type of the placenta, did not harbor replication (38). A study of pregnant macaques also failed to detect CHIKV in fetal tissue, despite presence in placental tissue and viremia in the mother (46).

Lymphocytes have been shown to be refractory to CHIKV infection (38) (37). However, macrophages have been shown to express CHIKV antigen and can allow for low levels of replication when infected in vitro at a high MOI (3, 37, 38). Infection of monocytes does not produce viable virions, although one study has detected CHIKV antigens in monocytes of infected patients (37) (47). CHIKV RNA was also detected in the macrophages of the joints many months after infection, a possible reason why some pts have chronic arthritis (48). Macrophages are known to be important for much of the muscle pathology of CHIKV infection, as mice treated with bindarit, an inhibitor of MCP-1, have decreased muscle damage compared to untreated mice, while viral titers between the groups are similar (49). When macrophages are depleted with clodronate prior to infection in the arthritis model, mice experience less ankle swelling, but have a delay in viral clearance, indicating the macrophage response is also helpful to the host (44).

Various mouse models have also been used to evaluate CHIKV-induced disease. When the autophagic proteins are reduced in Atg16L^{HM} mice, CHIKV-infected cells become more susceptible to apoptosis and mice have increased levels of lethality (50). Cells infected with CHIKV undergo rapid apoptosis, as confirmed by caspase-3 and TUNEL staining of infected cells (37). Interestingly, CHIKV-infected cells also induce autophagy pathways that delay the onset of apoptosis (50). CHIKV has also been used to infect mice lacking dendritic cell immunoreceptor (DCIR), which is found on monocytes, macrophages, dendritic cells, B cells and neutrophils. Compared to WT mice, DCIR$^{-/-}$ have increased inflammation and edema in the muscle, more severe pathology in the muscle, greater weight loss, and increased cytokines and chemokines in the serum (51). While viral loads were similar between DCIR$^{-/-}$ and WT mice early in infection, there was a delay in clearance by DCIR$^{-/-}$ mice at late time points, suggesting DCIR is important for the negative regulation of the inflammatory response in CHIKV-infected mice (51).

Several interferon stimulated genes (ISGs) have also been tested in knock-out mice. ISG15 is an ubiquitin-like molecule that can be conjugated to a variety of host proteins, and is important to many viral infections (52). ISG15$^{-/-}$ are highly susceptible to CHIKV infection, and generally die before five days post infection (42). However, these mice do not have increased viral loads compared to WT mice, although they do exhibit profoundly elevated cytokines in their serum, particularly IL-1β, IL-6 and TNFα (42). ISG15 is conjugated to its target proteins by the E1 ligase, Ube1L. However, Ube1L$^{-/-}$ mice do not have increased lethality or greater cytokine production that WT mice, indicating that ISG15 does not need to be conjugated for its effects on CHIKV.

The interferon regulatory factors IRF3 and IRF7 are transcription factors that act downstream of IFNAR, and are responsible for the production of more type I interferons as well as other ISGs. Single knockouts of either IRF3 or IRF7 do not show increased lethality, suggesting they have redundant

functions (53) (54). However, the IRF3xIRF7$^{-/-}$ mice displays increased lethality over WT mice, as well as increased viral load in the serum. Bone marrow chimera experiments have revealed having IRF3 and IRF7 in either the hematopoietic or non-hematopoietic compartments are sufficient for survival and decreased viral load (53).

III. CHIKV and interferons.

Type I Interferons

Type I interferons were first discovered by Isaacs and Lindenmann in 1957, and were remarkable for their antiviral properties (55). There is a single type II interferon, IFN-γ that is involved in control of tumors and anti-viral effects (56). Type III interferons are known as IFN-λ's or IL-28a/b and IL-29 and have also shown to have anti-viral activities. All of the types have similar structure, and are α-helix bundles, but bind to distinct receptors. We will focus on type I interferons, and use the IFN abbreviation to refer only to type I subsequently.

There are 16 IFN subtypes in humans, which include 12 α's and a single β, ε, κ, ω. Mice have an extra alpha in addition to the other subtypes. These are small proteins that include a signal sequence for export out of the cell. Although this work focuses on their role in anti-viral defense, type I interferons are involved with a variety of cell processes, such as cancer prevention, cell differentiation, dendritic and NK cell activation, T-cell proliferation and survival, apoptosis and angiogenesis (57). Some of these functions are attributable to differences in transcription factor activation in different cell types (57).

Each of the IFN subtypes have between 30-90% sequence identity with each other. The binding affinities of each IFN affects the strength of the anti-viral and anti-proliferative effects. IFN-β is the most

potent, followed by the IFN-α's. IFN-α and IFN-β are the most well-known, and modified forms are even used as therapy for hepatitis C and multiple sclerosis. IFN-β and IFN-α4 are produced in response to stimulation of PRRs, and act on a positive feedback loop to produce more type I interferons, including the other α-subtypes. IFN-β and IFN-α's have similar functions, but vary in their affinity to IFNAR, and thus are able to induce anti-viral and anti-proliferative effects to different degrees (58). IFN-ε is another recently characterized subtype, that was found to be constituitively expressed in the female reproductive tracts of humans and mice, and decreases severity of sexually transmitted infections in mice (59). Although IFN-ε acts like the other type I interferons in that it is able to induce ISG production, it is unique in that it cannot be induced by PRRs and lacks IRF and STAT binding sites in its promoter (59). Interestingly, IFN-δ and IFN-τ are present in cows and pigs, but not humans, and are important early in gestation for maintaining pregnancy (60). IFN-κ is another subtype in humans and mice that appears to have behavior similar to IFN-α and IFN-β in that it appears to be induced by IFN-β and viral infections, and also appears to have anti-viral properties. IFN-κ will be discussed further in Chapter 4.

Interferon Signaling

Type I interferons bind to the heterodimeric Interferon α/β receptor (IFNAR) that is found on nearly all mammalian cell types. This receptor is actually a heterodimer made up of the low affinity IFNAR1 and high affinity IFNAR2c transmembrane proteins (56). Binding of IFN causes IFNAR1 and IFNAR2c to come together, causing activation of their associated Janus family kinases, Jak1 and the tyrosine kinase, Tyk2. When brought in proximity of one another, the kinases transphosphorylate each other as well as the cytoplasmic tails of the receptor subunits. Phosphorylation sites on the intracellular tails of IFNAR recruit signal transducers and activators of transcription (STATs), most commonly STAT1 and STAT2. These are, in turn, also phosphorylated by Jak1 and Tyk2. STAT1 and STAT2, then form a heterodimer which binds

with IRF9 to produce a transcription factor known as interferon-stimulated gene factor 3 (ISGF3). ISGF3 can travel into the nucleus and bind to Interferon-stimulated response elements (ISRE) in the promoters of certain genes. Collectively, the genes induced after treatment with interferon are known as interferon-stimulated genes (ISGs). It is these ISGs that carry out the functions that IFNs are known for, particularly anti-viral and anti-proliferative effects. Even individual ISGs, such as ISG15, can have a dramatic effect in viral infection. Mice lacking ISG15, are highly susceptible to CHIKV infection (42). However, different cohorts of ISGs are induced by each virus, and different viruses are more or less susceptible to the actions of different ISGs (61).

Chikungunya Detection

Interferons are produced during CHIKV infection when the virus is detected by several pathogen recognition receptors (PRR). PRRs are an essential part of the innate immune response, and allow rapid response to newly introduced pathogens by recognizing common motifs in pathogens called pathogen associated molecular patterns or PAMPs. The first group of PRRs discovered were Toll-like receptors (TLRs), which are present on the cell-surface or in endosomes. Important for CHIKV infection are TLR3 which recognizes dsRNA, and TLR7 which recognizes ssRNA. TLR7 signals through the adaptor protein MyD88, while TLR3 signals in a MyD88-independent manner, using the adaptor TRIF (62). Activation of the TLRs results in NFκB activation, a transcription factor that enters the nucleus to cause transcription of pro-inflammatory genes in addition to IRF3 and IRF7 that initiate transcription of interferons. Other important sensors for CHIKV include the RIG-I like receptors (RLRs) RIG-I, and MDA5. The RLRs signal through a mitochondrial-bound adaptor, MAVS, to result in the transcription of pro-inflammatory genes and IFN (63).

Interferon Production during Chikungunya Infection

Type I interferons can be detected at high levels in the serum of CHIKV infected patients, with the lFN level correlating to serum viral load (64). Because plasmacytoid dendritic cells are known for their ability to make large amounts of IFN, these cells and PBMCs were stimulated with CHIKV, but failed to produce much IFN, in comparison to poly(I:C) (64). This data suggests that hematopoietic cells are not the source of IFN produced in CHIKV infection, which is supported by data suggesting these cells are refractory to CHIKV infection (37). Fibroblasts, which are easily infected, however, produce high levels of IFNb in response to infection (64). Further, bone marrow chimeras with WT mice receiving IFNAR bone marrow survived infection, while the reverse, IFNAR mice receiving WT bone marrow did not, indicating that IFN response from radio-resistant stromal cells is necessary for survival (64).

CHIKV is highly susceptible to the actions of type I interferons, as evident when adult IFNAR deficient mice are infected. In contrast to wild-type adult mice, $IFNAR^{-/-}$ mice are show lethality with as little as 20 pfu of CHIKV. $IFNAR^{-/-}$ mice die by 3 days post infection, and show high viral load in many organs, including liver, spleen, and brain, that are not infected in the WT mice or people (Figure 1.2) (38).

Interestingly, mice deficient in RIG-I, MDA5, or MyD88 did not show increased lethality to CHIKV infection, but did have higher viral loads than WT mice, indicating that RLRs and TLRs are all likely to contribute to the sensing of CHIKV infection (64). High levels of IFN can be detected just 24h after CHIKV infection, and is necessary to keep the infection under control (64).

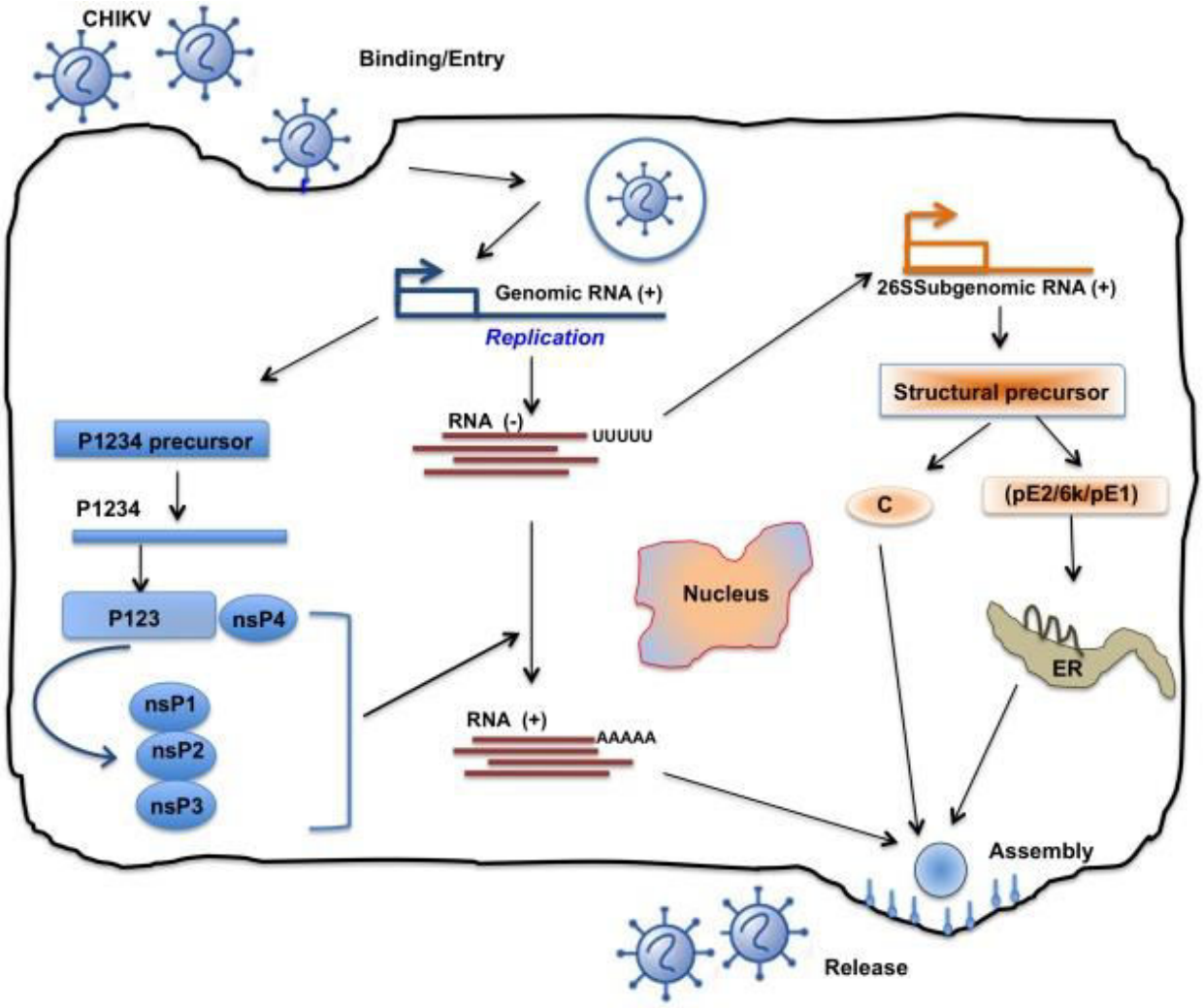

Figure 1.1. Summary of Chikungunya lifecycle. Taken from Thiboutot MM, et al. (2010)

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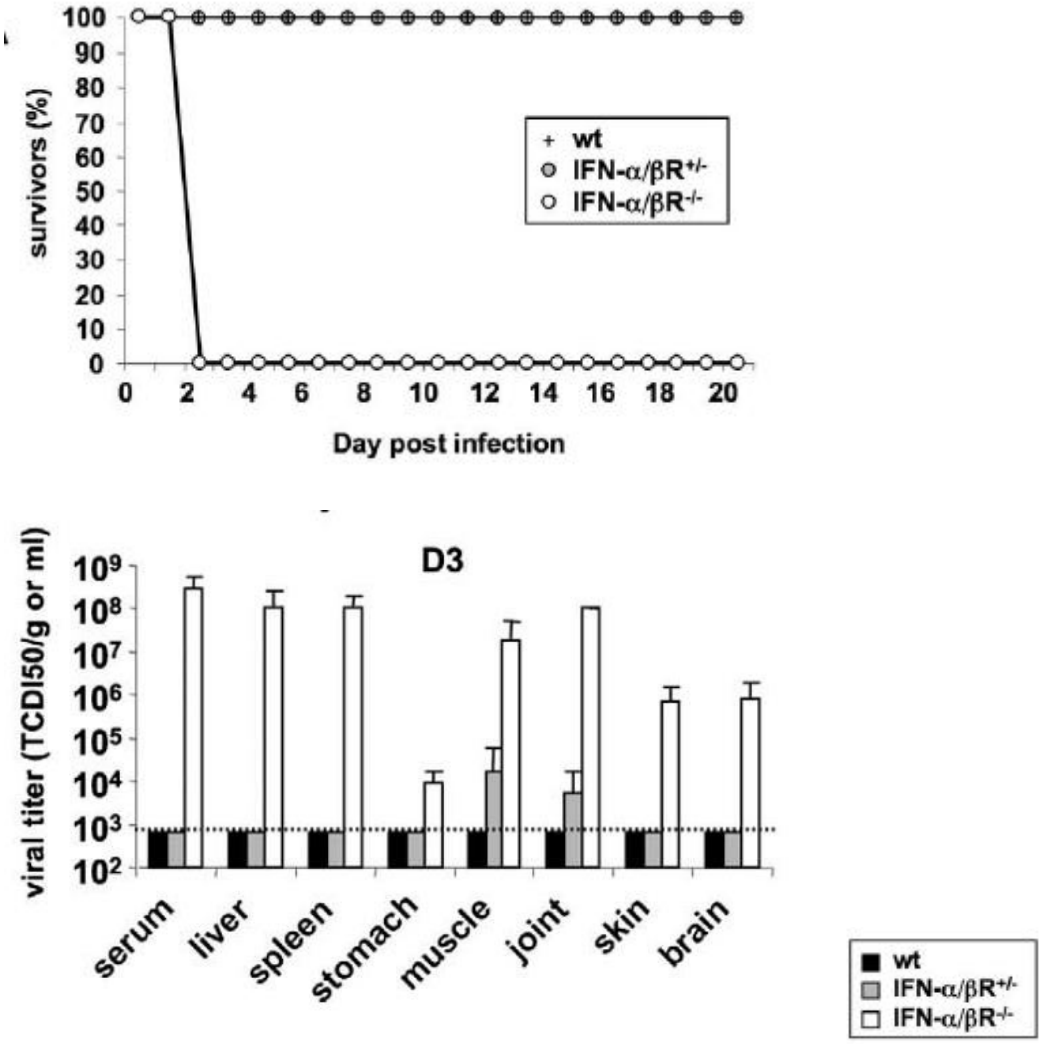

Figure 1.2 IFNAR mice have increased lethality and viral load during CHIKV infection compared to WT. Top panel) Mice were infected with 106 pfu of CHIKV-21 and monitored for survival for 21 days. Bottom panel) WT, IFNAR+/- and IFNAR-/- were infected with 106 pfu, 106pfu or 20 pfu, respectively. Organs were collected and titered by plaque assay 3 days post infection. Reprinted from Couderc T, et al. (2008) A mouse model for Chikungunya: young age and inefficient type-I interferon signaling are risk factors for severe disease. PLoS Pathog

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Chapter 2

Infection of myofibers contributes to the increased pathogenicity during infection with an epidemic strain of Chikungunya Virus

This chapter has been submitted to the Journal of Virology for publication.

Title: Infection of myofibers contributes to the increased pathogenicity during infection with an epidemic strain of Chikungunya Virus

Running Title: Infection of Myofibers Controls Severity of Muscle Pathology in CHIKV infected Mice

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Abstract

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

Introduction

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

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

The reasons for the increased severity during this outbreak are likely multifactorial and include the larger scale of the outbreak, more thorough documentation and follow-up by the medical community, and novel mutations that have accrued in the current circulating strain of CHIKV. The importance of these novel mutations has already been demonstrated by a recent study in which an alanine to valine mutation at residue 226 in the E1 protein of La Reunion strains of CHIKV was found to result in increased infectivity of a new mosquito vector, *Aedes albopictus*, the Asian tiger mosquito (12). The new vector is present in temperate regions and may have contributed to autochthonous outbreaks in France and Italy, and has potential to spread the disease in North America as well (13-15). It is therefore also possible that some of the novel mutations could contribute to the increased disease pathology observed during this ongoing outbreak.

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

CHIKV infection, and provides us with a small animal model to evaluate potential differences in disease pathogenesis between the CHIKV recombinant clones.

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

Methods and Materials

Mice. Mice were maintained at Washington University School of Medicine in accordance with all federal and University guidelines. For mouse studies the principles of good laboratory animal care were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the NIH. The protocols were approved by the Animal Studies Committee at Washington

University. All efforts were made to minimize suffering. C57BL/6 and IFNα/β Receptor 1 ($IFNAR^{-/-}$) mice on the C57BL/6 background were bred and maintained in our mouse colony. For neonatal experiments, mice were infected at 6 days of age. Litters were weight matched at the initiation of the experiments.

Virus Production. The construction of recombinant plasmids for the LR2006 OPY1, 37997, LR2006-GFP and 37997-GFP strains of CHIVK has been previously described (19, 20). Recombinant viral stocks were generated from viral cDNA clones by *in vitro* transcription of linearized cDNA templates followed by RNA transfection with Lipofectamine 2000 (Invitrogen) into baby hamster kidney-21 cells (BHK cells) as previously described (23). After 48 hours, the supernatant was collected, centrifuged, aliquoted and stored at -80°C. Viral stocks were titered by plaque assay as previously described (24). All work with live virus was performed in a Biosafety Level 3 facility and followed strict guidelines established by the Environmental Health and Safety committee at Washington University School of Medicine.

Cells. Murine embryonic fibroblasts (MEFs) were generated from C57BL/6 or $IFNAR^{-/-}$ mice and were grown in DMEM (Mediatech), supplemented with 10% FBS (Sigma), 1% Pen Strep (Mediatech), 1% L-Glutamine (Mediatech), 1% HEPES (Mediatech). MEFs were used prior to passage number 5 for these studies. Primary muscle fibroblasts were isolated from newborn mice as previously described (25). Briefly, skeletal muscle obtained from newborn mice was dissected and digested with collagenase (Worthington). Cells were cultured in F12 media (Invitrogen) with 10% FBS and 1% Pen Strep for two days before replating the cells for growth curve analysis. C2C12 cells were obtained from ATCC (ATCC® CRL1772™), and cultured as recommended. To obtain myofibers, C2C12 cells were plated at 5×10^4 cells/well in 24-well plates and were differentiated into myofibers by the substitution of 2% horse serum for FBS and then cultured for 10 days prior to infection as described below.

