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Characterization of the role of the innate immune system in the pathogenesis of chikungunya virus in mice

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Characterization of the role of the innate immune system in the pathogenesis of chikungunya virus infection in mice

by

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Dedication

Dedicated to my wonderful family, including my mother, Connie, my grandparents, Joyce and Ernie, my husband, Jason, and my three wonderful children, Gabrielle, Daniel and Nathan. Their endless love and support have made me a better person.

In memory of Stephen Carper, Ph.D., my first mentor, who taught me how to be a scientist. He was an amazing man and his wisdom and humor inspire me in many aspects of my life. I would not have achieved this without his belief in my abilities.

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Characterization of the role of the innate immune system in the pathogenesis of chikungunya virus in mice

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Chikungunya virus (CHIKV) is an alphavirus which causes a febrile illness in people that can induce a persistent and recurrent arthralgia. Recent outbreaks of CHIKV in tropical regions have caused millions of people debilitating illness. With no specific vaccine or anti-viral treatment, the development of an animal model and a better understanding of CHIKV pathogenesis is very important. The focus of my work was to develop a small animal model of CHIKV infection and to aid in elucidating the role of the immune system in CHIKV pathogenesis. Infecting young outbred mice resulted in an acute illness with severe myositis and inflammation in the skeletal muscle. This was used as a small animal model that aided in the study of the pathogenesis and the immune response of infected animals. By using GFP and luciferase bearing clones of CHIKV, it was found that the virus replicated in the lymph nodes and skeletal muscle of the mice. It was also shown that mosquito saliva, lower virus dose and footpad inoculation all cause decreased amounts of myositis as compared to needle inoculation in the skin of the back. Lastly, it was shown that certain immunosuppressants can

decrease the disease severity of CHIKV infection in mice, while decreasing interferon increases disease severity and mortality in mice. Dexamethasone was studied as a possible treatment option for CHIKV infection and was found to cause a decrease in the disease severity in CHIKV-infected mice. Overall, this work has shown that the immune system plays a complex role during CHIKV infection in mice, both clearing the virus and causing disease related pathology. Interferon is important in controlling viral replication, but macrophages and neutrophils may be key mediators of CHIKV-induced myositis.

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List of Abbreviations

Ae	*Aedes*
ALT	alanine transaminase
ALP	alkaline phosphatase
ABSL-3	animal biosafety level 3
ACL-3	arthropod containment level 3
AST	aspartate transaminase
BSL-3	biosafety level 3
CO_2	carbon dioxide
CHIKF	chikungunya fever
CHIKV	chikungunya virus
CTL	cytotoxic T-lymphocytes
dpi	days post infection
DTH	delayed type hyper-immune reaction
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
EEEV	eastern equine encephalitis virus
GETV	Getah virus
GFP	green fluorescent protein
Hg	*Haemagogus*
HI	hemagglutination inhibition
H&E	hematoxylin and eosin
hpi	hours post infection
HIAF	hyper-immune ascitic fluid

Ig	immuneglobulin
IVIS	*in vivo* imaging system
ic	infectious clone
IACUC	Institute for Animal Care and Use Committee
ICR	Institute for Cancer Research
INF	interferon
IL	interleukin
IP	intra-peritoneal
IT	intra-thoracic
LUC	luciferase
LCMV	lymphocytic choriomeningitis virus
MAYV	Mayaro virus
µL	microliter
MIDV	Middleburg virus
mL	milliliter
mM	millimolar
nm	nanometers
nM	nanomolar
NIAID	National Institute for Allergies and Infectious Diseases
NK	natural killer cells
nsP	non-structural protein
ONNV	O'nyong nyong virus
PFU	plaque forming units
PCR	polymerase chain reaction
RNA	ribonucleic acid

RRV	Ross River virus
SFV	Semliki Forest virus
SINDV	Sindbis virus
SC	subcutaneous
$TCID_{50}$	tissue culture infectious dose: 50%
TNF-α	tumor necrosis factor- alpha
UNV	Una virus
U.S.	United States
UTMB	University of Texas Medical Branch
VEEV	Venezuelan equine encephalitis virus
WEEV	Western equine encephalitis virus