Viral Growth Curves. MEFs, primary connective tissue fibroblasts, and C2C12 cells were grown and/or differentiated as described above. For viral growth curves, MEFs were plated at 1×10^5 cells/well and all

other cell types were plated at 5×10^4 cells/well in 24-well tissue culture treated plates and allowed 24h to adhere. For interferon sensitivity assays, cells were then either pretreated with interferon-β (PBL Interferon) at the indicated dose or mock treated with media only for 24 hrs. Cells were infected at the indicated multiplicity of infection (MOI). Cells were rinsed with DMEM with 1% FBS, after which diluted virus was added and allowed to adhere for 1h. The virus was then aspirated and replaced with fresh media. At each time point, a plate was frozen at -80°C and underwent three freeze-thaw cycles before titering on BHK cells by standard plaque assay.

Viral Studies. Six to nine day old pups were infected with between 5×10^4 and 1×10^5 pfu of the indicated recombinant CHIKV clones (37997 or LR2006 OPY1) diluted in 15 µl of PBS and injected intradermally (i.d.) to mimic a mosquito bite, using a Hamilton syringe and 30G needle, into the left upper pectoral area. For survival experiments pups were monitored daily for clinical signs of disease (including ruffling of fur, altered gait, and hind limb paralysis) and lethality for 21 days post-infection. For viral titers the mice were infected as described above and the various tissues (injection site, serum, muscle, brain) were harvested into PBS and stored at -80° C. Injection site refers to the skin and underlying connective tissue and fat at the site of infection. Serum samples were pooled when collected with each sample containing serum from two mice from a single experiment. For muscle titers, the hind limb ipsilateral to the site of infection was skinned and the hamstring was collected. For the analysis of viral loads the samples were homogenized with 100 µl of 1.0 mm-diameter zirconia-silica beads using a MagnaLyzer (Roche) by alternating two pulses of 7000 rpm for 30 seconds each, with chilling on ice for 30s. The homogenates were serially diluted and titers were determined by plaque assay on BHK cells. For serum collection, pups were bled at the designated time post-infection into BD Serum Separator tubes. These were spun for 9 minutes at 12,000 × g then frozen at -80°C until they were titered.

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

Cytokine/Chemokine analysis. Sera were harvested from infected mice at the indicated time points and stored at -80°C for analysis. Serum cytokines levels were measured using Luminex technology with the Bioplex Pro Mouse Cytokine 23-Plex Assay (BioRad).

Immunofluorescence. For immunofluorescence experiments the mice were infected as described above but we utilized recombinant clones for the 37997 and LR2006 OPY1 strains expressing green fluorescent protein (GFP) that behave identically to parental viruses (19). For intradermal infections, 1×10^5 pfu of either GFP strain was injected into the skin of the upper left chest of six-day old pups. For intramuscular (i.m.) injections, 2.5×10^4 pfu of either the GFP-expressing, or parental recombinant strains

was injected directly into the left hamstring of six-day old pups. Tissue was harvested from the mice at the indicated times and fixed in 4% paraformaldehyde for 24 hours at 4°C. It was then rinsed with PBS for 24h and allowed to equilibrate in 30% sucrose for 2h before embedding in OCT. Sections were cut using a cryostat and blocked with 5% Normal goat serum (Sigma). Sections were stained with an anti-Collagen IV antibody (Abcam) and with an anti-GFP antibodies (Abcam). The slides were developed with a donkey anti-rabbit 546 secondary antibody using AlexaFluor (Invitrogen).

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

rtPCR. Mice were infected with 1×10^5pfu i.d. Tissues were excised and flash frozen in liquid nitrogen. Tissues were homogenized using the Magnalyser (Roche) and RNA was extracted using the RNeasy Fibrous Tissue Kit (Qiagen). RNA was used to run real-time PCR on the infected tissues, normalizing to GAPDH (IDT). MCP-1 and RANTES gene expression assay was purchased from Applied Biosystems.

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

Results.

CHIKV strains 37997 and LR2006 OPY1 display similar growth and sensitivity to an interferon beta induced antiviral state in primary MEFs. .

A previous comparison of the sequences of the 37997 and the LR2006 OPY1 strains of CHIKV revealed 162 amino acid differences between these two strains (22). Since these differences could impact the ability of these viruses to replicate, we first wanted to evaluate their growth in mammalian cells prior to evaluating potential differences in pathogenesis. Both BHK cells and MEFs were infected with the two strains of CHIKV at a low MOI and viral growth over time was analyzed. Both the LR2006 OPY1 and the 37997 strains of virus grew with similar kinetics in BHK cells (data not shown). We also observed no difference in the viral growth rate or in the peak viral titers achieved in MEFS infected with either strain at any of the time points analyzed (Fig. 2.1A).

Previous studies in other alphaviruses has shown that mutations controlling the sensitivity of a virus to type I interferons could impact upon its *in vivo* pathogenesis (26, 27). Mutations mapped to the 5' non-translated region, nsP1, and nsP2 genes in Sindbis virus have been found to affect interferon sensitivity (26, 27). There are residue differences in these genes between the LR2006 OPY1 and 37997 strains (22). Therefore, we next tested whether the two strains of CHIKV displayed similar *in vitro* sensitivity to type I IFNs. C57BL/6 MEFs were either mock treated or treated for 24 hrs prior to infection with IFN-β at a dose that would induce an antiviral response and partially inhibit viral replication. The cells were then infected and viral growth was monitored at several time points post-infection. As seen in figure 1B, IFN pretreatment was able to equivalently inhibit both the LR2006 OPY1 and the 37997 strain of CHIKV (Fig. 2.1B, dotted lines). We also observed a similar increase in replication of the two CHIKV strains following the infection of $IFNAR^{-/-}$ MEFs, with the virus growing to titers that were nearly 100 fold higher than in B6 MEFs (Fig. 2.1C). As expected, pretreatment of these cells had no impact on CHIKV replication. Together, these results indicate that the LR2006 OPY1 and the 37997 recombinant clones of CHIKV displayed similar growth kinetics in mammalian cells and they displayed similar sensitivity to IFN *in vitro*.

The LR2006 OPY1 strain of CHIKV induces more severe disease in comparison to the 37997 strain of CHIKV.

Next, we evaluated the pathogenesis of these two clones using a neonatal murine model of CHIKV infection. In this model, the infection of wild type mice less than 10 days of age results in the development of a disseminated infection with significant lethality (16, 28). We infected 6 day old C57BL/6 pups i.d. in the upper left chest with 5×10^4 PFU of either the LR2006 OPY1 or the 37997 strain of CHIKV. Infection with either strain of CHIKV resulted in significant lethality with a similar median onset of lethality of 10 days for both viruses, with 54% of the LR2006 OPY1 infected pups and 68% of the 37997 infected pups succumbing to infection (Fig. 2.2A). However, despite the similarity in lethality induced by both strains of CHIKV, we observed more severe clinical signs of disease in the LR2006 OPY1 infected pups as compared to the 37997 infected mice. While mice infected with either strain of CHIKV displayed ruffled fur, sluggishness, and altered gait, the LR2006 OPY1 infected mice often developed hind limb weakness and paralysis, which was rarely seen in the 37997 infected mice (data not shown). This severe weakness was first observed at 7 days post-infection (dpi) and lasted throughout the course of infection in surviving mice.

To further investigate the differences in the clinical course induced by the two strains, we next evaluated viral dissemination and replication of the two clones in six day old mice at different times post-infection. An analysis of the injection site, which included the skin and underlying connective tissue, revealed similar viral loads at all of the time points analyzed, with peak titers obtained at 1 dpi and a steady decrease in viral replication until 9 dpi, when replicating virus was no longer detected (Fig. 2.2B). It has been previously reported in the neonatal model of CHIKV infection that mice develop viremia, resulting in viral dissemination to multiple tissues. Mice infected with either strain of CHIKV

developed viremia, with similar viral levels detected in the LR2006 OPY1 and the 37997 infected mice at both 1 and 3 dpi (Fig. 2.2C). However, mice infected with the 37997 strain cleared the virus from the serum by 4 dpi. In contrast, mice infected with the LR2006 OPY1 strain maintained persistent viremia until 7 dpi when serum viremia was no longer detected.

The hind limb weakness and ataxic gait we observed in the LR2006 OPY1 infected mice could be due to CHIKV infection impacting upon either the central nervous system or the muscle of these mice, or both. Therefore we evaluated viral loads in both the brains and the muscle of mice infected with either the LR2006 OPY1 or the 37997 strains. While we did observe an increase in viral titers in the LR2006 OPY1 infected mice at 3 and 4 dpi, by 5 dpi similar titers were observed in mice infected with either CHIKV strain, and both strains were cleared by 12 dpi in surviving mice (Fig. S1, supplemental material). Furthermore, histological evaluation of the brains from these mice at the various times post-infection revealed no significant pathology induced by either strain of CHIKV (data not shown). In contrast to the brain, an analysis of viral titers in the muscle revealed a striking difference between the two strains. We found that infection with either strain of CHIKV resulted in detectable viral titers in the muscle as early as 1 dpi (Fig. 2.2D). Consistent with our clinical observations, however, the LR2006 OPY1 infected mice had significantly greater viral burden in their muscle. By 3 dpi the mice infected with the LR2006 OPY1 strain of CHIKV displayed greater than 100-fold more virus in their muscle than the 37997 strain, even though serum viral loads were similar between the two strains at this time point. The increased viral burden observed in the muscle of the LR2006 infected mice persisted until at least 9 dpi, with the LR2006 OPY1 infected mice having between 3-4 logs higher viral titers in their muscle (Fig. 2.2D). Mice infected with the 37997 strain had peak replication at 5 dpi with a mean titer of $3.0\ 10^4$ (pfu/mL); while the LR2006 OPY1 strain peaked at 6 dpi with a mean titer of $6.17\ 10^7$(pfu/mL) (Fig. 2.2D). While the 37997 strain was largely cleared from the muscle by 9 dpi, mice infected with the LR2006 OPY1 strain continued to have greater than 10,000 fold more virus, even at 9 dpi. Even increasing the infecting

dose of 37997 to 20X the dose (2×10^6 pfu i.d.) did not result in an increase in viral loads detected in the muscle of the infected mice (Fig. S2, supplemental material). To determine if the adaptive immune response played a role in clearing the viral infection from the muscle we also infected $Rag^{-/-}$ mice with either strain of CHIKV and observed no differences in viral loads as compared to WT mice (Fig. S3, supplemental material). Therefore, consistent with the hind limb weakness observed in the mice infected with the LR2006 OPY1 strain of CHIKV, we observed increased peak viral loads and prolonged replication in the muscle of mice infected with the LR2006 OPY1 strain.

LR2006 and 37997 elicit similar serum cytokine and chemokine profiles during the course of infection.

We have previously seen that neonatal mice infected with CHIKV develop a systemic inflammatory response (24). Furthermore, in a neonatal model of Sindbis infection, this systemic inflammatory cytokine response likely contributes to their lethality (29). We therefore wanted to determine if there was a difference in the cytokines induced by either the 37997 or LR2006 OPY1 strain of CHIKV that may account for the differences in pathogenesis that we observed. We analyzed 23 cytokines and chemokines in the serum of infected mice at 1, 3, 5, and 7 dpi. As previously observed, several cytokines were induced by CHIKV infection, including IL-1α, IL-1β, IL-6, IL-10, and G-CSF (Fig. 2. 3A). Both CHIKV strains induced elevated levels of IL-1β, IL-6, and RANTES, cytokines found to be associated with severe disease in human patients afflicted with CHIKV (18). Surprisingly, despite the increased viral loads detected in the LR2006 OPY1 infected mice during the course of viral infection, we observed no significant difference in the cytokine levels between each strain at any time point we analyzed. We did observe a trend toward an increase in several chemokines including KC, MCP-1, MIP-1α, MIP-1β and RANTES at 1 dpi in mice infected with the 37997 strain of CHIKV (Fig. 2.3A), although these differences did not reach statistical significance. To determine whether elevations in these

chemokines were also present in the muscle, where we observed the differences in viral titers, we analyzed the hind limb muscle for expression of MCP-1 and RANTES RNA by RT-PCR (Fig. 3B). RNA levels of both chemokines were elevated over mock-infected levels as early as 1dpi. However, we observed no differences in the induction of either MCP-1 or RANTES RNA between the two strains of CHIKV. At 7dpi, the RNA levels of both chemokines were further elevated, with mice infected with LR2006 expressing similar levels of MCP-1 and RANTES RNA than those infected with the 37997 strain, despite the increased viral burden present in these mice at this time (Fig. 2.3B). Together, these results indicate that both the 37997 and the LR2006 OPY1 strains of CHIKV induce a systemic cytokine and chemokine response in this neonatal model, however, no significant differences were noted in the responses induced by the two viral strains.

LR2006 OPY1 strain of CHIKV infects myofibers and results in greater hind limb muscle damage than the 37997 strain of CHIKV.

To further characterize the disease induced by these two CHIKV strains, we next performed a histological analysis of the muscle of infected mice. Muscle from the hind limb distal to the site of infection was collected at various times post-infection and stained with H&E. These slides were evaluated by a pathologist in a blinded fashion. Muscle sections were given a severity score from 0 (normal) to 5 (most severe) as described in the Materials and Methods. An analysis of the muscle from CHIKV infected mice revealed significant pathological changes (Fig. 2.4A-B). In mice infected with the 37997 strain we observed focal areas of myositis and necrosis, with the majority of the muscle being largely undamaged. The peak damage seen was at 9 dpi for 37997 infected mice (Fig. 2.4B). Consistent with the profound muscle weakness observed clinically and the increased viral titers, muscle from mice infected with the LR2006 OPY1 strain displayed widespread muscle destruction beginning at 5 dpi and

lasting until at least 12 dpi, with peak damage occurring at 12 dpi (Figure 2.4A-B). In these samples there were dense inflammatory infiltrates accompanying massive myonecrosis (mean score = 5 at 12 dpi, Fig. 2.4A). Therefore, this histological data supports the clinical findings that the LR2006 strain of CHIKV is inducing more severe muscle disease in these mice.

To further evaluate the differences seen in the muscles of these mice we next determined if there were differences in viral tropism between the two strains. Previous work has shown that CHIKV replicates in the connective tissue fibroblasts and myoblasts in both mice and humans (16). While other studies have noted myofiber necrosis and attributed this to infection of those fibers by CHIKV, previous studies have not detected infection of the myofibers (30). We utilized recombinant CHIKV strains in which GFP expression was driven by a subgenomic promoter in either the 37997 or LR2006 OPY1 backbone. By utilizing these viruses only cells that harbor active CHIKV infection will express GFP. Mice were infected i.d. with either the 37997-GFP or LR2006-GFP-expressing CHIKV strain and at different times post-infection the hind limb muscle was evaluated by immunofluorescence. Similar to what has been previously reported, we detected GFP staining in the connective tissue fibroblasts surrounding the muscle fibers following infection with either the LR2006 or 37997 strain of CHIKV (Fig. 2.5A, left panels) . Staining was detected as early as 3 days post-infection and increased throughout the course of infection until myofibers were destroyed. Quantification of GFP+ fibroblasts revealed a similar quantity of cells were infected following infection with either strain of CHIKV (Fig. 2.5B). Surprisingly, in mice infected with LR2006 OPY1 we also detected GFP+ staining of the myofibers, particularly at late time points after infection, although GFP+ fibers can be seen as early as 3 dpi. In contrast, there is almost a complete absence of GFP+ myofibers in the hind limb of mice infected with 37997-GFP (Fig. 2.5A, 5C). This data suggests that the ability of the LR2006 OPY1 strain to gain access to the myofibers and establish infection results in increased viral loads in the muscle and increased muscle damage, and likely contributes to the increased pathogenicity of this CHIKV strain.

During intramuscular injection, both strains are able to infect myofibers.

The inability of the 37997 strain to infect the myofibers following an intradermal infection could be due to an inability of the virus to directly infect the myoblasts/myofibers, an inability to grow in the myoblasts, or difficulty in gaining access to this compartment. To test these possibilities we first evaluated the ability of the LR2006 OPY1 and 37997 strains to infect and replicate in primary connective tissue fibroblasts and in myoblasts and myofibers. Primary muscle connective tissue fibroblasts were isolated from neonatal mice and infected with either strain of CHIKV at an MOI of 0. 05. Viral growth was assessed at various times post-infection. Both strains grew at similar rates, and reached comparable peak titers after 48 hours post-infection (Fig. 2.6A). This supported our *in vivo* observation that both strains infected and replicated within the connective tissue fibroblast compartment (Fig 2.5A, B. We next evaluated the ability of the LR2006 OPY1 and 37997 strains to infect and replicate within myoblasts and myofibers. We first used the C2C12 myoblast cell line to assess growth *in vitro*. Both CHIKV strains were able to infect the C2C12 cells and an analysis of viral growth revealed that both strains displayed similar growth kinetics (Fig. 2.6B). Next, myoblasts were differentiated into myofibers, forming a mixed culture of both myoblasts and myofibers. They were also infected with a low MOI, and produced comparable growth between the strains (Fig. 2.6C). These results suggest that both viral strains have the ability to infect connective tissue fibroblasts, myoblasts, and myofibers. Furthermore, it appears that both strains display similar growth characteristics *in vitro*. Therefore it doesn't appear that the 37997 cannot infect and/or replicate in the myoblasts, but rather this viral strain may not be able to gain access to this compartment *in vivo*.

To determine whether the difference in viral replication was due to the inability of 37997 to infect myofibers *in vivo*, intramuscular injections were performed by injecting 2.5×10^4 pfu directly into

the left hamstring of six day old mice. Hind limb muscle from the site of injection was collected and stained by IF. In contrast to the intradermal infection, following the direct intramuscular administration of virus, both 37997-GFP and LR2006-GFP were able to infect myofibers, as shown at 3 dpi (Fig. 2.6D), and the quantification of the number of GFP + myofibers were similar between the two viral strains (Fig. 6E). At later time points, massive tissue destruction is seen in mice infected by either strain, and few GFP+ myofibers remained (data not shown). This finding was further supported by an analysis of viral loads in the muscle. Mice were infected with the parental strains of LR2006 OPY1 and 37997, which lack GFP, and viral titers were analyzed in the infected muscle at different times post-infection. Following direct inoculation the two strains reached similar viral titers with only a 3.54-fold increase in titers in the LR2006 OPY1 vs. 37997 infected muscle, as compared to the 1000-fold difference seen during id administration (Fig. 2.6F). Following direct inoculation the 37997 strain reached peak titers of 6.77×10^5 pfu/mL, much higher than its peak viral load following administration by the id route, despite using a lower inoculating dose of 2.5×10^4 pfu (Fig. 2.6F). The ability of the 37997 strain to replicate to high viral titers following direct i.m. inoculation indicates that both viral strains display similar fitness once they gain access to the muscle fiber compartment. During the course of these direct intramuscular infections we once again noted that the contralateral leg of the mice infected with the LR2006 OPY1 strain of CHIKV developed severe weakness while in the mice infected with the 37997 strain of CHIKV we did not detect contralateral leg weakness. When the contralateral leg was titered, we found that similar to the i.d. route of infection, the LR2006 OPY1 infected mice achieved significantly higher viral titers in their contralateral leg than the titers obtained in the 37997 infected mice, with viral titers reaching 5.5×10^6 pfu/mL in the LR2006 OPY1 infected mice at 5 dpi as compared to titers of only 1.5×10^4 pfu/mL in the 37997 infected mice (Fig. 2.6G). Thus, injecting virus at a different location, in this case directly into the muscle, does not alter its ability to spread throughout the host. Taken together these results demonstrate that both the LR2006 OPY1 and the 37997 strains of CHIKV have the ability to replicate

within the primary cells of the muscle, namely the connective tissue fibroblasts and the myoblasts when access is provided. The differences in pathogenesis that we have seen in our model appear to reflect the ability of the LR2006 OPY1 strain to access the myoblast/myofiber niche, which it can do following either i.d. or i.m inoculation. In contrast, the 37997 strain of CHIKV, while able to establish viremia and infect the connective tissue fibroblasts, fails to establish a robust infection of the myoblasts/myofibers unless it is provided direct access via i.m inoculation. This results in decreased viral loads and diminished muscle damage.

Discussion

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

In this study we compared the pathogenesis of two different CHIKV strains, one isolated during the La Réunion outbreak in 2006 and another isolated from Senegal in 1983. Importantly, both viruses utilized in these studies were generated from recombinant clones to minimize viral heterogeneity. The clones were not mouse adapted and represent viruses that were actively circulating when isolated. Neonatal mice infected with the La Reunion recombinant strain (LR2006 OPY1) developed more severe disease with progressive hind limb weakness and severe myonecrosis compared to the Senegal strain (37997). 37997 and another strain from the 2006 outbreak (DHS-4263) were also compared in a macaque model, where increased viremia was also seen with the DHS-4623 strain (30). Mild muscle

fiber necrosis was also seen in several animals, but muscle titers or pathology during acute infection was not performed (30). In the murine arthritis model, more severe joint swelling was seen in mice infected with the LR2006 OPY1 strain than those infected with an Asian isolate from the 1960's (31). Together, this data supports our findings of increased disease severity with LR2006 OPY1.

The most striking difference in the disease induced by each strain was the very high muscle titer in the mice infected with LR2006 OPY1 that resulted in severe myonecrosis (Fig. 2.2D,2.4). Both strains are able to spread from the site of infection and establish infection in the distal muscle by infecting connective tissue fibroblasts early in infection. CHIKV has been shown to infect this cell type in murine models and in human biopsy specimens (16). However, as early as 3 dpi, when viral loads in the serum were similar between the two strains, mice infected with the LR2006 OPY1 strain had viral titers within their hind limb muscles that were 1000-fold greater than that observed in the 37997 infected mice. This increased viral load persisted until at least 9 dpi. Most strikingly, this increase in viral replication correlated with the ability of the LR2006 OPY1 strain, but not the 37997 strain, to infect the myofibers within the muscle.

There are several hypotheses that could explain the difference in behavior between 37997 and LR2006 OPY1 strains. These include differences in viral fitness, viral tropism, access to myofibers. A recent study of Ross River virus identified a single amino acid change within the E2 protein (Y18H) that resulted in a dramatic reduction in muscle titers and pathology. This switch to histidine resulted in a decrease in the fitness of this virus in mammalian cells, resulting in reduced replication in both murine and human cells, but an increased ability to replicate in mosquito cells (32). An alignment of the E2 proteins from the LR2006 OPY1 and 37997 strains revealed that both have a histidine at this position. Furthermore, while the LR2006 strain does display an increase in viral replication within the muscle and induces more damage, we have not observed differences in viral fitness. Similar replication between the

two strains was observed in several mammalian cell types including BHK, MEFs, and connective tissue fibroblasts (data not shown, Fig. 2.1, 2.6), suggesting the fitness of the virus does not explain our *in vivo* phenotypes.

Previous to this study, direct infection of myofibers by CHIKV had not been shown, though evidence of myofiber fragmentation and necrosis have been reported (30). Since we observed the infection of myofibers following i.d. administration of the LR2006 OPY1 strain but not the 37997 strain, differences in cellular tropism could also contribute to the altered pathogenesis. Mutations in the LR strain could result in increased receptor binding and/or replication within myoblasts or myofibers. However, our in vitro studies demonstrated that both strains were able to infect connective tissue fibroblasts, myoblasts, and myofibers, and the viruses grew with similar kinetics and to similar titers (Fig. 2.6). Furthermore, the administration of the 37997 strain directly into the muscle resulted in myofiber infection and dramatically reduced the differences in viral titers seen between the two strains (Fig 2.6). Interestingly, while direct i.m. administration of the two strains equalized the viral loads within the injected muscle and allowed for 37997 to infect the myofibers, spread of the virus to the contralateral muscle was more efficient in the LR2006 strain, once again resulting in viral loads that were greater than 100-fold higher than seen with the 37997 strain (Fig. 2.6). This data suggests that the LR2006 OPY1 strain may have increased ability to access myofibers.

Previous studies evaluating the ability of gene therapy viral vectors, such as HSV-1 and adenoviruses, to infect skeletal muscle have shown that the basal lamina can function as a physical barrier to limit myofiber infection in adult muscle. Intact skeletal muscle was not permissive to HSV-1 infection of myofibers, but isolated myofibers cultured *in vitro* could be infected (33). The disruption of the basal lamina through the utilization of genetically defective mice (dy/dy) which have abnormal basal lamina synthesis, allowed for myofiber infection *in vivo* (34). One potential explanation for the

differential infection of myofibers with our viruses is that during the course of the LR2006 OPY1 infection there is an alteration in the production of cytokines or proteases that can damage the basal lamina surrounding the muscle fibers, allowing the LR2006 OPY1 strain to establish infection. Proinflammatory cytokines and chemokines are induced in animal models of CHIKV, and in human studies elevated levels of IL-6, IL-1β, and RANTES have been associated with more severe disease manifestations (18). Infection with either the LR2006 OPY1 or the 37997 strain of CHIKV did induce a proinflammatory cytokine and chemokine response, however despite the prolonged serum viremia following LR2006 OPY1 infection, no significant differences were observed between the two strains during the course of infection (Fig. 2.3). Alternatively, within the context of the intact muscle architecture, differential induction in the expression of receptors for CHIKV on myoblasts or myofibers could account for differences in infectivity. Until the CHIKV receptor is identified this later hypothesis cannot be formally tested. Whether the prolonged serum viremia we observed during the LR2006 OPY1 infection also contributes to the myofiber infection by allowing the LR2006 OPY1 virus to reach higher concentrations along the basal lamina is unknown. However, the prolonged viremia cannot account for the entire muscle phenotype, as LR2006 OPY1 mice already have high titers at 3 dpi, when both strains cause similar serum viremia (Fig. 2.2).

The prolonged serum viremia observed following LR infection could have many impacts upon CHIKV pathogenesis. An increase in the length of time that a patient exhibits high serum viremia would prolong the period of time in which a patient can transmit the infection to a new mosquito host and subsequently to another patient. It has previously been shown that an alanine to valine mutation within the LR strain resulted in increased infectivity of the Asian tiger mosquito, *Ae. albopictus*. Therefore, a prolonged period of serum viremia coupled with the ability of this strain to replicate to high titers in *Ae. albopictus* could contribute to the ease with which CHIKV has spread to infect many on La Réunion, and ultimately millions in Southeast Asia. A prolonged viremic phase could also contribute to more severe

disease manifestations, especially in the elderly and neonates with already diminished immune systems. A prospective cohort study of patients on La Reunion found that those patients with chronic disease had higher viral loads compared to their counterparts who recovered fully, suggesting a deficit in viral clearance (35). Future studies evaluating whether the ability of CHIKV to access and infect myofibers contributes to more severe disease manifestations, such as the development of arthritis or the maintenance of chronic infection, will be important to further our understanding of CHIKV pathogenesis and in the identification of therapeutic interventions.

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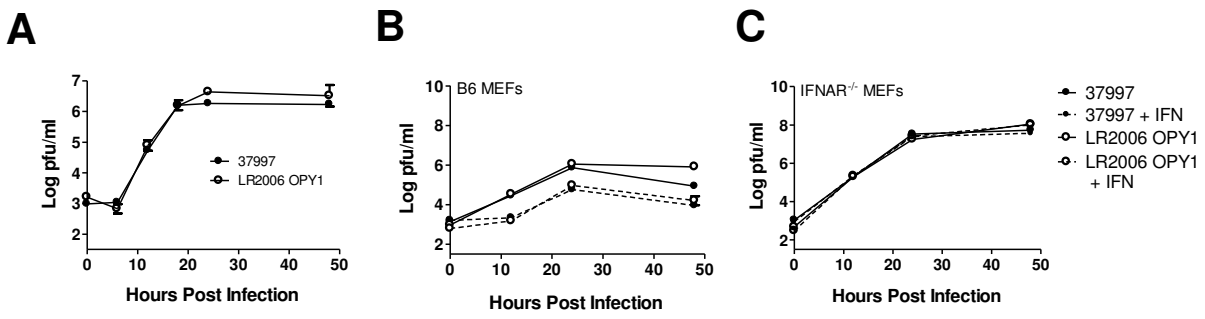


Figure 2.1. Recombinant CHIKV strains LR2006 OPY1 and 37997 Display Similar Growth Characteristics *In Vitro*. A) WT MEFs were infected at an MOI of 0.05 for 1 hr and analyzed for titer by plaque assay at several time points post infection. B) WT MEFs or C) IFNAR-/- MEFS were pretreated with 8 U/mL of IFNβ for 24hrs before infection, then infected at an MOI of 0.05 for 1 hour and titered by plaque assay as in A. Growth curves were performed in two independent experiments, each in duplicate.

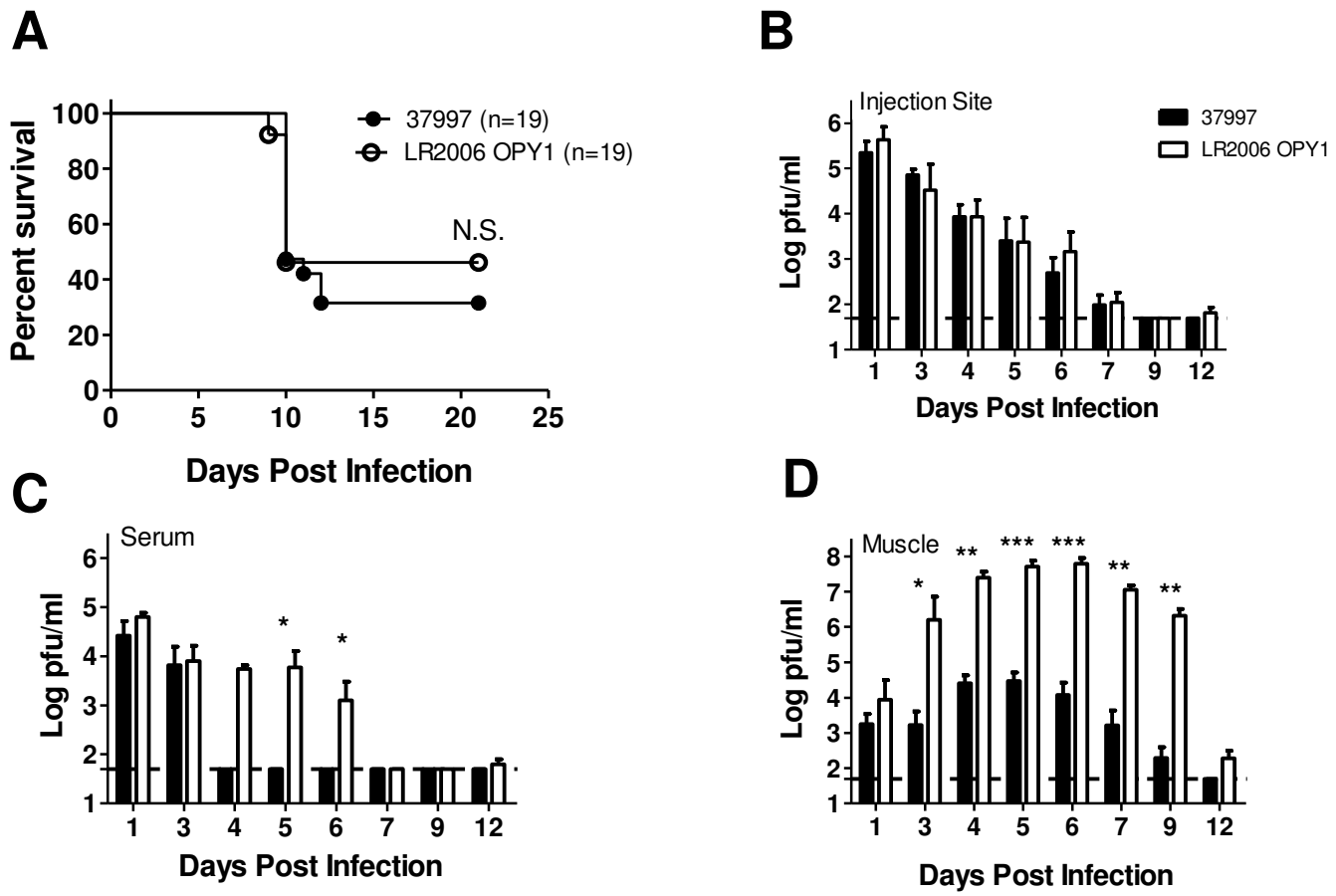
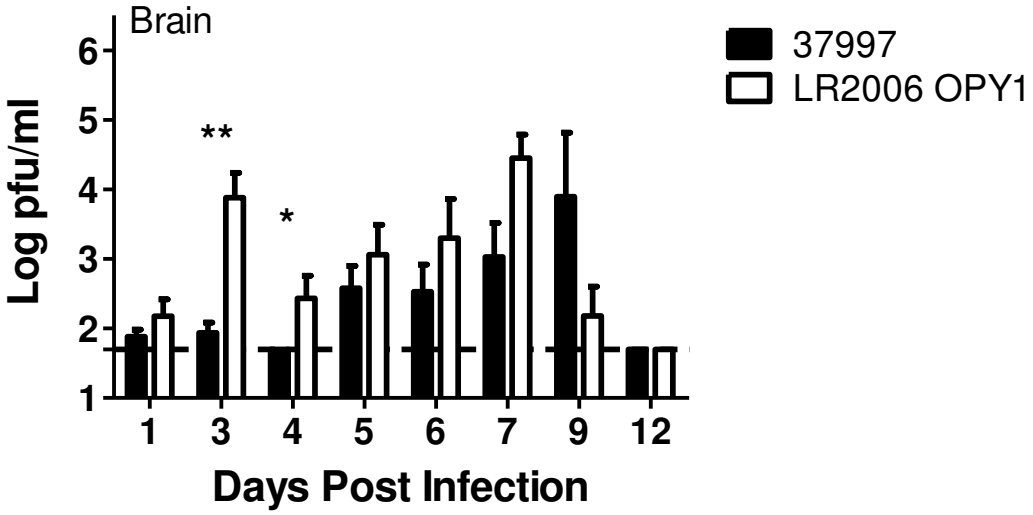


Figure 2.2. LR2006 OPY1 achieves higher muscle titers and persistence in the serum. Pups were infected i.d. at six days of age with 5×10^4 pfu of either LR2006 OPY1 or 37997 CHIKV strains, A) Infected mice were monitored for clinical signs of disease and death each day until 21 days post-infection. B-D) Infected mice were harvested at the indicated time points and B) injection site, C) serum, and D)distal hind limb muscle were collected and titered by plaque assay. N = 5-8 pups per group per time point, except for at 12 dpi where N = 4 pups per time point. The dashed line indicates the limit of detection for the assay. Significance for survival was evaluated by both the Log Rank and the Gehan-Breslow-Wilcoxon tests. Statistical significance for B-D was evaluated with the Mann-Whitney test. * p = 0.01-0.05; ** p= 0.001-0.01; *** p< 0.001



Supplemental Figure 1. A comparison of CHIKV replication in the brains of mice infected with either LR2006 OPY1 or 37997. Pups were infected at six days of age with 5x104 pfu i.d. with either the LR2006 OPY1 or 37997 strains. At the indicated time post-infection, the calvarium was opened and the half of the brain ipsilateral to the side of infection was collected for titering. The dashed line on each graph indicates the limit of detection

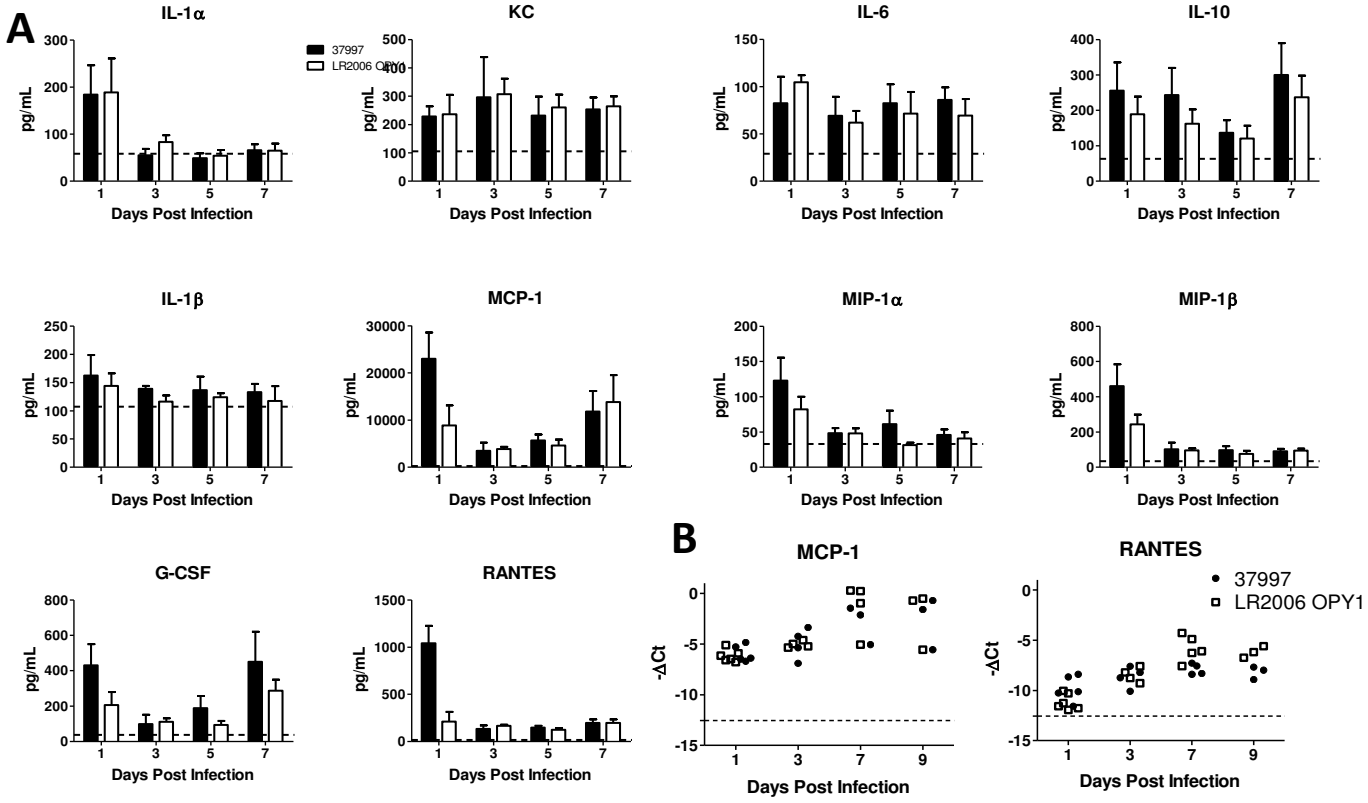
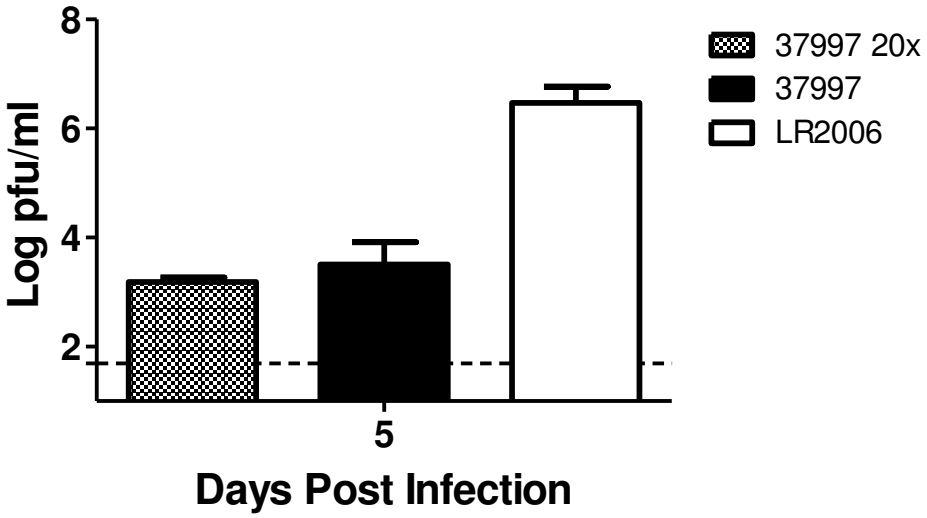
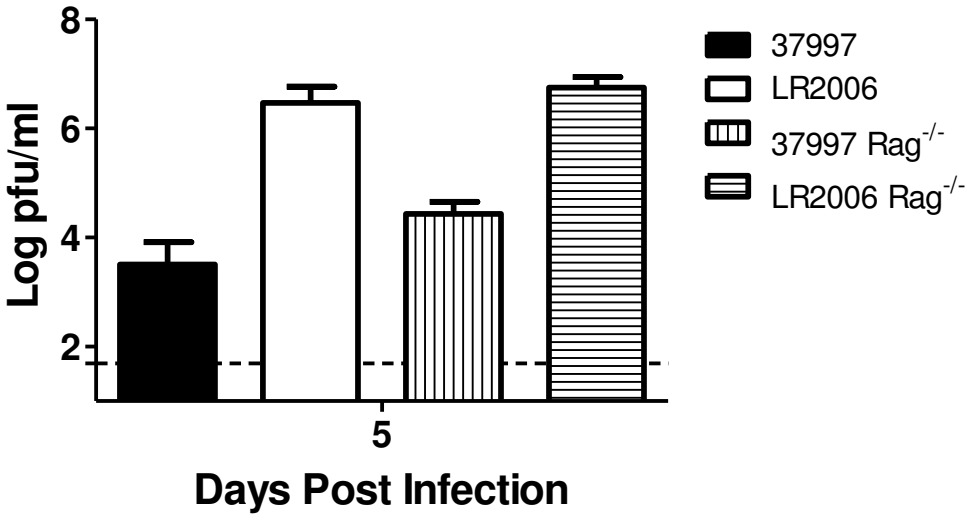


Figure 2.3. Similar cytokine and chemokine profiles are induced by both strains of CHIKV. A) Six day old pups were infected with 5×10^4 pfu of either CHIKV strain or mock treated with PBS i.d. Serum was collected at the indicated times and analyzed using a Bioplex Pro mouse 23-plex array. Dashed line indicates level of cytokine or chemokine in mock infected samples. N=4 per time point. Samples didn't reach statistical significance at any time points analyzed. B) Mice were infected with 1×10^5 pfu of either CHIKV strain. RNA was extracted from hind limb muscle and rtPCR was performed. Samples were normalized to GAPDH. Dashed line indicates RNA levels in mock infected mice.