Chapter 1: Introduction

Objectives

Because of the threat to global health from chikungunya virus (CHIKV) and other arthralgic alphaviruses, understanding the pathogenesis of these viruses is important to aid in development of specific therapeutics and effective vaccine candidates. Since their pathogenesis appears to be linked to the cells of the immune system, this has implications in anti-viral and vaccine development. CHIKV infection causes a febrile illness in people called “chikungunya fever” (CHIKF). The objective of my work was to utilize a mouse model of CHIKV infection to study the role of macrophages in the pathogenesis of CHIKF. Four aspects of CHIKV pathogenesis were studied including: 1) development of a small animal model of CHIKF; 2) identify the sites of viral replication and persistence in mice; 3) elucidate the role of mosquito bite inoculation in CHIKV infection in mice and 4) evaluate immune suppression as a treatment strategy for CHIKV infection.

Alphaviruses

Genomics and proteomics of the genus Alphavirus

Viruses in the family *Togaviridae* are classified into two distinct genera, *Alphavirus* and *Rubivirus* (Kuhn 2007). The genus *Alphavirus* includes approximately 30-40 viruses in seven different serogroups (Kuhn 2007, Luers et al. 2005). The type member of the genus *Alphavirus* is Sindbis virus (SINDV), which is included in the Western Equine Encephalitis antigenic complex. Alphaviruses infect mammals, birds and fish and are found worldwide including

Antarctica. Most are transmitted by arthropod vectors, with the few exceptions being those that infect fish (Kuhn 2007). The mosquito vectors that these viruses utilize are diverse and include medically important species such as *Aedes aegypti* and *Aedes albopictus*. Some alphaviruses have been included on the NIAID list of priority pathogens because of their biothreat potential, their ability to produce high titers of virus and their threat to human health (NIAID et al. 2011).

Alphaviruses are enveloped viruses that have a positive sense, single stranded genome that is approximately 11kb in length (Ryman et al. 2008). They are spherical with icosahedral symmetry (Griffin 2007). The *Alphavirus* genome has a 7-methylguanasine on the 5'end and is polyadenalated on the 3'end (Kuhn 2007). The genome has two open reading frames which contain the genes for the non-structural proteins on the 5' end and structural proteins on the 3' end (Figure 1). There are four non-structural proteins (nsP1, 2, 3 and 4) that are the machinery needed for RNA replication and processing of the structural genes. These proteins also encode viral enzymes needed for post translational processing. The five structural proteins (capsid, E1, E2, E3 and 6K) are responsible for receptor binding, translocation, membrane fusion and association with the viral RNA.

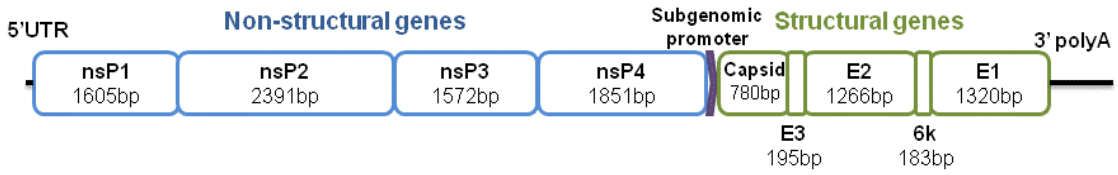


Figure 1: CHIKV genome map

Alphaviruses enter into the host cell by attachment to a receptor by the E2 protein and viral/host cell membrane fusion by the E1 protein (Sourisseau et al. 2007). Preliminary work with SINDV has suggested that heparin sulfate may be a cell surface receptor which is widely expressed on vertebrate cells (Byrnes et al. 1998, Kuno et al. 2005). Once fusion has occurred, genome replication can take place in the cytoplasm. The positive strand RNA genome is used directly as mRNA for the translation and synthesis of the non-structural proteins (Figure 2).