Supplemental Figure 2. Increasing the dose of 37997 strain doesn't impact upon its ability to replicate in the distal muscle. WT pups were infected at six days of age with $2x10^6$ pfu (37997 20x) or $5x10^4$ pfu (37997 and LR2006 OPY1), and hind limb muscle collected at 5 days post infection was titered by plaque assay.



Supplemental Figure 3. Lack of adaptive response does not change low 37997 titer at 5 days post infection. WT and Rag knockout mice were infected with 5 x 10^4 pfu of either strain, and hind limb muscle was harvested at 5 days post infection titered by standard plaque assay.

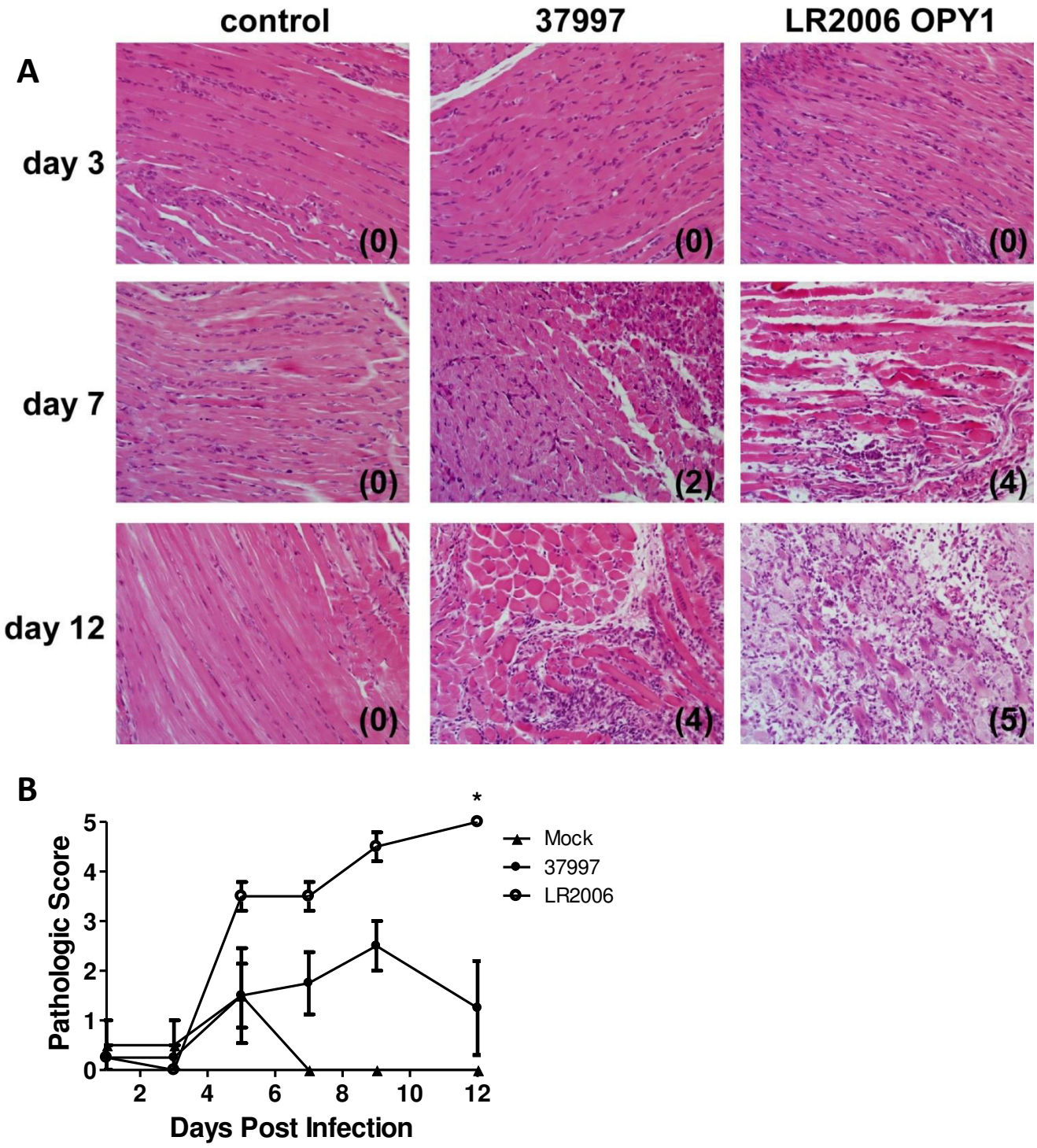

control
37997
LR2006 OPY1
A
day 3
day 7
day 12
(0)
(0)
(0)
(0)
(2)
(4)
(0)
(4)
(5)
B
Pathologic Score
Days Post Infection
Mock
37997
LR2006
*

Figure 2.4. The LR2006 OPY1 strain induces dramatic myositis and necrosis. Six day old pups were infected with 5×10^4 pfu of CHIKV i.d. or were mock treated with PBS. The hind limb was isolated on the indicated days post infection and fixed in 10% formalin for 48h. Sections were submitted for embedding, sectioning and staining. A) The pathology of each sample was scored by a pathologist on a scale of 0 -5 as described in the Materials and Methods. Representative H&E sections at several days post infection at a magnification of 40× are shown, with the pathological score indicated in parantheses. B) Graphical representation of the scores of all of the histological sections. * p = 0.01-0.05

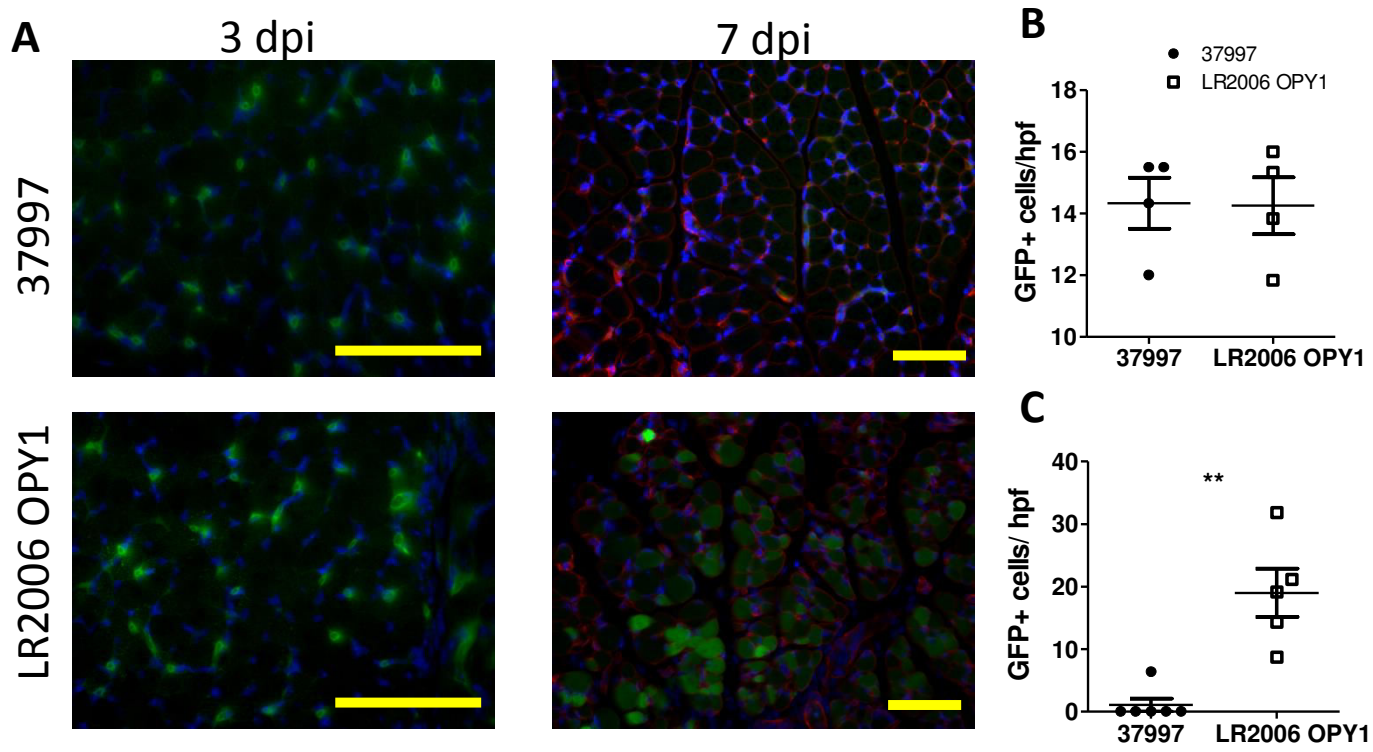


Figure 2.5. Intradermal infection results in infection of myofibers by LR2006 OPY1, but not by 37997.

Mice were infected with either 37997-GFP or LR2006 OPY1-GFP i.d. at a dose of 5×10^4pfu. Muscle from the hind limb were stained for collagen IV (red), GFP (green) or nuclei (blue). A) Left panel muscle harvested at 3dpi (40× magnification), right panel,7dpi (20× magnification). B) Connective tissue fibroblasts were counted in 40× high power fields at 3 dpi. C) Myofibers per 20× high power field at 7 dpi. Scale bar indicates 100 µm.

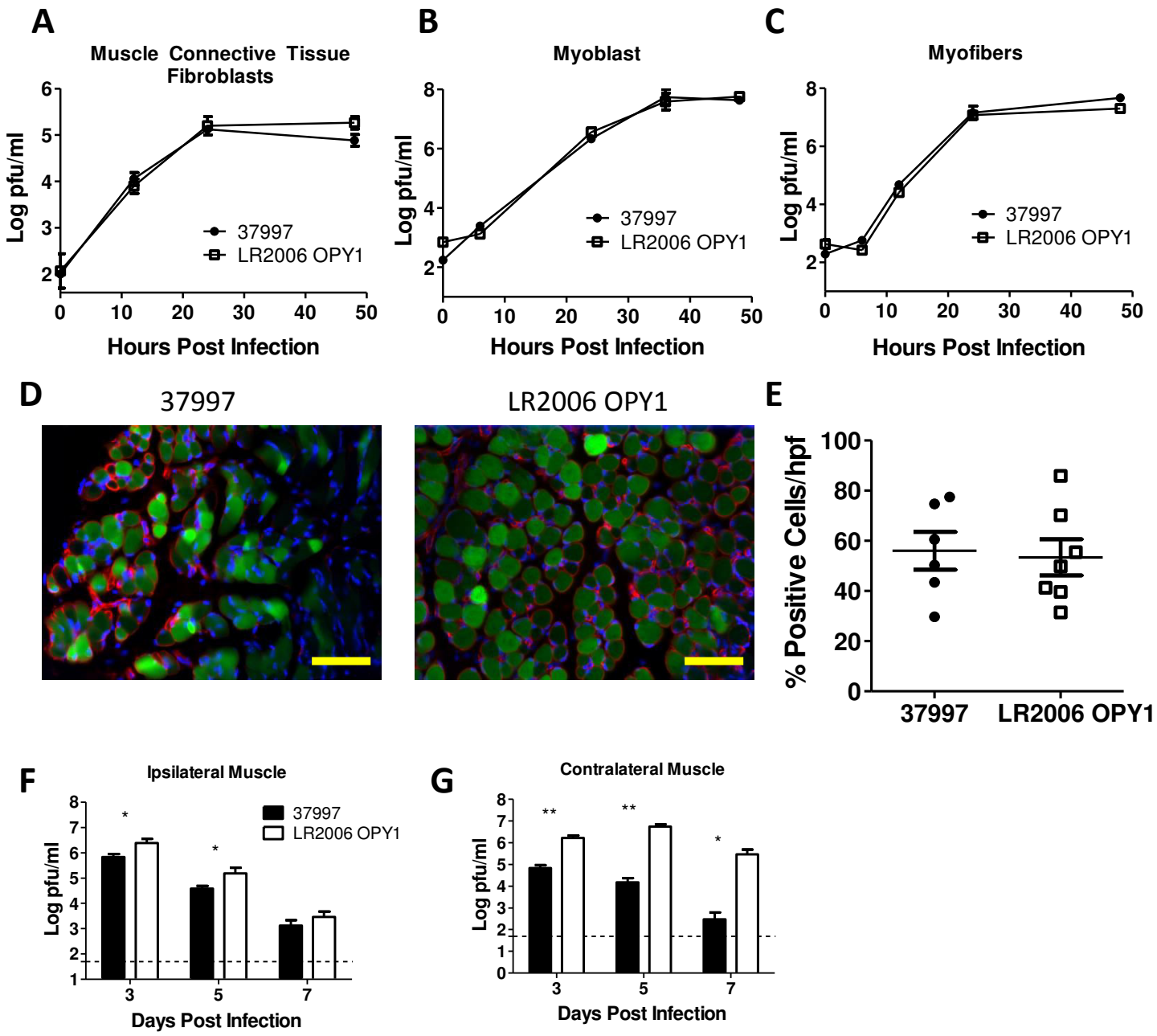

A
Muscle Connective Tissue Fibroblasts
Log pfu/ml
Hours Post Infection
37997
LR2006 OPY1
B
Myoblast
Log pfu/ml
Hours Post Infection
37997
LR2006 OPY1
C
Myofibers
Log pfu/ml
Hours Post Infection
37997
LR2006 OPY1
D
37997
LR2006 OPY1
E
% Positive Cells/hpf
37997
LR2006 OPY1
F
Ipsilateral Muscle
Log pfu/ml
37997
LR2006 OPY1
Days Post Infection
G
Contralateral Muscle
Log pfu/ml
Days Post Infection

Figure 2.6. Both strains can infect muscle when given intramuscularly. A) Primary muscle fibroblasts B) C2C12 myoblasts and C) differentiated C2C12 myofibers were infected with either LR2006 OPY1 or 37997 at an MOI = 0.05, and titered at various times post infection by plaque assay. D, E) Mice were infected in the hind limb with 2.5 x 10^4 pfu LR2006 OPY1-GFP or 37997-GFP and sacrificed at 3 days post infection. D) Frozen sections of hind limb muscle was stained for collagen IV (red), GFP (green), nuclei (blue). Scale bar indicates 100 μm. E) Quantification of GFP+ myofibers per high power field. F,G) Mice were infected in the hind limb with the parental strains at 5×10^4 pfu and F) muscle ispsilateral and G) contralateral to the site of infection were harvested at the indicated days and titered by plaque assay. * p = 0.01-0.05; ** p= 0.001-0.01; *** p< 0.001

Chapter 3

Genetic determinants responsible for myofibers infection

Introduction

The latest CHIKV outbreak has appeared to be more severe than past outbreaks, as discussed in Chapter 1. There are several possible reasons for this increase in virulence, including the greater size of the latest epidemic, better reporting of symptoms as well as evolution of the virus. Our comparison of two CHIKV strains, LR2006 OPY1 and 37997 showed that while both strains grew similarly in vitro, they had dramatic differences in pathogenesis due to their genetic differences. Mice infected with the LR2006 OPY1 strain showed prolonged serum viremia and elevated titers in the muscle. Further analysis revealed the difference in muscle titer was likely due to the LR2006 OPY1's ability to infect myofibers following administration by the intradermal route, while 37997 did not. However, when injected directly into the muscle, both strains had similar titers and were able to infect myofibers. This indicates the LR2006 OPY1 strain has an enhanced ability to infect myofibers during intradermal infection, meant to mimic a mosquito bite. Given that both strains are able to infect myofibers when no longer under the constraints of an intradermal infection, the issue is not simply one of tropism. Instead, the virus could gain an advantage at any stage when traveling from the injection site to the distal muscle.

As discussed in Chapters 1 and 2, changes in the CHIKV viral genome have already been linked to changes in virus behavior. A mutation in the E1 protein from an alanine to a valine at position 226 resulted in increased infectivitity of a new mosquito vector, Ae. albopictus (1). Mutations in other alphaviruses, such as Sindbis Virus, have been shown to alter a variety of viral properties, such as interferon sensitivity, virulence, as well as replication kinetics (2-4). Thus, an analysis of the residue differences between LR2006 OPY1 and 37997 could reveal a lot about the mechanisms of pathogenesis of CHIKV.

There are 162 residue differences between 37997 and LR2006 distributed throughout every gene of the CHIKV genome, though only 91 of these are non-synonymous. Any number of these mutations, or combinations of these could result in a change in tropism. We have devised three

methods of investigating the genetic changes responsible for the phenotype we see. The first involves creating chimeric viruses where a region or gene in one strain has been replaced with the same region from the other strain, thus transferring the genetic changes of a specific region at one time. This method may allow us to identify areas of interest, and possibly even to a single residue responsible.

A similar technique was used to identify areas of the viral genome responsible for increased muscle damage induced by a related alphavirus, RRV. In this study, an area of the nsp1 protein as well as in the E2-E3 region were both found to cause increased muscle damage (5). A follow-up study identified a single residue in the E2 region that controlled muscle damage (4). This method was also used to map a single residue responsible for persistence in MNV (6).

The next method involves checking the phenotype of an 'intermediate' strain, Ag41855 that was isolated in Uganda in 1982. Although this strain is fairly dissimilar from 37997, it is more closely related to LR2006 OPY1 with only 23 residue differences (7). Ag41855 is also a member of the East, Central and South African clade of CHIKV, like LR2006 OPY1. 37997, however, is a member of the West African clade that diverged from the ECSA clade long ago. Thus, Ag41855 represents an example of what LR2006 OPY1 may have recently evolved from.

Our final strategy involves passaging 37997 in neonatal mice to see if it will acquire a mutation that would result in high muscle titers. CHIKV has been passaged in tissue culture to produce an attenuated strain for vaccine development (8). Interestingly, the mutations acquired that were responsible for attenutation were in the E2 protein (9). Further, when murine cytomegalovirus is passaged in BL6 mice, escape mutants that evade detection by NK cells quickly develop (10). Thus, repeated passage of virus can result in genetic changes that alter the disease phenotypes seen.

Although none of these methods have allowed us to identify a region of interest responsible for the increased muscle damage and titers that we observed with the LR2006 OPY1 strain, they have each

revealed interesting information about CHIKV. First, the chimeric viruses have allowed us to determine that the E2 protein is not responsible for our myofibers infection phenotype, despite being implicated in tropism for other alphaviruses. Secondly, 41855 has a phenotype that is intermediate to that of 37997 and LR2006 OPY1. While this does not allow us to narrow down the residues responsible for the increased muscle titers observed with LR2006 OPY1, it may provide insight into the evolution of the current outbreak. Finally, passaging the 37997 strain *in vivo* did not yield a virus that displayed the increased replication in muscle as compared to 37997. However, it did result in a virus that appears to be significantly more virulent in mice. This could provide us with a new tool useful for investigating CHIKV pathogenesis in adult mice.

Methods and Materials.

Chimeric Virus Plasmid Construction. Chimeric viruses were constructed as previously described. Briefly primers (Table 3.2) were constructed that flanked a region of interest, and were able to bind to both 37997 and LR2006 OPY1. These primers were used to amplify from one plasmid that will be referred to as the insert. The PCR segment was included in a PCR reaction with the target plasmid, referred to as the backbone. After the PCR was completed, the parental backbone was digested with DpnI. The remaining PCR reaction was transformed and colonies were screened by PCR and sequencing.

Mice. Mice were maintained at Washington University School of Medicine in accordance with all federal and University guidelines. For mouse studies the principles of good laboratory animal care were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the NIH. The protocols were approved by the Animal Studies Committee at Washington University. All efforts were made to minimize

suffering. C57BL/6 and IFNα/β Receptor 1 ($IFNAR^{-/-}$) mice on the C57BL/6 background were bred and maintained in our mouse colony. For neonatal experiments, mice were infected at 6 days of age. Litters were weight matched at the initiation of the experiments.

Virus Production. The construction of recombinant plasmids for the LR2006 OPY1, 37997, LR2006-GFP and 37997-GFP strains of CHIVK has been previously described (11, 12). Recombinant viral stocks were generated from viral cDNA clones by *in vitro* transcription of linearized cDNA templates followed by RNA transfection with Lipofectamine 2000 (Invitrogen) into baby hamster kidney-21 cells (BHK cells) as previously described (13). After 48 hours, the supernatant was collected, centrifuged, aliquoted and stored at -80°C. Viral stocks were titered by plaque assay as previously described (14). All work with live virus was performed in a Biosafety Level 3 facility and followed strict guidelines established by the Environmental Health and Safety committee at Washington University School of Medicine.

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

the site of infection was skinned and the hamstring was collected. For the analysis of viral loads the samples were homogenized with 100 µl of 1.0 mm-diameter zirconia-silica beads using a MagnaLyzer (Roche) by alternating two pulses of 7000 rpm for 30 seconds each, with chilling on ice for 30s. The homogenates were serially diluted and titers were determined by plaque assay on BHK cells. For serum collection, pups were bled at the designated time post-infection into BD Serum Separator tubes. These were spun for 9 minutes at 12,000 × g then frozen at -80°C until they were titered.

Muscle Passage. A six day old pup was infected with $1x10^5$ pfu of 37997. On day 3 post infection, hind limb muscle was collected in PBS with 100 µl of 1.0 mm-diameter zirconia-silica beads using a MagnaLyzer (Roche) by alternating two pulses of 7000 rpm for 30 seconds each, with chilling on ice for 30s. The homogenates were serially diluted and titers were determined by plaque assay on BHK cells. 30uL of the muscle homogenate with the highest titer was used for the next intradermal injection, and hind limb muscle was collected, titered and passaged again, for a total of four passages. A single round of muscle passage was amplified in BHK cells, by adding 100uL of muscle homogenate from passage four to a 125 cm2 flask of BHKs that were 80% confluent. After 48h of infection, little CPE was observed and fresh media was added and media containing virus was harvested at 72h. This was titered then used for injection of following mice.

Results.

Region of interest has not yet been identified.

To map the high muscle titer phenotype to a region a specific region, chimeric viruses were made by replacing a gene from one strain in the backbone of the other. In this manner, the genes nsp1,

the first half of nsp3, nsp4, E1 and E2 proteins were tested for the ability to confer a change in viral load in the hind limb, compared the the viral load of the parental strain used for the backbone (Table 3.1). The remaining nsp2, second half of nsp3, capsid, 6k and E3 chimeric viruses were not constructed due to difficulties cloning. None of the chimeras tested resulted in a change of viral load in the muscle. However, the negative data are still informative, and set CHIKV apart from other alphaviruses.

Because the E2 protein of CHIKV is responsible for receptor binding upon cells we began by creating chimeric viruses to determine if the E2 could confer the high-titer phenotype. First, a chimeric virus was made by replacing the E2 from 37997 in the backbone of LR2006 OPY1, referred to as 3e2. Our neonatal mouse model was used to screen for successful swaps by checking hind limb muscle titers at 5 dpi, when there is a 1000-fold difference in titer between the strains. For the E2 swap to contain a region of interest, the muscle titers must decrease to levels comparable with 37997, however this was not the case (Fig. 3.1). In case the swap is directional, we also created a chimeric virus (5e2R) that contained the E2 from LR2006 OPY1 in the backbone of 37997 and looked for an increase in hind limb muscle titers. However, this was not seen, indicating that tropism for muscle fibers is controlled by a novel mechanism in CHIKV.

The nsp1 gene has also been implicated in controlling muscle damage in RRV (5). Thus, we made chimeras inserting segments of the nsp1 from LR2006 OPY1 into the backbone of 37997 and screened for an increase in hind limb titers relative to those of 37997 (Fig. 3.2) These strains representing the first half of nsp1 (1n10R) and the second half (1n15R), exhibited an low titer phenotype, similar to the parental strain. These results indicate that the nsp1 is not responsible for the high titer phenotype, unless a swap of the entire nsp1 protein may reveal an increased titer phenotype due to cooperation between residues. The high muscle titer phenotype does not appear to be caused by nsp1 in CHIKV, unlike in RRV.

Virus Ag41855 has an intermediate phenotype

The CHIKV Ag41855 recombinant clone has 23 residue differences between LR2006 OPY1 (7). Thus, if this strain also displayed increased replication in the muscle following i.d. administration it could be a useful tool in narrowing down residues that are responsible for our high titer muscle phenotype. Six day old mice were infected with $5x10^4$ pfu of Ag41855, and hind limb muscle was harvested and titered at various times post infection. Fig 3.3 shows that at 3 dpi, titers of Ag41855 are more similar to those from 37997. However, by 7dpi, the titers observed in the muscle of mice infected with Ag41855 were similar to those observed after infection with LR2006 OPY1. It is important to note that Ag41855 exhibited a small-plaque phenotype in vitro in BHKs, indicating the mutations carried by this viral strain may have an impact on viral fitness and growth kinetics (data not shown). Given this observation, we decided not to pursue further studies with this strain to attempt to identify residue responsible for the increase in muscle damage and replication.

Serial Passage of 37997 has resulted in a more virulent, low muscle titer virus

The next approach we took was to serially passage the 27997 strain in muscle to see if we generated a viral isolate with an increase in muscle replication. We thought this approach may help in identifying residues responsible for the increased tropism for myofibers, since we anticipated that if successful we would have fewer differences between this adapted virus and 37997. 37997 was serially passaged from the hind limb of neonatal pups. Each hind limb was harvested and homogenized, titered by plaque assay and used for intradermal infection for the next passage. The virus underwent four passages, none of which were associated with an increase in hind limb titers (Fig. 3.4). Further, because of the low titers in the muscle, we were not able to give a full dose of $5x10^4$ pfu from the homogenized

muscle alone. However, even at the low titer passages, where pups received between 260 and 1147 pfu mortality was still observed. The final passage was then amplified in BHK cells to a high titer stock and used to infect mice with $5x10^4$ pfu i.d. This was still not associated with an increase in muscle titer, but resulted in 100% lethality, where the majority of mice infected with the parental strain would probably still survive (Fig. 3.5). Thus, the muscle passages may have resulted in an adaptation that increases virulence in mice, without increasing hind limb titer.

Discussion.

During the CHIKV outbreak, the virus has adapted to spread more efficiently as well as to increase disease severity. In particular, the E1 and E2 proteins of alphaviruses have been shown to be responsible for a variety of changes that effect virus behavior. Because the E2 protein of alphaviruses is thought to be responsible for receptor binding, it was a good candidate for our muscle phenotype. Further, the E2 was also found to be responsible for controlling muscle pathology in RRV. A single residue in the E2 protein of RRV, Y18H, was found to control growth in mammalian versus mosquito cells. A strain that expressed a histidine at this position grew to higher titers in mosquito cells, but inflicted little damage in mouse hind limb muscle. When this residue was mutated to a tyrosine, the virus grew less well in mosquito cells, but better in mammalian cells and induced significant hind limb pathology (4). Thus, we swapped the E2 proteins from each of our CHIKV strain, but did not see a change in titers from the parental strains. Thus, it appears there is a different mechanism applicable to CHIKV than RRV.

Although the myofiber phenotype initially appeared to be a matter of entry to the myofiber, in vivo and intramuscular injection revealed that 37997 can also infect myofibers, thus the issue might not be one of only entry within that cell type. Several CHIKV genes have been implicated in immune

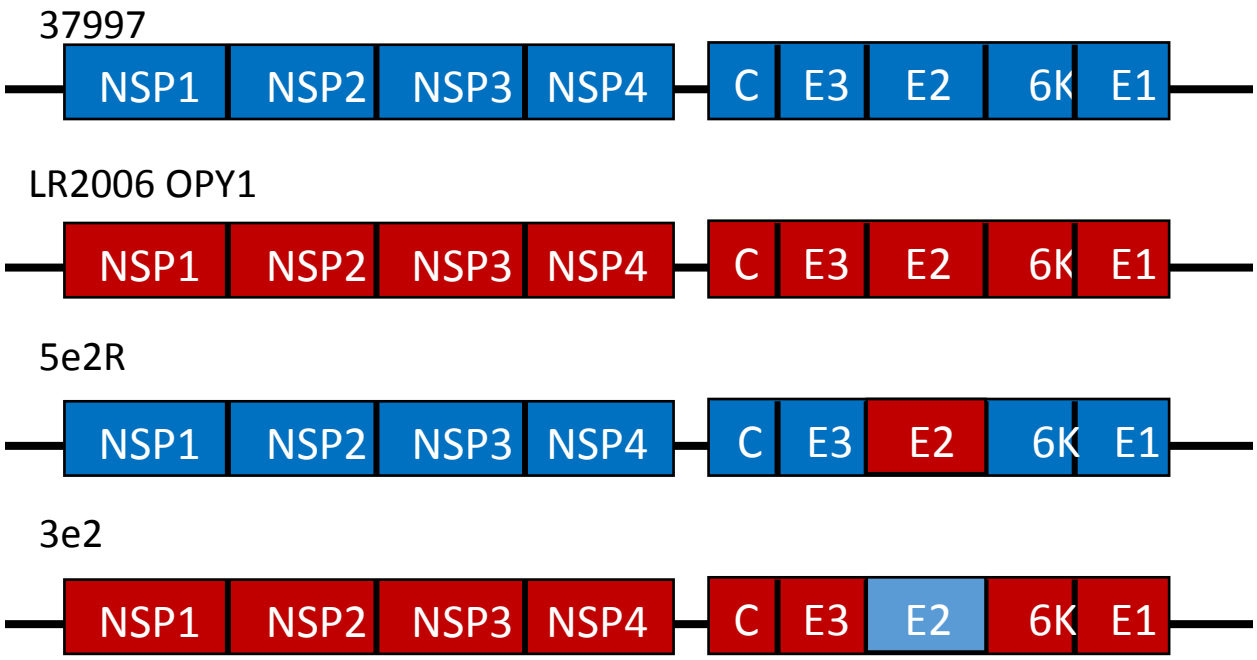
evasion. Subversion of the immune response by LR2006 OPY1, for example, could allow more efficient spread to myofibers without the restriction of the immune response. Recently, the nsp2 protein of Old World alphaviruses was found to mediate ubiquitin-mediated degredation of Rpb1, a subunit of the host RNA Polymerase II (15). Absence of Rpb1 results in loss of transcription of host genes, and the cell is unable to mount an effective anti-viral response. It is unlikely this mechanism is different between 37997 and LR2006 OPY1, as intramuscular titers were similar, suggest that LR2006 OPY1 does not have an advantage in subverting the host response. Due difficulty constructing these strains, the nsp2 chimeras were not tested, but would be worthwhile to test as other, yet undetermined mechanisms may be at play.

Another candidate gene is nsp3, which has hyper-variable regions that have been shown to affect tropism in Venezualan Equine Encephalitis Virus (16). A chimeric virus of the first half of the nsp3 gene did not result in a change in titer from the parental strain, but the second half or the full protein chimera should be tested before this gene can be ruled out. It is also possible that more than one mutation in separate genes is responsible, making the residues responsible difficult to isolate. While this approach has been used to identify viral determinant in other systems it is important to note that the generation of chimeric viruses can result in attenuation of the virus, making the utilization of this approach challenging.

Our next method, the Ag41855 to LR2006 OPY1 comparison did not reveal a difference in viral load that could help us narrow down mutations responsible for our high titer muscle phenotype. It would also be useful to investigate a strain closer the divergence of the West African and East Central and South African Clades of CHIKV to learn more about when the high titer virus has arisen and also other adaptations that may have taken place. Recombinant strains were used for these studies to eliminate the heterogeneity of clinical isolates. Recombinant strains also allow us to create chimeric viruses. Additional strains should be tested for high viral load and myofiber infection as recombinant

clones become available. Further, it would be interesting to look more closely at differences in serum viremia between LR2006 OPY1 and Ag41855, as the prolonged serum viremia seen with LR2006 OPY1 could be independent of the muscle phenotype.

Viruses with an RNA genome, such as CHIKV, are known to have high rates of mutation due to an error-prone RNA-dependent RNA polymerase. Arboviruses, however, are under selective pressure from both their mammalian and insect hosts, and thus mutations that arise must be viable in both hosts in order to flourish. As a result, arboviruses tend to accumulate fewer mutations than their single-host RNA virus counterparts. In our experiments, we used a single host, the neonatal mouse to determine if we could acquire a mutation that would result in a high-titer virus in the muscle. While this was not the case after four passages, it does appear our CHIKV virus has adapted in some way, even at low doses that would not have killed mice if from the parental strain are used. Because there was no pressure to select for high titer viruses in the muscle specifically, it is more likely that our strains have mutated to survive the immune response of the mouse host. Future studies including the sequencing of this viral strain to determine the residue differences that have been accumulated and additional pathogenesis experiments to determine why this new viral strain is more pathogenic will be important future experiments. This information could provide useful information as to immune evasion mechanisms and clearance of the host. A more virulent strain of CHIKV may also allow us to infect older mice in future experiments. Although these studies did not reveal a region responsible for the myofibers phenotype, they do provide promising avenues to further investigate the pathogenesis of CHIKV.


37997
NSP1
NSP2
NSP3
NSP4
C
E3
E2
6K
E1
LR2006 OPY1
NSP1
NSP2
NSP3
NSP4
C
E3
E2
6K
E1
5e2R
NSP1
NSP2
NSP3
NSP4
C
E3
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NSP1
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C
E3
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6K
E1

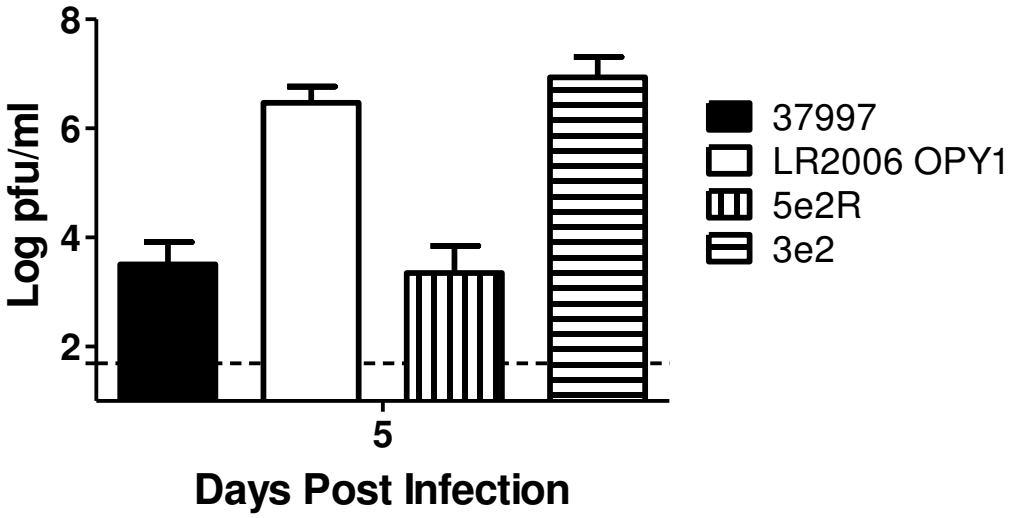
8
6
4
2
Log pfu/ml
5
Days Post Infection
37997
LR2006 OPY1
5e2R
3e2

Figure 3.1 The E2 protein alone is not responsible for high titers in hind limb muscle. Chimeric viruses were constructed by replacing the E2 from one strain in the backbone of the other. 3e2 contains the E2 from 37997 on the backbone of LR2006 OPY1 and 5e2R contains the E2 from LR2006 OPY1 on the backbone of 37997. Six day old pups were infected with 5x104pfu of either strain and hind limb muscle was harvested at 5 dpi and titered by plaque assay. Dashed line indicates the limit of detection. n=3-4. Statistical significance was evaluated with the Mann-Whitney test, groups were not statistically different.

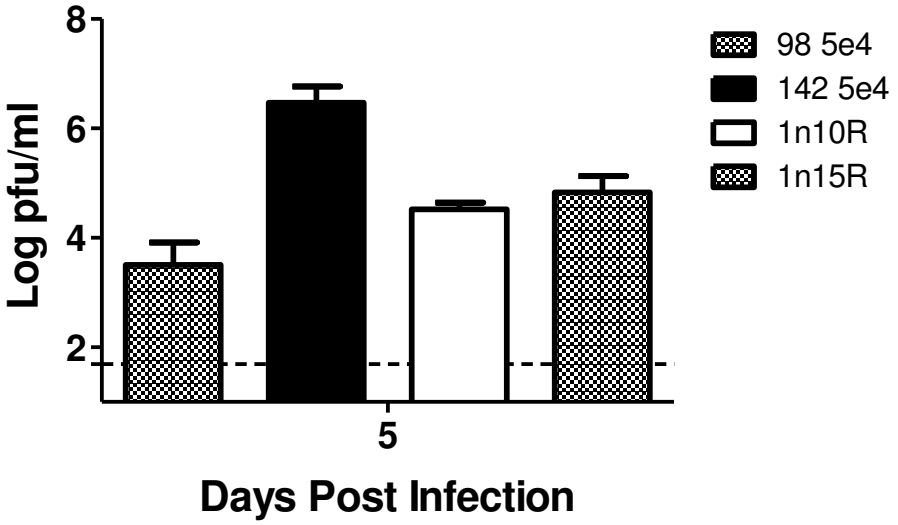

Figure 3.2 Chimeric viruses of the nsp1 may have an intermediate phenotype. Chimeric viruses were constructed swapping either the first half of nsp1 (1n10R) or the second half (1n15R) from LR2006 OPY1 into the backbone of 37997. Six day old pups were infected with 5x104pfu of each strain and hind limb muscle was harvested at 5 dpi and titered by plaque assay. Dashed line indicates the limit of detection. n=3-4. Statistical significance was evaluated with the Mann-Whitney test, groups were not statistically different.

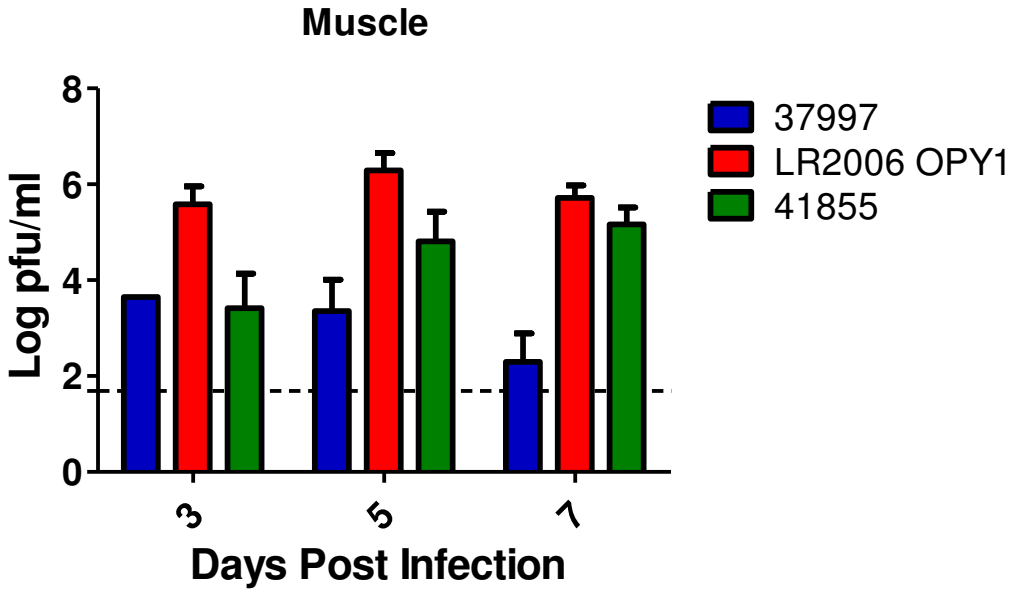

Figure 3.3 CHIKV 41855 an intermediate muscle titer phenotype. Six day old pups were infected with 5x104pfu of either strain and hind limb muscle was harvested at 5 dpi and titered by plaque assay. Dashed line indicates the limit of detection. n=3-4. Statistical significance was evaluated with the Mann-Whitney test, groups were not statistically different.

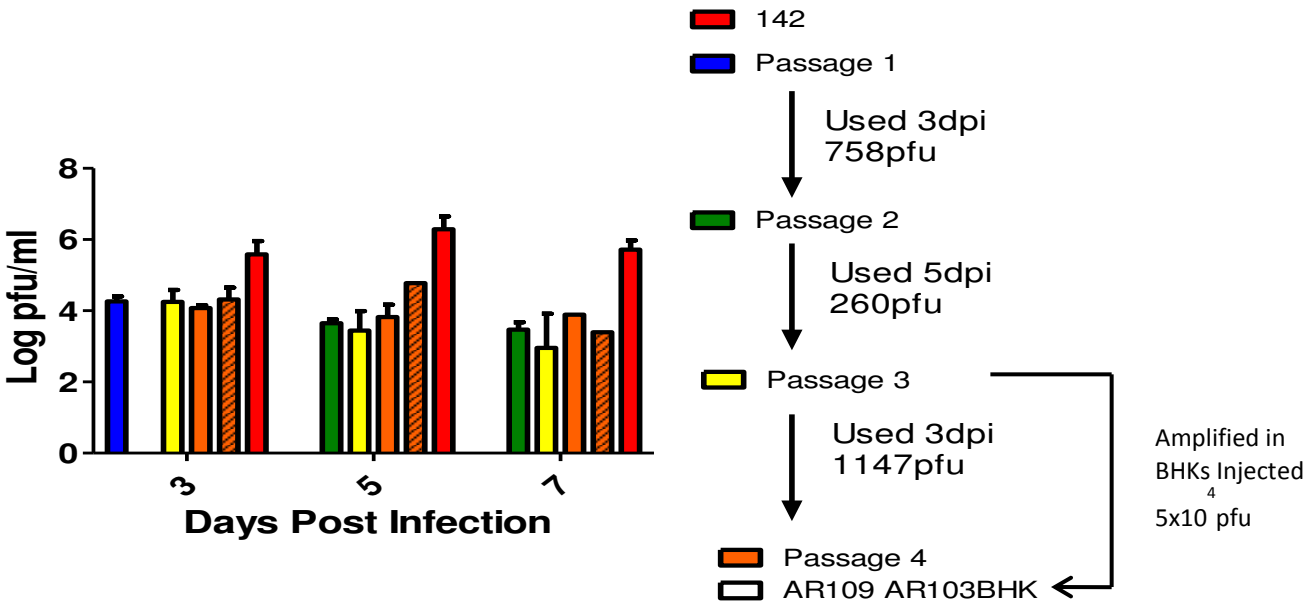

Figure 3.4. Passaging 37997 did not result in higher muscle titers. Mice were infected with 5x104 pfu of the parental strain, 37997. Hind limb was collected at several days post infection, homogenized, and titered. 30uL of the homogenate was used for an intradermal infection for the next passage. After the third passage, a portion of the homogenate was amplified in BHKs before the next passage.