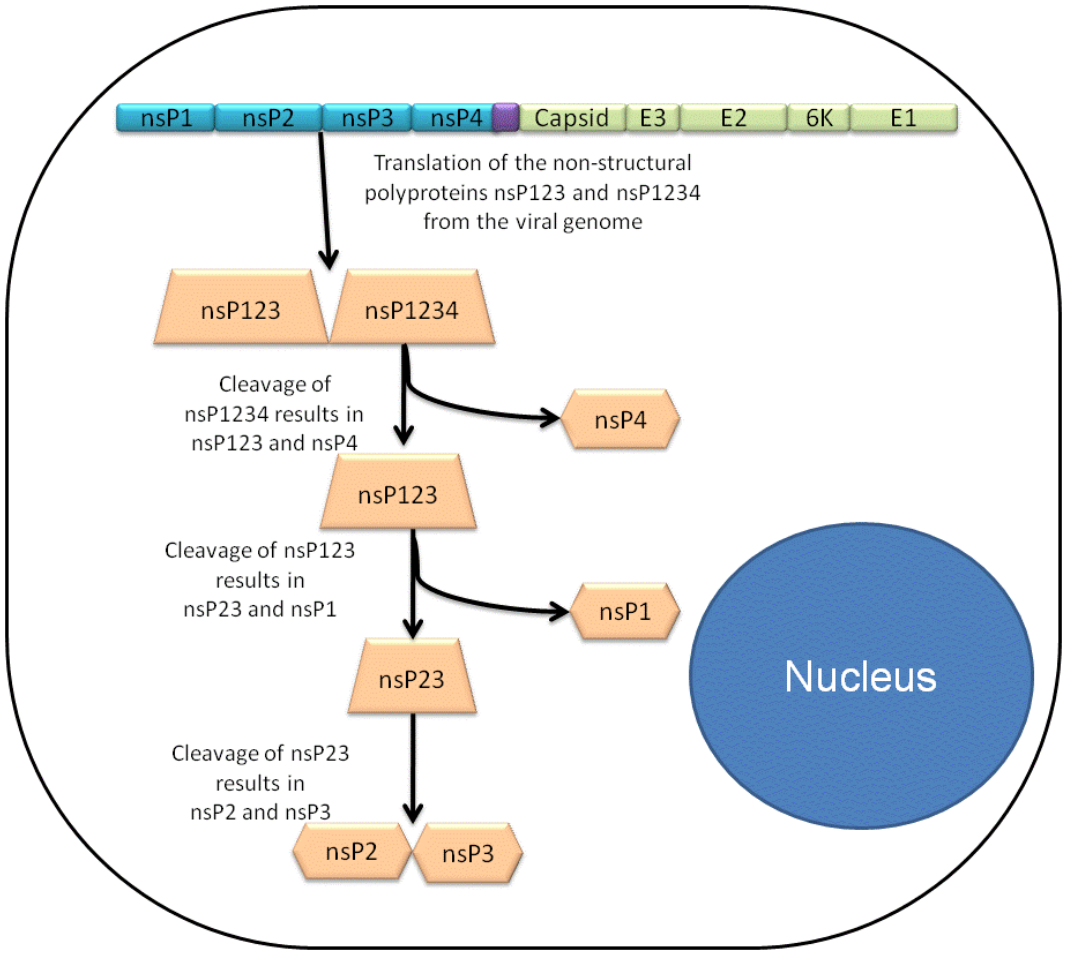


Figure 2: Non-structural protein synthesis of alphaviruses

These proteins are first synthesized as a single polyprotein. Two polyproteins are produced, nsP123 and nsP1234, with nsp1234 made in much smaller concentrations. The polyproteins are cleaved by proteinase activity on the nsP2 protein with nsP4 being cleaved first. The protein complex, nsP123-nsP4 begins the synthesis of the negative RNA strand. Next, nsP1 is cleaved from the