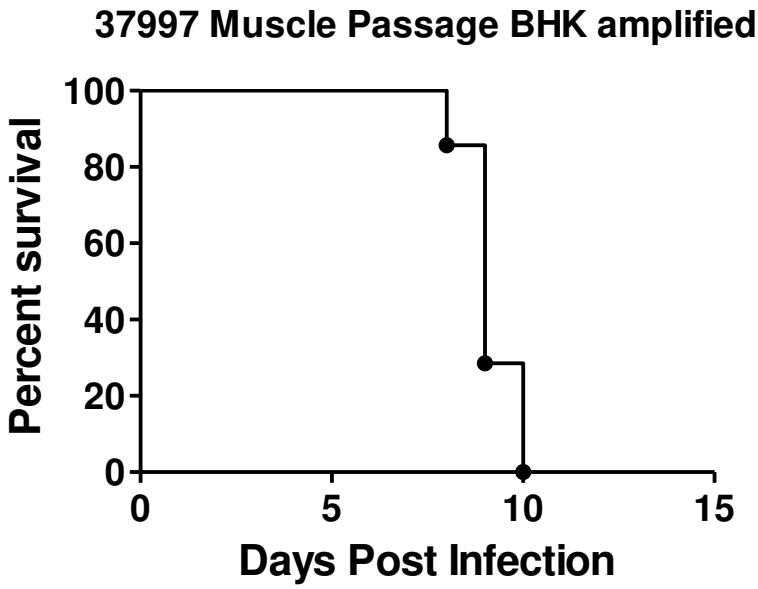

Figure 3.5. After passage 37997 is more virulent than the parental strain. The parental strain was used to infect six-day-old pups with 37997, and the hind limb muscle was collected after three dpi, homogenized and used for intradermal infection of new pups. This was repeated for a total of three passages. After the third passage, the passaged virus was amplified in BHKs and used to infect six day old pups at a dose of $5x10^4$pfu. These pups were monitored for survival. n=7

	Residue Differences	Reverse Clone	Titer	Muscle Titer compared to Parental Strain
E1	26	7e1	$1.82x10^7$	Same
E2	14	5e2R 3e2	$6.14x10^6$ $1.39x10^8$	Same Same
Nsp1 - 1st Half	5	1n10R	$1.80x10^6$	Same
Nsp1 - 2nd Half	6	1n15R 7n15	$4.85x10^6$ $1.19x10^7$	Same NT
Nsp2 - 1st Half	8	13n20	$1.37x10^6$	NT
Nsp2- 2nd Half	13	23n25	$4.83x10^4$	NT
Nsp3 - 1st Half	8	2n30R 7n30	$2.45x10^7$	Same
Nsp3 - 2nd Half	40	NG		
Nsp4 - 1st Half	15	3n40R	$3.95x10^7$	Same
Nsp4 - 2nd Half	11	1n45R	$3.17x10^7$	Same
Capsid/E3	12	NG		

Table 3.1. Chimeric viruses did not reveal a region responsible for the high titer phenotype. Chimeric viruses constructed with the LR2006 OPY1 gene inserted in the 37997 backbone are referred to with an "R" under clone name. The remaining chimeric viruses were also constructed with the 37997 gene inserted in the LR2006 OPY1 backbone. NG = not generated. NT = not tested.

Segment	Forward Primer (5’ → 3’)	Reverse Primer (5’ → 3’)
E2	AATGTCTATAAAGCCACAAGAC	ACGTTGACAGACTCTGAGAA
E1	GCGCGTACGAACACGTAAC	GCCTGCTAAACGACACGCA
Nsp1 – 1st half	GGATCCTGTGTACGTGGAC	GTTGACCCGACTGAGAACAG
Nsp1 – 2nd half	CTGTTCTCAGTCGGGTCAAC	ACGTGGTCTGTTGGTTGGG
Nsp2 – 1st half	CCCAACCAACAGACCACGT	TCCACGATAGTCAATTTGCAG
Nsp2 – 2nd half	CTGCAAATTGACTATCGTGGA	GTTCTTCGCGATGTCCATGC
Nsp3- 1st half	GCATGGACATCGCGAAGAAC	TTATGCTTGTGACATGGTTCAT
Nsp3 – 2nd half	ATGAACCATGTCACAAGCATAA	GAATATGTACCCACCTGCC
Nsp4 – 1st half	GGCAGGTGGGTACATATTC	CCTGCACTTTAGGTCTTTCC
Nsp4 – 2nd half	GGAAAGACCTAAAGTGCAGG	GTGCGTACCTATTTAGGACC
Capsid and E3	GGTCCTAAATAGGTACGCAC	GTCTTGTGGCTTTATAGACATT

Table 3.2 Primers used for creating chimeric viruses

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Chapter 4

Differential roles of interferon kappa and interferon beta in chikungunya virus infection

Introduction

The type I interferon response is a crucial part of the innate immune response to many viral infections, as discussed in Chapter 1. Type I interferons include 16 subtypes in humans, including 12 IFN-α's and a single IFN-β, IFN-ω, IFN-ε and IFN-κ. Although much has been studied about IFN-β and the IFN-α subtypes, little is known about the other subtypes present in humans and mice, IFN-ω, IFN-κ and IFN-ε. Recently, IFN-ε was characterized and was found to have a novel function in protecting the reproductive epithelium from pathogenic challenge (1). Although IFN-ε still appears to induce ISGs like other IFNs, it is unique in that it was found to be constitutively expressed in the female reproductive tract. Additionally, IFN-ε doesn't appear to have STAT or IRF binding sites in its promoter, and instead appears to be regulated by hormones. This data suggests that gaining a better understanding of the regulation and function of previously uncharacterized IFN subytpes may provide important insight into the overall IFN response.

IFN-κ is another IFN subtype that has not been thoroughly characterized. The few studies that have been performed focused on the human system. IFN-κ was found to be expressed primarily from human keratinocytes, and at lower levels in monocytes and dendritic cells (2). In many of these initial studies, IFN-κ behaved similar to other IFNs. For example, IFN-α's are produced after stimulation with IFN-β, and keratinocytes were able to produce IFN-κ after stimulation with IFN-β or IFN-γ. IFNs are also induced during infection, and IFN-κ expression was also reported from kerotinocytes infected with EMCV. Keratinocytes stimulated with IFN-κ were able to produce ISGs such as PKR and STAT1, although a complete evaluation of the genes that were induced downstream of IFN-κ was not performed. Further, IFN-κ expression prevented cell lysis after EMCV infection, suggesting an anti-viral role for IFN-κ. Type I interferons also play an immunomodulatory role, important for maturation of APCs. Dendritic cells stimulated with IFN-κ were able to produce TNFα and IL-10 (3). These data suggest IFN-κ can behave like a typical type I interferon.

IFN-κ has also been linked to several disease processes, but its precise role remains unclear and reports have been contradictory. In the initial reports on IFN-κ, monocytes and dendritic cells expressing IFN-κ were detected in biopsies of lesions from patients with psoriasis and atopic dermatitis, but not in healthy skin (3). However, another group reported decreased expression of IFN-κ in patients with psoriasis and atopic dermatitis, but they were able to detect increased IFN-κ levels in biopsy samples from patients afflicted with lichen-planus and contact dermatitis (4). Downregulation of IFN-κ, as well as other ISGs, was seen in keratinocytes infected with HPV (5). Finally, certain SNPs in the IFN-κ gene were found to be associated with high levels of type I IFNs in the serum of lupus patients (6).

Type I interferons are also important for the control of Chikungunya Virus, an emerging arbovirus that causes fever, rash and polyarthralgia in infected patients. Mouse models are being used to investigate the pathogenesis of CHIKV. IFNAR$^{-/-}$ mice, which lack the type I IFN receptor and therefore cannot respond to type I IFNs, are highly susceptible to CHIKV, with the mice developing high viral loads in many tissues and 100% mortality seen within 2-3 days post (7). High levels of IFNs have been observed in both CHIKV-infected humans and mice, likely as a result of viral detection by multiple pathogen recognition receptors (8, 9). While type I IFNs clearly play a critical role during CHIKV pathogenesis, the roles of individual IFN subtypes during CHIKV infection have not been characterized. Since CHIKV is transmitted by inoculation into the skin following mosquitoe bite and infection often causes rash it is possible that disease pathogenesis may be regulated by IFN-κ. This study characterizes both the roles of IFN-β and IFN-κ during CHIKV infection. As expected, IFN-β$^{-/-}$ mice were more susceptible to CHIKV infection than WT, but not nearly as susceptible as IFNAR$^{-/-}$ mice. IFN-β$^{-/-}$ mice displayed increased viral titers in several organs as compared to WT mice with a dramatic increase noted in the brains of mice at the end of the infection. Surprisingly, IFN-κ$^{-/-}$ mice were resistant to CHIKV infection as compared to WT mice. Despite the difference in mortality, titers between the two strains were similar in multiple organs throughout the course of the infection. Both WT and IFN-κ$^{-/-}$ mice

induced similar expression of IFN-β during infection. IFN-$\kappa^{-/-}$ mice appeared to have greater infiltrates into the hind limb muscle, which could facilitate survival of these mice, despite causing greater muscle pathology. Recombinant tools are being developed to further characterize the functions of IFN-κ.

Methods and Materials.

Mice. Mice were maintained at Washington University School of Medicine in accordance with all federal and University guidelines. For mouse studies the principles of good laboratory animal care were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the NIH. The protocols were approved by the Animal Studies Committee at Washington University. All efforts were made to minimize suffering. IFN-κ-/- and were generated by EuComm by the method of BAC recombineering. IFN-β-/-, IFN-βxIFN-$\kappa^{-/-}$ and IFN-κ-/- were provided courtesy of Michael S. Diamond, C57BL/6 and IFNα/β Receptor 1 (IFNAR$^{-/-}$) IFN-β-/-, IFN-βxIFN-$\kappa^{-/-}$ and IFN-κ-/- mice on the C57BL/6 background were bred and maintained in our mouse colony. For neonatal experiments, mice were infected at 6 days of age. Litters were weight matched at the initiation of the experiments.

Virus Production. The construction of recombinant plasmid for the LR2006 OPY1, 37997 has been previously described (10, 11). Recombinant viral stocks were generated from viral cDNA clones by *in vitro* transcription of linearized cDNA templates followed by RNA transfection with Lipofectamine 2000 (Invitrogen) into baby hamster kidney-21 cells (BHK cells) as previously described (12). After 48 hours, the supernatant was collected, centrifuged, aliquoted and stored at -80°C. Viral stocks were titered by plaque assay as previously described (8). All work with live virus was performed in a Biosafety Level 3 facility and followed strict guidelines established by the Environmental Health and Safety committee at Washington University School of Medicine.

Viral Studies. Six to nine day old pups were infected with 1×10^5 pfu of recombinant CHIKV clone LR2006 OPY1 or VEEV TC83 diluted in 30 µl of PBS and injected intradermally (i.d.) to mimic a mosquito bite, using a Hamilton syringe and 30G needle, into the left upper pectoral area. For survival experiments pups were monitored daily for clinical signs of disease (including ruffling of fur, altered gait, and hind limb paralysis) and lethality for 21 days post-infection. For viral titers the mice were infected as described above and the various tissues (injection site, serum, muscle, brain) were harvested into PBS and stored at -80° C. Injection site refers to the skin and underlying connective tissue and fat at the site of infection. Serum samples were pooled when collected with each sample containing serum from two mice from a single experiment. For muscle titers, the hind limb ipsilateral to the site of infection was skinned and the hamstring was collected. For the analysis of viral loads the samples were homogenized with 100 µl of 1.0 mm-diameter zirconia-silica beads using a MagnaLyzer (Roche) by alternating two pulses of 7000 rpm for 30 seconds each, with chilling on ice for 30s. The homogenates were serially diluted and titers were determined by plaque assay on BHK cells. For serum collection, pups were bled at the designated time post-infection into BD Serum Separator tubes. These were spun for 9 minutes at 12,000 × g then frozen at -80°C until they were titered.

Histopathology. Six day old pups were infected with 1×10^5 pfu i.d. of either CHIKV recombinant clone or mock infected with PBS i.d. in the left upper pectoral area. Mice were sacrificed at the indicated day post-infection the hind limb and brain were fixed with 10% formalin for 48 hrs at RT, then rinsed with PBS for 15 min and stored in 70% ethanol at 4°C until submitted for embedding in paraffin blocks and sectioning. The sections were stained with hematoxylin and eosin to examine for histopathology. Embedding, sectioning, and staining were performed by the Anatomic and Molecular Pathology Histology Core at Washington University in St Louis.

rtPCR. Mice were infected with 1×10^5pfu i.d. Tissues were excised and flash frozen in liquid nitrogen. Tissues were homogenized using the Magnalyser (Roche) and RNA was extracted using the RNeasy

Fibrous Tissue Kit (Qiagen). RNA was used to run real-time PCR on the infected tissues, normalizing to GAPDH (IDT). *IFNk* and *IFNb1* gene expression assay primers and probes were provided by Helen Lazear.

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

Results.

Poly(I:C) treatment results in greater induction of Ifnb1 transcript than Ifnk in neonatal mice.

To characterize basal levels of *Ifnk* as well as induction after treatment with an RNA analog known to induce type I interferons, six day old WT pups were given 30ug of poly(I:C) intraperitoneally or left untreated, and various tissues were harvested for RNA after 18hours (Fig 4.1). The highest levels of basal expression of *Ifnk* was seen in the liver, thymus, small intestine, large intestine and stomach. The greatest increase in *Ifnk* expression after poly(I:C) was seen in the liver and thymus of 9.0-fold and 8.5-fold, respectively. The remaining organs did not show an increase in *Ifnk* levels from untreated mice. In contrast, levels of IFN-β were higher at baseline, and increases in expression with poly(I:C) treatment were seen in all organs tested. These results suggest that the regulation of IFN-κ may be different from that of IFN-β.

IFN-β and IFN-κ play distinct roles during CHIKV infection.

To first characterize the role of individual IFN subtypes on CHIKV pathogenesis we infected 6 day old mice (WT, IFNAR-/-, IFN-β-/- and IFN-κ-/- mice) with $1x10^5$ pfu of the CHIKV strain LR2006 OPY1

and monitored mice for lethality (Fig 4.2). IFNAR$^{-/-}$ mice displayed a dramatic increase in susceptibility to CHIKV infection and succumbed to infection by 3 days post infection, as previously described (7). We found that while IFN-β$^{-/-}$ mice survived for several days longer than IFNAR$^{-/-}$, they also display increased susceptibility to infection with nearly 100% lethality seen by 12 day pi. This was in constrast to WT mice, which died between days 10-15 pi. with an overall lethality of 74%. Therefore as expected IFN-β plays a significant role as an antiviral IFN during this response but does not account for the entire type I interferon response during CHIKV infection.

To determine if IFN-κ also played an antiviral role we also infected IFN-κ$^{-/-}$ mice with CHIKV. Surprisingly, we found that IFN-κ$^{-/-}$ mice had only 13% mortality following CHIKV infection, and the few mice that succumbed to infection did so around 10 dpi (Fig 4.2). This data is supported by unpublished work by Helen Lazear, in which she observed a similar protective effect in mice infected with Influenza A/PR8. In these experiments IFN-κ$^{-/-}$ mice were resistant to influenza A induced lethality as compared to WT mice. Since the deletion of IFN-β and IFN-κ gave the opposite results we next wanted to ascertain which phenotype was dominant. IFN-βxIFN-κ$^{-/-}$ mice were bred and then infected with CHIKV as described above. The IFN-βxIFN-κ$^{-/-}$ mice mirrored the survival of IFN-β$^{-/-}$ mice alone (Fig 4.2), suggesting that while the lack of IFN-κ can help mice survive CHIKV infections, it is not enough to overcome the deleterious effect of the loss of IFN-β. It is important to note that IFN-κ$^{-/-}$ mice were not protected against all viruses tested. When mice were infected with Venezualan Equine Encephalitis Virus or West Nile Virus, IFN-κ$^{-/-}$ displayed similar survival to WT mice (Fig 4.3 and unpublished work by H. Lazear). These viruses differ from CHIKV and Influenza A by their ability to infect neurons, suggesting that the tropism of the virus may affect whether IFN-κ has an impact on pathogenesis.

IFN-β$^{-/-}$ and IFN-βxIFN-κ$^{-/-}$ have increased viral loads during infection, particularly in the brain

IFNAR-/- mice are thought to die from rampant viral growth (7). To see whether this was the case in our mice lacking IFN subtypes we analyzed the serum, injection site, muscle and brain for viral burden. Viral loads of IFN-$\beta^{-/-}$ and IFN-βxIFN-$\kappa^{-/-}$ mice were similar in the serum, and persist while WT mice clear virus from the serum (Fig. 4.4). IFN-$\beta^{-/-}$ and IFN-βxIFN-$\kappa^{-/-}$ mice also die before they can clear the serum viremia. A similar pattern is seen in the injection site, titers of all IFN-$\beta^{-/-}$ and IFN-βxIFN-$\kappa^{-/-}$ mice start high, but are slowly cleared throughout the course of the acute infection, but at a slower rate than WT mice. IFN-$\beta^{-/-}$ and IFN-βxIFN-$\kappa^{-/-}$ mice also have very high titers in the hind limb muscle prior to death. In the brain, IFN-$\beta^{-/-}$ and IFN-βxIFN-$\kappa^{-/-}$ mice initially have low titers, but these increase at 5 dpi and 7dpi, indicating these mice may be dying because of their inability to control the virus in the brain. A similar phenotype was seen when IFN-$\beta^{-/-}$ mice were infected with West Nile Virus, where WT mice infected with CHIKV are able to control replication of WNV but IFN-$\beta^{-/-}$ mice cannot (13). This data shows that IFN-$\beta^{-/-}$ is particularly important in controlling CHIKV access and replication in the brain.

WT and IFN-$\kappa^{-/-}$ have similar viral loads, despite differences in lethality

A possible reason for survival of IFN-$\kappa^{-/-}$ is decreased fitness of the virus in the host. If CHIKV is not able to replicate as well in mice that lack IFN-κ, there may be less damage that could lead to greater survival. Thus, we were interested in looking at viral loads in these mice. In the serum, injection site, hind limb muscle and brain, WT and IFN-$\kappa^{-/-}$ had similar viral loads (Fig 4.4). In the serum and injection site, both WT and IFN-$\kappa^{-/-}$ mice have high titers early in infection that decreases with time, until levels are near the limit of detection at 7 dpi. In the hind limb muscle, both WT and IFN-$\kappa^{-/-}$ mice have very high viral loads, that peak at 5 dpi and then decrease at 9 and 12 dpi in surviving mice. Although it appears IFN-$\kappa^{-/-}$ mice may clear virus faster in the hind limb muscle, this difference is not significant. WT and IFN-$\kappa^{-/-}$ mice have limited growth in the brain, and reach peak titers at 5 dpi, and then decrease until virus

can no longer be detected in surviving mice. These data suggest that the survival of IFN-κ$^{-/-}$ mice is not mediated by differences in viral load compared to WT mice.

IFN-κ$^{-/-}$ *and WT mice induce similar ifnb1 expression during CHIKV infection*

IFN-κ$^{-/-}$ mice could also be surviving by decreasing the competition for the IFNAR. If IFN-κ is normally present and occupies the IFNAR preventing the more potent beta from binding, the removal of IFN-κ would result in an increase in IFNAR signaling induced by IFN-β or other IFN subtypes, and this new anti-viral state could result in less death in IFN-κ$^{-/-}$ mice. This would also result in increased IFN-β expression, as IFN-β is produced in a positive feedback loop after binding to IFNAR. At baseline we found that adult IFN-κ$^{-/-}$ mice had similar levels of *Ifnb1* and *Ifna* subtypes compared to WT mice (Lazear, unpublished data). Next, we looked at *Ifnb1* production in WT and IFN-κ$^{-/-}$ mice in both the injection site and hind limb muscle (Fig. 4.5). In the injection site at 1 dpi, when viral loads are high, we observed high expression of *Ifnb1*transcripts which decreased as the viral load decreased at 7 dpi. We observed no difference in the transcript levels induced between WT and IFN-κ$^{-/-}$ mice. The hind limb muscle appears to have a similar phenotype, with low viral load and consequently low *Ifnb1* transcript at 1dpi and high viral load and higher transcript levels detected at 7 dpi. However, as in the injection site, there was no difference in *Ifnb1*transcript levels between WT and IFN-κ$^{-/-}$ mice. These data, along with similar viral loads that we observed in WT and IFN-κ$^{-/-}$ mice, suggest that the loss of IFN-κ does not result in increased binding and or signaling of IFN-β.