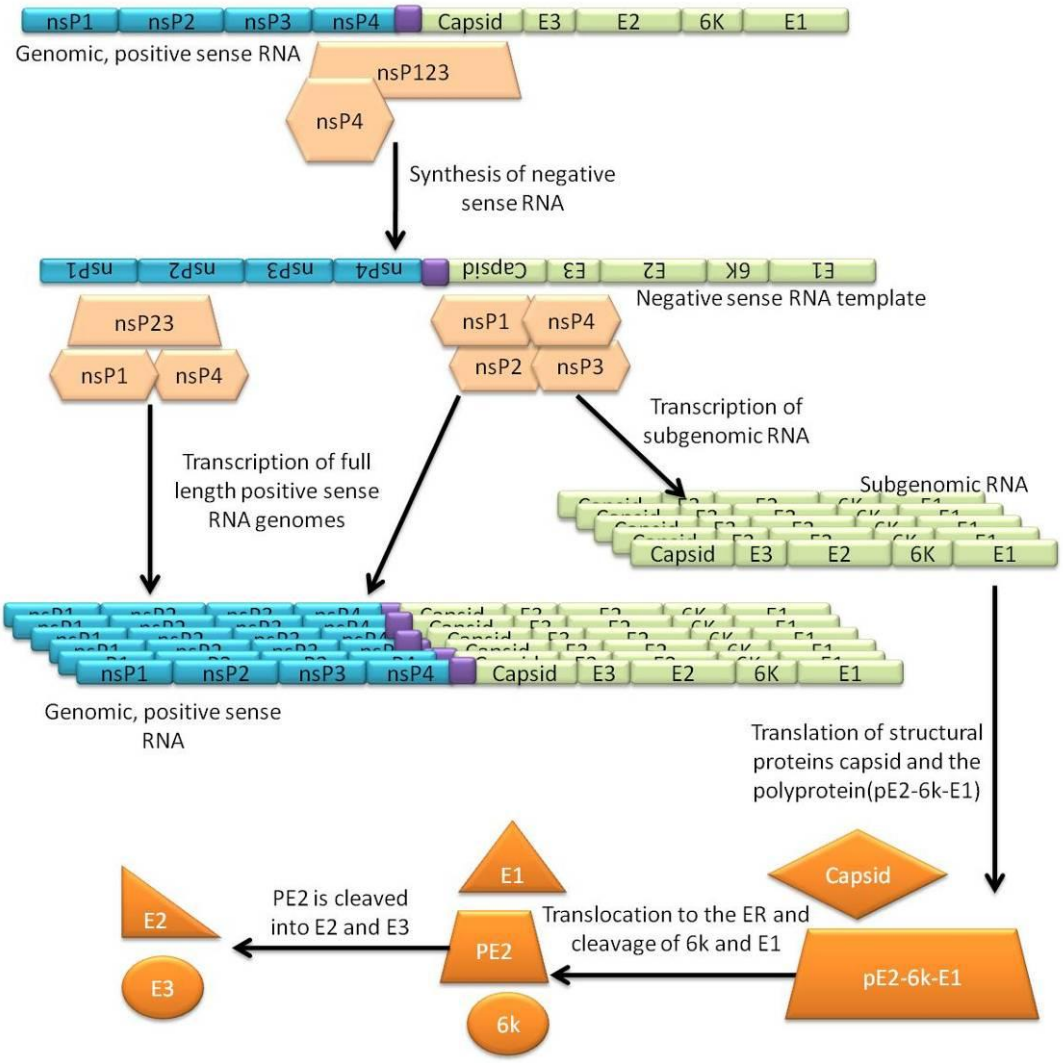


Figure 3: Structural protein synthesis of *Alphaviruses*

polyprotein which initiates the synthesis of full length positive strand RNA. This is made in much higher quantities than the negative strand RNA.

Once nsP23 is cleaved, the protein complex containing all four non-structural genes begins synthesis of subgenomic RNA for the structural proteins as well as continuing the synthesis of full length positive sense RNA. The subgenomic RNA contains the genes encoding the structural proteins. The regulation of synthesis of the viral RNA and proteins is tightly controlled by the concentrations of the non-structural proteins. As concentrations of the individual non-structural proteins increase, the rate of formation of the positive sense RNA is increased and complementary strand synthesis is decreased in rate (Galbraith et al. 2006).