Next, we looked at induction of *Ifnk* transcripts during infection in WT mice, as previous reports were able to see induction after EMCV infection. In the injection site, we observed high levels of *Ifnk* in mock-infected mice, however, these levels did not appear to change after CHIKV infection at either 1 or 7 dpi (Fig. 4.6). In the hind limb muscle, *Ifnk* levels are low in mock-infected mice and CHIKV-infected

mice at 1 dpi. At 7 dpi, there appears to be a slight increase in *Ifnk* transcript. Whether this increase is enough to affect the course of CHIKV infection is unclear. Further, the constitutive expression of IFN-κ may be more important than IFN-κ that is induced late in infection, when mice are close to death.

IFN-$\kappa^{-/-}$ *mice may have more infiltrates in hind limb muscle*

Since we did not observed a difference in the viral loads in the IFN-$\kappa^{-/-}$ mice as compared to WT mice we next looked at the hind limb histology of mice after infection to determine if there was a difference in cellular infiltrates. Interestingly, while muscle from WT and IFN-$\kappa^{-/-}$ looked similar early in infection, later during the infectious course, the IFN-$\kappa^{-/-}$ mice appeared to have more infiltrates into the hind limb (Fig. 4.7). WT and IFN-$\kappa^{-/-}$ mice both had areas of tissue infiltrates with muscle fiber fragmentation and edema visible with a predominantly mononuclear infiltrate. In addition, IFN-$\kappa^{-/-}$ mice had areas of dense infiltrates that were more pervasive than in WT. A further characterization of the cell types that are infiltrating and their functionality is needed.

Development of recombinant tools

Given the unexpected observation the mice lacking IFN-κ are protected from viral induced lethality in some viral models a further characterization of the biological properties of the IFN subtype is warranted. Very few reagents are currently available. To generate these reagents we first developed recombinant IFN-κ that contains a 3xFLAG tag, 6xHis tag and a tev cleavage site for removal of tags. Transient transfection of this construct into 293T cells resulted in the production of protein that reacts with both an anti-FLAG Ab as well as to a commercially available anti- IFN-κ antibody (Fig. 4.8). To

determine if these proteins were functional, 293T cells were transfected with constructs for IFN-β, IFN-κ or MULT1. After 48h, supernatants from these transfections were used to stimulate MEFs, which were then lysed and blotted for STAT1 phosphorylation, which is known to occur downstream of IFNAR engagement. Commercially purchased IFN-β, recombinant IFN-β and IFN-κ were able to cause STAT1 phosphorylation in WT MEFs, but not IFNAR$^{-/-}$ MEFs (Fig 4.9) showing that the protein is functional and can only activate STAT1 through the IFNAR receptor. Supernatant containing MULT1 did not result in STAT1 phosphorylation. The IFN-κ plasmid and protein is also being used to develop neutralizing antibodies in IFN-κ$^{-/-}$ mice. These reagents will be crucial for the further analysis of IFN-κ function.

Discussion.

IFN-κ appears to have a novel function during CHIKV infection, with the lack of IFN-κ resulting in the unexpected survival of CHIKV-infected mice. However, this effect was not confined to CHIKV, as protection from lethality was also seen in IFN-κ$^{-/-}$ mice infected with influenza A virus. Although these viruses function differently and have drastically different tropism, they are united in their ability to cause immunopathology. For influenza A, pathology is not caused by the virus inducing cell death, but rather thought to be due to an exuberant host response within those animals. This also appears to be a possible mechanism for CHIKV-associated pathology. When WT mice are treated with bindarit, an inhibitor of MCP-1, they no longer see the profound infiltrates that are evident in untreated mice (14). Viral titers are the same with or without treatment, indicating that the muscle damage is due to the infiltrating cells (14).

Contradictory to their resistance, IFN-κ$^{-/-}$ mice actually appeared to have more damage and more infiltrates in their hind limb muscle. However, the nature of these infiltrates is not well understood, while they appear to be mainly monocytes, macrophages and neutrophils, their function

could be impaired by the loss of IFN-κ. Furthermore, a recent study found that suppressive macrophages delay clearance of CHIKV (15). The specific profile of infiltrates may provide clues as to the mechanism of IFN-κ. A preliminary analysis of cytokines and ISGs in the hind limb muscle by RT-PCR has demonstrated no differences between IFN-$\kappa^{-/-}$ and WT mice, but additional time points need to be analyzed and the serum levels of cytokines and chemokines have not yet been measured.

There are several possible mechanisms to explain the increased resistance to CHIKV in IFN-$\kappa^{-/-}$ mice. First, IFN-κ could function as a dominant negative that occupies IFNAR to prevent IFN-β binding. Thus, in the absence of IFN-κ, we would expect to see increased signaling through the IFNAR, and greater levels of *Ifnb1* transcript in IFN-$\kappa^{-/-}$ mice, which is not the case (Fig 4.5). Furthermore, if this were true, we would also expect the increased IFN-β signaling to result in a heightened anti-viral state, and lower viral load in IFN-$\kappa^{-/-}$ mice versus WT mice, which is not true (Fig 4.4). A second hypothesis is that IFN-κ differs in its expression and regulation from IFN-β, as is the case with IFN-ε. However, analysis of the promoter elements of IFN-κ show that it does appear to have IRF and STAT binding sites, and should be able to be induced like IFN-β and IFN-α.

A final hypothesis is that IFN-κ binds to the receptor at a different affinity, and thus can induce different genes. The evidence for this hypothesis in the literature is limited. A variety of IFNs bind to the same receptor complex, but are able to produce different biological effects. Some of this is cell-type dependent. For example, IFNAR binding typically results in STAT1 and STAT2 phosphorylation, but can also activate all of the other STATs depending on the cell type (16). The specificities of each of these transcription factors may differ, allowing for a difference in gene induction. Several studies have reported differential gene induction by different type I IFNs in the same cell type. The gene β-R1 was reported to be induced differentially by IFN-β, and not by IFN-α (17). However, when cells were treated with 250x greater concentration of IFN-α2, expression of the gene was detected. Thus, the affinity of each IFN appears to regulate the level of the response to IFN, but may not actually differ in gene

induction within a certain cell type. Differential action of IFN-α were also reported with T cell motility (18). IFN-α2 was able to stimulate T-cell migration, but the higher-affinity IFN-α8 was not. At the doses used, IFN-α2 was able to induce IRF7 expression, but IFN-α8 was not, even though both IFNs were able to induce the ISG MxA. The mechanism behind the differing actions of type I interferons is not yet known.

A recent study compared the affinities of IFN subtypes to their ability to induce the anti-proliferative and anti-viral responses that IFNs are known for (19). The varying affinities affected the rate of IFNAR downregulation from the cell surface, which can affect signaling and thus the degree of gene expression. Each subtype contacts the receptor in the same points, but it is the nature of the contacts that determine the overall affinity for the receptor. Early work suggests that IFN-κ probably has much lower affinity than IFN-β, and this will be analyzed more closely by our collaborators.

To compare the gene expression of IFN-κ and IFN-β, microarrays will be performed on cell types relevant to CHIKV and influenza A infection, such as muscle connective tissue fibroblasts as well as tracheal epithelial cells. We would expect the same concentration of IFN-κ and IFN-β to induce similar genes, with IFN-β expressing at higher levels due to increased affinity for the receptor. It may be necessary to treat cells with high levels of IFN-κ to max out the response, so as not to confuse the lower induction of genes with IFN-κ with the lack of induction. It has also been shown that stimulation of IFN-β results in downstream activation of accessory signaling pathways such as p38, PI3K and NF-kB (Rani 2005). The differing affinity of IFN-κ may lead to differing activation of these accessory pathways. IFN-κ is a type I interferon with a novel function during viral function. Further investigation into the mechanism by which it is protective could lead to new therapeutics for viral infections.

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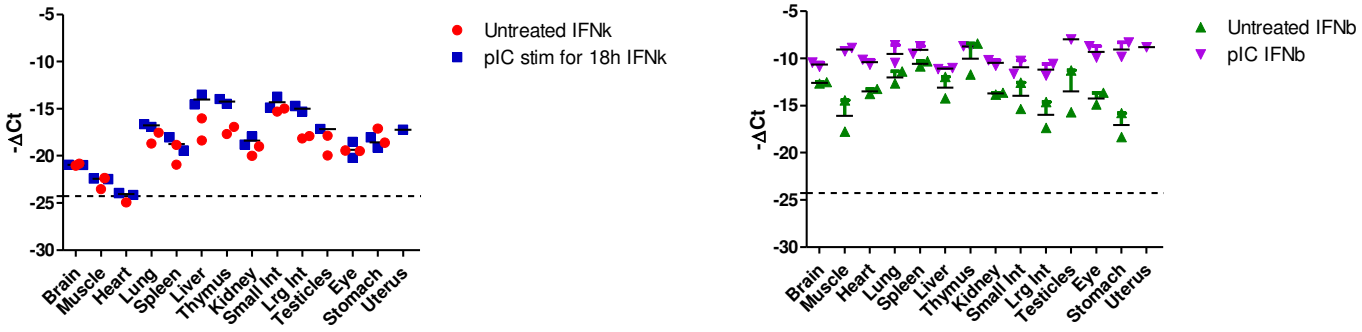

Figure 4.1. *Ifnk* RNA levels are minimally induced after treatment with poly (I:C). Six day old pups were given 30ug of poly(I:C) intraperitoneally or left untreated. Tissues were harvested 18h later and flash frozen until RNA extraction. rtPCR was perfomed using primers/probes for *Ifnk* and *IFNb1*, and normalized to GAPDH. The dashed line represents the limit of detection.

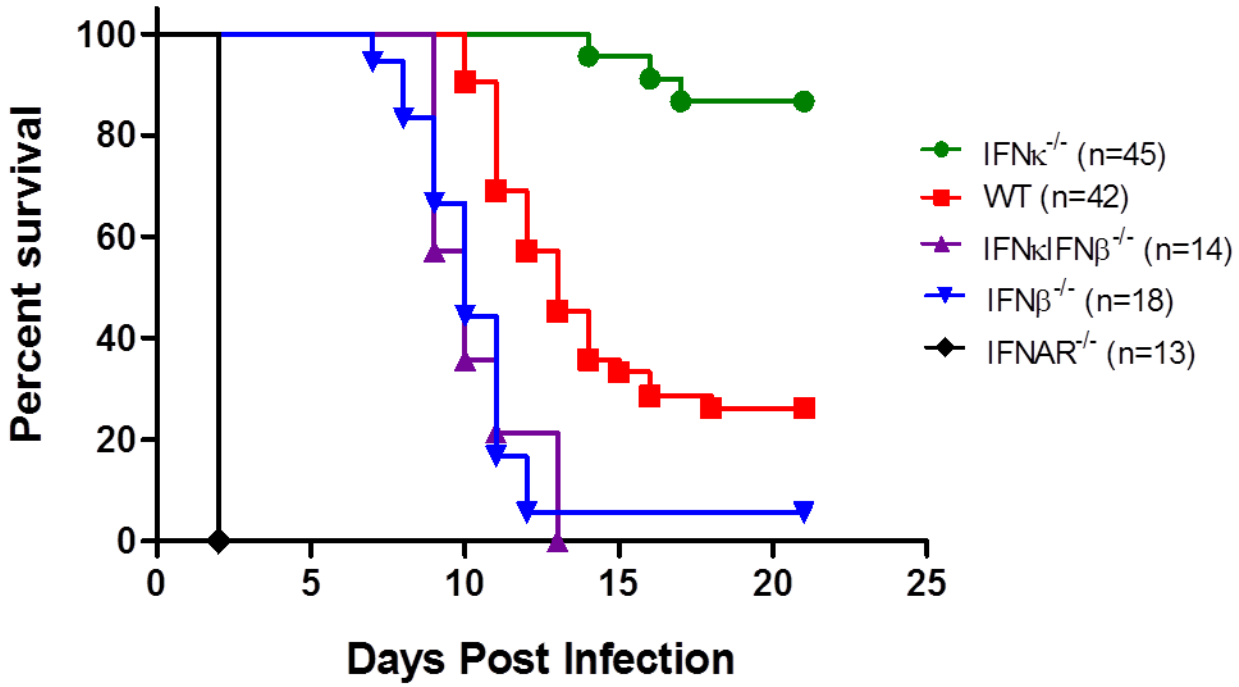

Figure 4.2. IFN-κ$^{-/-}$ mice are more resistant to CHIKV-induced lethality than WT mice. Six-day-old pups of each genotype were infected with 1x10^5 pfu of LR2006 OPY1 and were followed for lethality for 21 days.

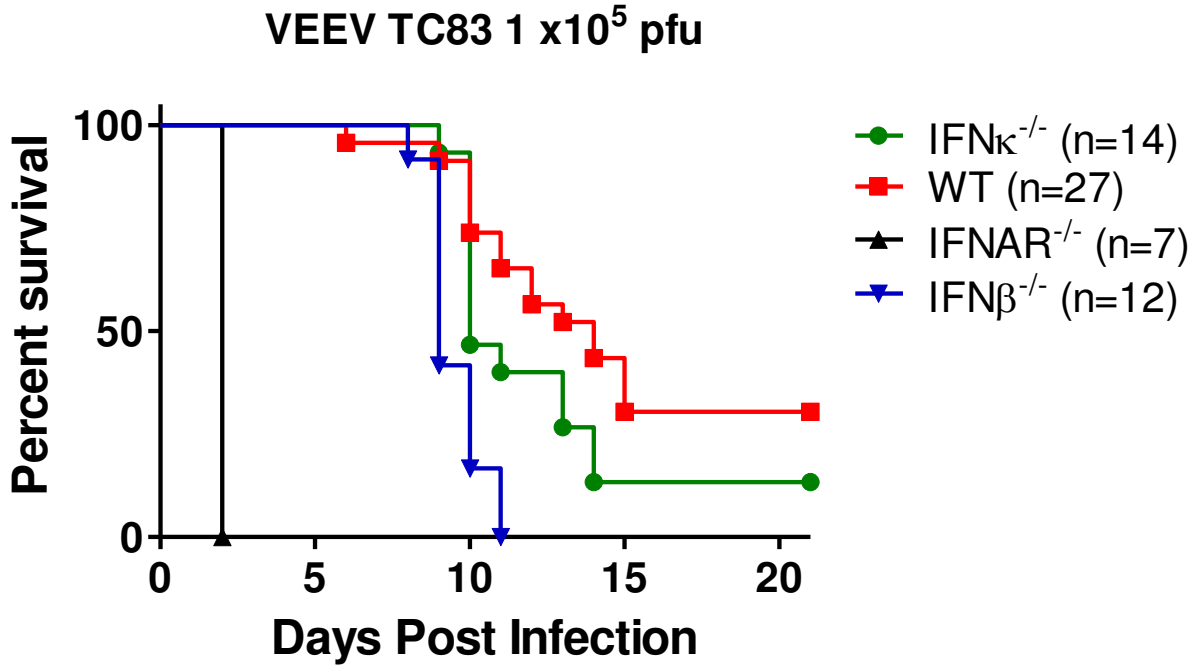

Figure 4.3. WT and IFN-κ$^{-/-}$ mice have similar lethality to VEEV infection. Nine day-old pups of the indicated genotype were infected with 1x10^5 pfu of VEEV TC83 and monitored for survival for 21 days.

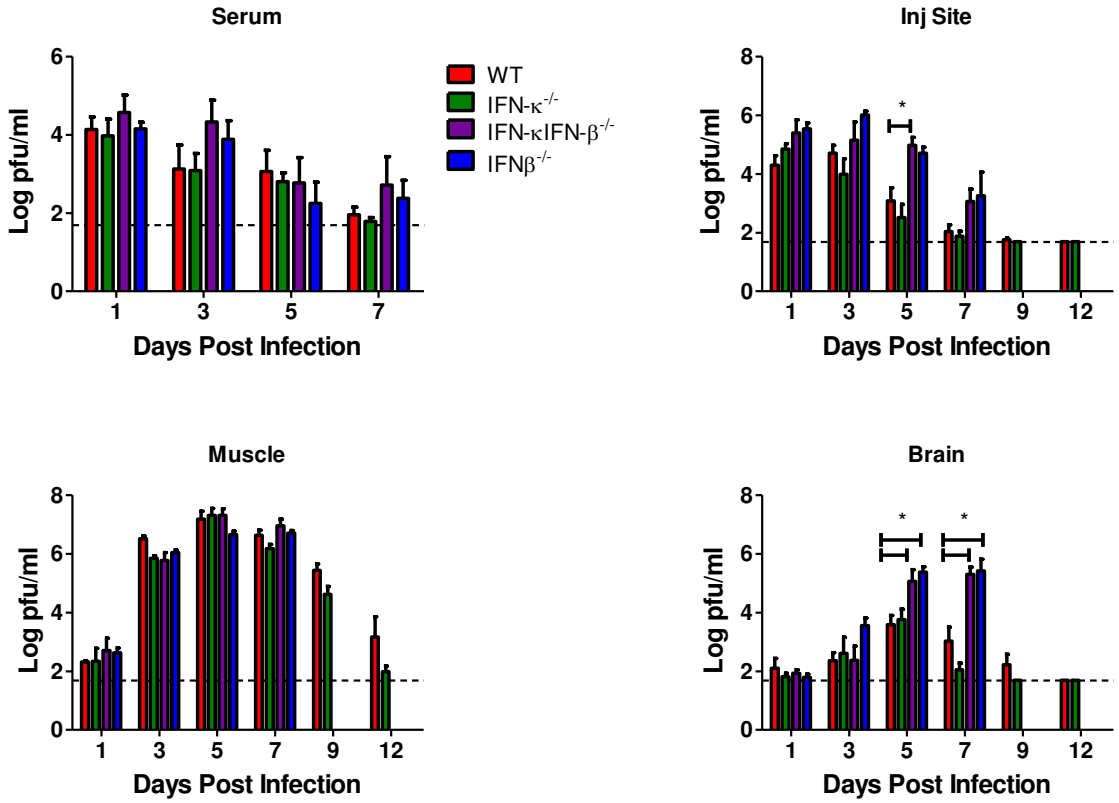

Figure 4.4. WT and IFN-κ$^{-/-}$ mice have similar viral loads. Six day old pups of each genotype were infected with 1x10^5 pfu of CHIKV LR2006 OPY1. Tissues were harvested at the indicated days post infection and titered by plaque assay. 8 mice were titered per genotype per time point. n=3-7. Statistical significance was evaluated with the Mann-Whitney test. * p = 0.01-0.05

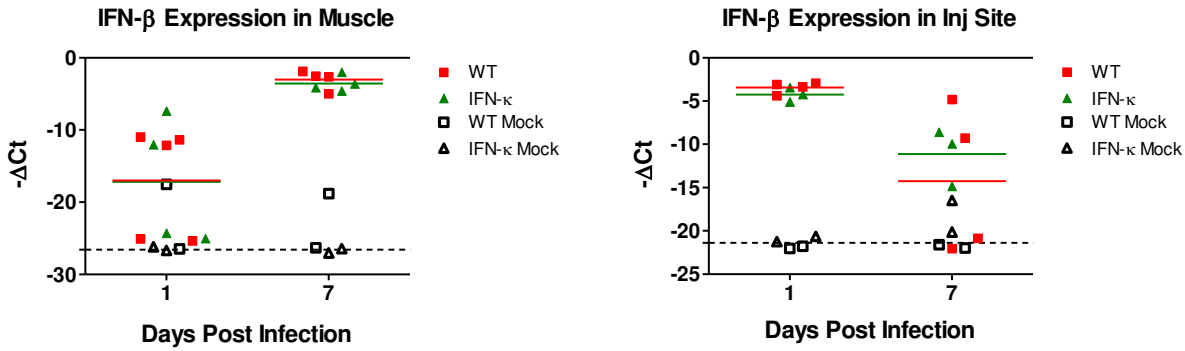

Figure 4.5. WT and IFN-κ$^{-/-}$ mice have similar *Ifnb1* expression during CHIKV infection. Mice were infected with 1x10^5 pfu of CHIKV or 30uL of PBS and tissues were harvested and flash frozen until RNA extraction. rtPCR was performed and *Ifnb1*transcript levels were normalized to GAPDH. The dashed line represents the limit of detection. n=3-5 for CHIKV-infected samples, n=2 for mock samples

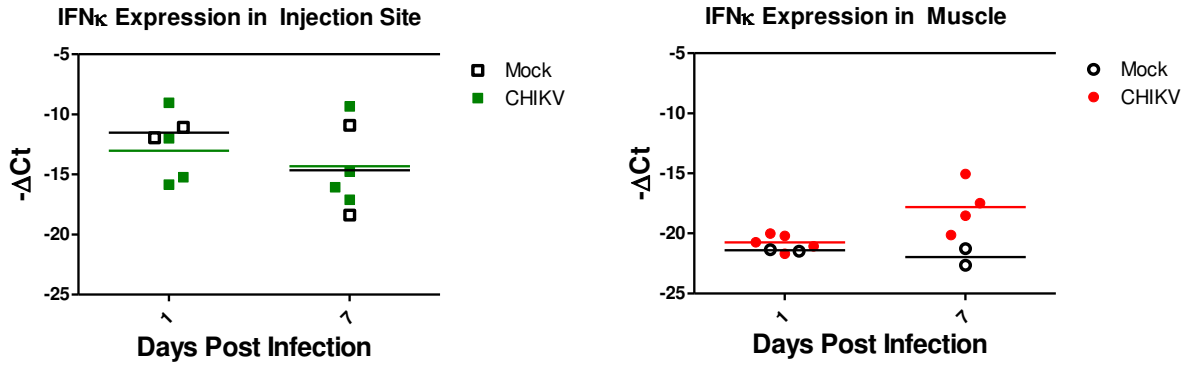

Figure 4.6. *Ifnk* transcripts are slightly increased in the muscle after infection. Mice were infected with $1x10^5$ pfu of CHIKV or 30uL of PBS and tissues were harvested and flash frozen until RNA extraction. rtPCR was performed and *Ifnk* transcript levels were normalized to GAPDH. The dashed line represents the limit of detection.

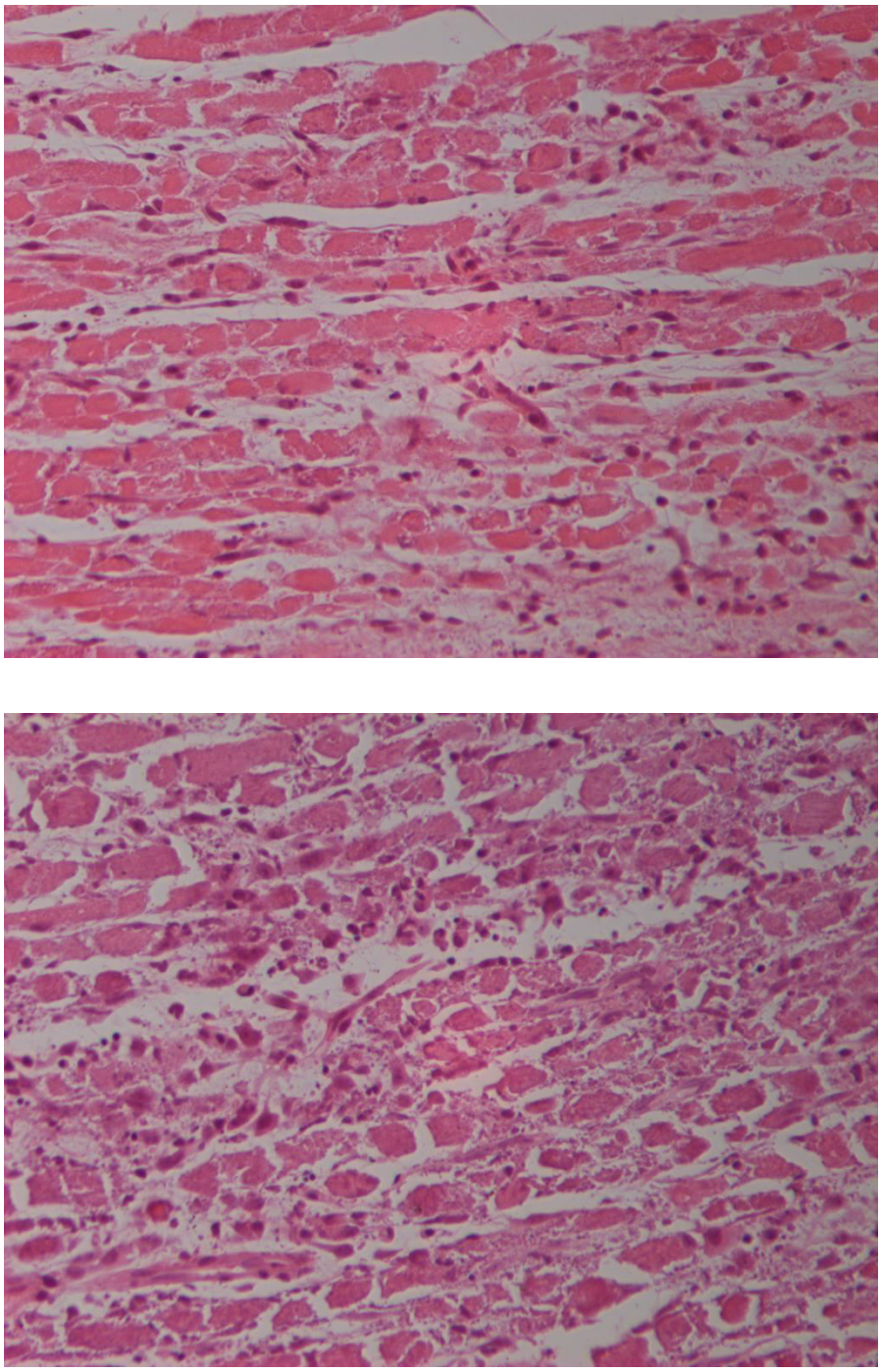

Figure 4.7. IFN-κ$^{-/-}$ mice appear to have an increase in cellular infiltrates after CHIKV infection. Mice were infected with 1x10^{5} pfu of CHIKV, and their hind limb was harvested at 9 days post infection and formalin-fixed for 48h. Samples were submitted to the Developmental Biology Histology Core for paraffin embedding, sectioning and H&E staining. Photos are representative of four mice per genotype.

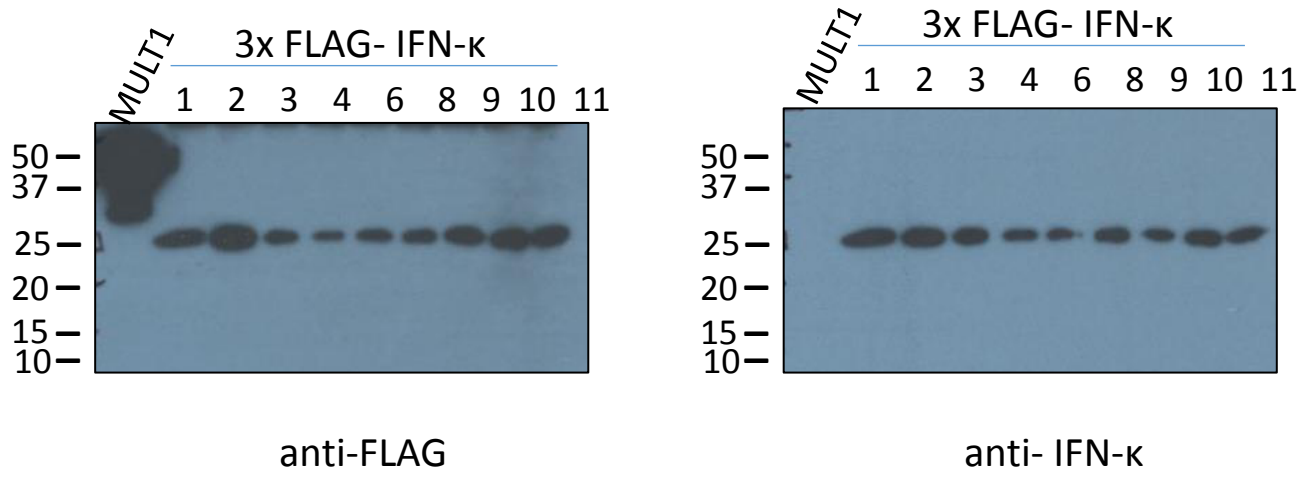

Figure 4.8. Recombinant IFN-κ protein reacts with anti-FLAG and anti- IFN-κ antibodies by Western.

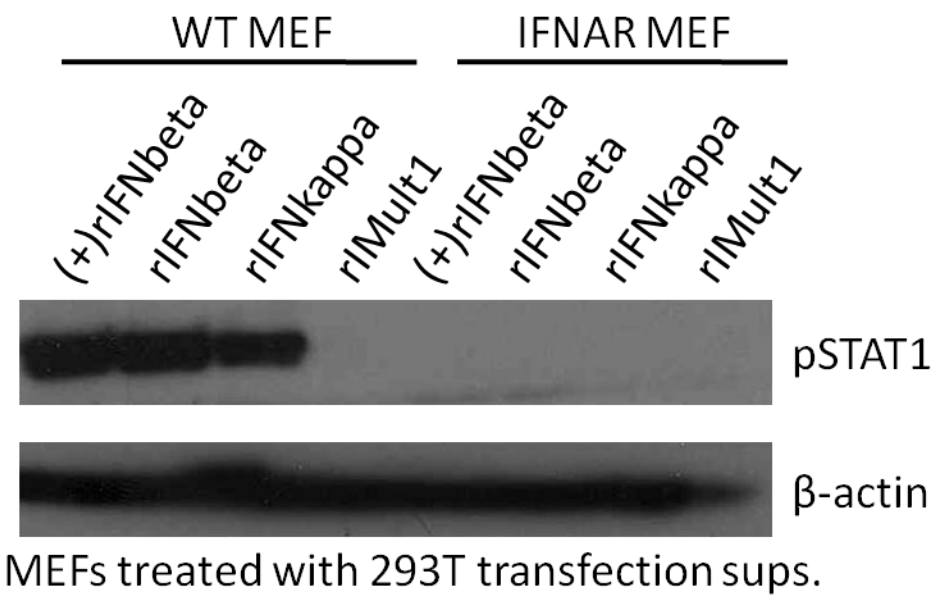

Figure 4.9. Recombinant IFN-κ stimulates STAT1 phosphorylation in WT MEFs in a IFNAR1 dependent manner. 293T cells were transfected with contructs for IFN-β, IFN-κ or MULT1. After 48h, supernatants from these cultures, plus commercially available IFN-β were used to treat WT or IFNAR-/- MEFs for 30 minutes, after which the MEFs were lysed and prepared for Westerns. Samples were blotted for pSTAT1 as well as β-actin. This figure was done by Lindsey Cook.

Chapter 5

Summary and Future Directions

I. Summary

This work explores both viral and innate immune factors that affect the severity of CHIKV disease in mice. Chikungunya Virus began receiving attention recently, because of an epidemic that began in Kenya in 2004, and spread throughout the Indian Ocean area to India and Southeast Asia, ultimately afflicting millions (1). A typical course of infection involves high fever and incapacitating arthritis. However, during the most recent outbreak, there were increased reports of severe symptoms, including encephalitis and hepatitis. For the first time, mortality due to CHIKV infection was also observed, particularly in the elderly with co-morbid conditions (2). Because previous outbreaks had been infrequent and of limited scope, there has been limited attention to CHIKV. More work must be done to investigate the pathogenesis of CHIKV to better be able to design effective therapies and vaccines.

The apparent increase in disease severity of CHIKV infection likely has a multitude of causes. First, this outbreak was the first in a Westernized medical system. This may have allowed for improved observations and record keeping to document CHIKV induced symptoms and clinical findings. Next, this appears to be the largest epidemic recorded, and thus a wider variety of symptoms of CHIKV may be observed. Further, a genetic change in CHIKV E1 A226V resulted in more effective transmission by a new mosquito vector, Ae. albopictus, which helped increase spread throughout Africa and Southeast Asia (3). This vector is also present in Europe and North America, potentially exposing naive populations to CHIKV infection. Already this vector has been attributed to an outbreak in Italy in 205 people(4). Thus, CHIKV appears to be genetically adapting for the newest outbreak. Thus, it is also possible that genetic changes may also have contributed to the increase in disease severity that is currently being observed.

To test this, we compared the pathogenesis of two strains, 37997 and LR2006 OPY1. 37997 was isolated from a mosquito colony in Senegal in 1983 and is a part of West African clade. LR2006 OPY1 was isolated in 2006 from a febrile patient infected on La Reunion and represents a member of the East Central and South African (ECSA) clade. First, we showed that both strains grow similarly in mammalian cells, and respond similarly to interferon pre-treatment. Next, we infected neonatal mice with either strain and observed similar survival, though there was a clinical difference in mice infected with LR2006 OPY1. These mice developed hind limb weakness, but those infected with 37997 did not. Upon further investigation we found the LR2006 OPY1 mice had higher viral loads in the hind limb muscle and prolonged viremia in the serum. GFP-expressing viruses were used to examine differences in tropism. While both strains were able to infect connective tissue fibroblasts within the muscle as previously reported, only LR2006 OPY1 was able to infect myofibers as well when given intradermally. Myofibers can be seen as early as 3 dpi, when the great difference in titer is seen. Interestingly, *in vitro*, both strains replicate similarly in several cell types in the muscle, including connective tissue fibroblasts, myoblasts and even myofibers. This indicates that both strains are able to infect these cells, but only LR2006 OPY1 is able to access the myofibers niche *in vivo*. So, next we did the intramuscular infection, where muscle was injected directly into the hind limb and there was GFP+ cells observed in both. Thus, there seems to be an issue of access to the myofibers in vivo. Several methods to identify the mutations responsible for this phenotype were undertaken, but these approached did not allow us to identify the responsible mutations.

Next, we looked at the host response to CHIKV, and the roles of different type I interferon subtypes in CHIKV infection. Interferons are known to be crucial for the clearance of CHIKV, and mice lacking the ability to respond to IFN were highly susceptible (5). As expected, mice lacking IFN-β were more sensitive to CHIKV than WT mice, however we observed that IFN-$\kappa^{-/-}$ mice were more resistant. This unexpected finding was also seen in infections with influenza A. IFN-$\beta^{-/-}$ mice had higher viral loads

as we would expect, but IFN-$\kappa^{-/-}$ mice instead of having lower viral loads, had similar levels as WT. Further IFN-$\kappa^{-/-}$ mice were able to induce similar levels of IFN-β as WT mice. We did notice increased infiltrates and muscle destruction in the hind limbs of IFN-$\kappa^{-/-}$ mice, which appears contradictory to the increased survival of these mice. Recombinant protein and neutralizing antibodies are being developed to further investigate the function of IFN-κ.

II. Future Directions

Myofiber infection

Previous studies of CHIKV tropism showed muscle connective tissue fibroblast and occasional myoblast infection, but not myofiber infection. The viral adaptations that allow LR2006 OPY1 to infect myofibers is not yet known, despite our efforts. First, progress should be made to complete the mapping of the increased disease severity with LR2006 OPY1 and 37997. Because there has been some difficulty with the construction of chimeric viruses by the technique used, a new approach may need to be developed. This could involve testing of additional CHIKV strains from before and during the current epidemic for high titers and myofibers infection. An alignment of multiple strains with variable genetic make-up could result in trends common to those strains that do infect myofibers, versus those that do not. Individual residue substitutions could then be made in those strains that do not infect myofibers to test whether this property can be conferred. However, few CHIKV strains are available as recombinant clones and are instead clinical isolates that contain heterogeneous populations of viruses. As recombinant clones become available, they should be tested for myofibers infection.

Additionally, little is known about myofiber biology. Some work has been done with the mdx mouse model of muscular dystrophy, to investigate the properties of myofibers that lead to necrosis in muscular dystrophy patients. Myofibers in mdx mice were able to express chemokines such as MCP-1

and RANTES that attract macrophages, while myofibers in healthy mice did not (6) (7). Interestingly, many of these chemokines are also produced in CHIKV, and were observed to be upregulated in the hind limb muscle during CHIKV infection. Mdx mice treated with TNFα blocking antibodies were able to prevent myofibers necrosis, which may be a potential therapeutic in severe cases of CHIKV (8). Further work to look at the response of the myofibers to CHIKV infection is important to learning about CHIKV pathogenesis. Though outside of scope of this work, elucidating a receptor for CHIKV is also necessary to further field of CHIKV pathogenesis.

The mechanism by which LR2006 OPY1 is able to infect myofibers is yet unknown. It is possible that LR2006 OPY1 is able to evade the interferon response and spread unrestricted to myofibers, while 37997 is not. However, this is not supported by the intramuscular injection data, when both strains are able to infect myofibers. In models of gene therapy, the basal lamina of myofibers was found to restrict entry of HSV-1 (9). Damage to this barrier allowed for myofibers infection, and it is possible that LR2006 OPY1 has developed a mechanism to disrupt this barrier. To test whether there is increased damage to myofibers in mice infected with LR2006 OPY1 or 37997, we can use Evans Blue Dye, which is absorbed by damaged cells. We would expect to see increased uptake early in infection in mice infected with LR2006 OPY1. Evans Blue Dye absorption is an established method used to assess alphavirus-induced muscle damage (10).

Muscle Passage

Despite failure to elicit a high muscle titer virus, the passage of muscle in the hind limb resulted in a more virulent version of 37997. This passaged strain is able to cause lethality in pups even at very low doses. The passaged strain should be characterized like LR2006 OPY1 and 37997 to evaluate for changes in pathogenesis. First, the fitness should be evaluated by growth curves in vitro, as well as viral

loads in vivo. Changes in viral loads from the parental strain, perhaps in the CNS, could contribute to increased lethality. Further, the host response to the passaged strain should be evaluated, in terms of interferon, cytokine and chemokine production. Next, the passaged strain should be purified and sequenced, and a recombinant strain should be made from the resulting sequenced virus to be used in further studies of CHIKV, perhaps also in adult mice where additional tools are available. This virus has not yet been tested in mice older than six days of age, and additional passages may be needed before adult mice are susceptible.

Like human newborns, neonatal mice are highly susceptible to infection by CHIKV. However, while human adults are still susceptible to the actions of CHIKV, adult mice are not for undetermined reasons. Many differences in the neonatal adaptive immune response versus the adult have been documented, such as bias towards Th-2 responses and fewer numbers of T-cells (11). Little work has been done to characterize the innate immune responses of neonates. The responses of PRRs to stimuli on neonatal cells have been investigated, but results have been inconsistent between studies and stimuli (12). The susceptibility to CHIKV changes rapidly in neonatal mice, as six-day-old pups have significant mortality, while 12 day-old pups have none. Thus, a comparison of six and 12 day-old pups in terms of response to viral infection would provide useful information about the maturation process of neonates. Important areas to consider are viral spread, cytokine production and immunopathology. These cells can potentially be cultured to further test responses of specific cell type and stimuli, though there is a risk of losing the neonatal behavior of the cells in vitro. According to UNICEF's 2009 report on maternal and newborn health, 4 million children under the age of six months die every year due to infections, this is an important area of study with great potential.

Function of IFN-κ

Our experiments in IFN-κ-/- mice reveals a novel role for IFN-κ in CHIKV and influenza A infection. However, the data do not provide a clear mechanism by which these mice are surviving CHIKV infection, as they have similar viral loads as WT mice and greater muscle destruction. Thus, looking at IFN-κ's function in a more global sense could provide some insight as to mechanism. First, reagents are being developed to facilitate studies. Recombinant IFN-κ has been developed and is being optimized for greater production. Neutralizing antibodies are also being made in IFN-κ-/- mice. These antibodies will be useful to confirm our in vivo results by blocking IFN-κ in WT mice. These can also be useful in staining tissue sections to look for IFN-κ localization. Although we see high expression of IFN-κ in the skin of WT mice, staining would be useful to confirm that keratinocytes are the source, as in humans. Further, if there is an antibody that could also be used in intracellular staining by flow, IFN-κ expression in immune cell populations, such as dendritic cells and monocytes could also be easily studied. The localization and basal expression of IFN-κ could provide important information about the function of IFN-κ, as localization was the key to understanding IFN-ε. It would also be useful to investigate IFN-κ in the immune infiltrates in hind limb muscle.

The most likely hypothesis is that the affinity of IFN-κ for IFNAR results in differential gene induction compared to IFN-β. To test this, microarray studies should also be performed, comparing IFN-κ gene expression to that of IFN-β. Cell types that are important for CHIKV or influenza A infection should be arrayed first, such as muscle connective tissue fibroblasts and tracheal epithelial cells. Because there is limited evidence of different genes being induced by different subtypes of IFN that all bind to the same receptor, there is a risk of microarrays revealing various degrees of expression of the same genes when comparing IFN-κ to IFN-β. However, there may still be a second unknown receptor that IFN-κ can bind in addition to IFNAR. It is also possible that the accessory pathways and differences in affinity between IFN-κ and IFN-β do lead to differences in gene expression that could help us explain our CHIKV

model. Should differences in gene induction result, a detailed analysis of IFN-κ binding to IFNAR as well as downstream signaling should be performed.

This work provides a detailed analysis of viral and host factors that affect disease severity. Although there is much progress to be made in the field of CHIKV pathogenesis, this work aids in the understanding of CHIKV biology. Future studies investigating the mechanism of myofibers infection and IFN-κ function may be helpful in the ultimate goal of designing therapeutics to treat Chikungunya virus.

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