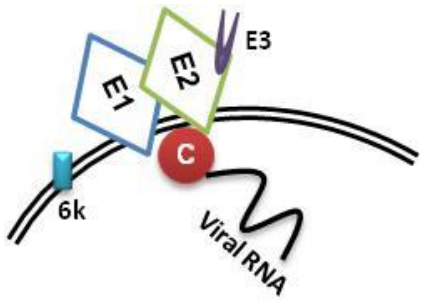


Figure 4: Mature alphavirus virion.

Depiction of the location of the viral structural proteins on the lipid membrane of the mature virion.

Translation of the structural proteins is initiated from the subgenomic RNA (Figure 3). The capsid protein is transcribed first and cleaved immediately from the polyprotein. The structural polyprotein, pE2-6k-E1 is translocated to the ER for further processing. pE2, 6k and E1 are cleaved and pE2 is translocated to the membrane, where it is cleaved into the E2 and E3 proteins. The 6k protein is membrane bound and is believed to be important for conformational folding of the E1 protein (Yao et al. 1996). The E2 and E1 proteins make up the protein spikes that cover the alphavirus (Figure 4). CHIKV monoclonal antibodies bind both the E1 and E2 proteins, confirming their location on the outer surface of the virion within the

envelope (Warter et al. 2011). The capsid protein is found closely associated with the genomic RNA and helps in packaging of the virus. It has been believed that the E3 protein is released from infected cells and not in the mature virion, but a recent report states that the E3 protein can be found peripherally attached to the E2 protein (Simizu et al. 1984, Voss et al. 2010).

Semliki Forest antigenic complex

The Semliki Forest antigenic complex contains viruses that cause febrile arthralgic disease in people and animals. CHIKV is a member of the Semliki Forest antigenic complex, which also includes other arthralgia causing alphaviruses, including Ross River (RRV), Mayaro (MAYV), Getah (GETV) and O'nyong-nyong (ONNV) (Table 1) (Luers et al. 2005). The arthralgic diseases in people that are caused by these viruses are clinically indistinguishable from one another (Tesh 1982). Representative viruses from this complex are found in Africa, Asia, South America, Australia and the islands of the Indian Ocean. CHIKV, ONNV, RRV, MAYV and UNV cause arthralgic disease in humans

Semliki Forest virus (SFV) can also cause encephalitis, fever and arthralgia. GETV causes an arthralgic disease in horses, but does not cause illness in people (Kono 1988). Middelburg virus (MIDV) has been isolated in mosquito pools and once from a dead horse. Bebaru virus has not been associated with disease in people and little is known about it. UNV and MAYV are the only two members found in the western hemisphere and occur in South America.

Table 1: Viruses in the Semliki Forest Antigenic complex

Adapted from (Griffin 2007)

Virus	**Geographic Distribution**	**Clinical Presentation (Animal disease)**
Bebaru	Asia	Undefined
Chikungunya (CHIKV)	Africa, Southeast Asia	Fever, arthritis, rash
Getah (GETV)	Asia	None (Horse)
Mayaro (MAYV)	South America	Fever, arthritis, rash
Middlelburg (MIDV)	Africa	None (Horse)
O'nyong-nyong (ONNV)	East Africa	Fever, arthritis, rash
Ross River (RRV)	Australia, South Pacific	Fever, arthritis, rash
Semliki Forest (SFV)	Africa	Fever (Horse)
Una (UNV)	South America	Fever, rash, arthralgia (Horse)

Phylogenetic analyses of alphaviruses are fairly consistent on the grouping of the Semliki Forest antigenic complex, except for MIDV and Barmah Forest virus (Figure 5). MIDV and Barmah Forest viruses have recently been classified in the Semliki Forest clade of viruses due to protein and genome sequences (Luers et al. 2005). MIDV can be classified as its own antigenic group, but within the Semliki Forest antigenic group it is most closely related to UNV. CHIKV and ONNV have a 74.5% nucleotide similarity and historically ONNV was classified as a CHIKV strain, but recently was classified as a distinct virus (Powers et al. 2000). CHIKV and RRV share 60.8% nucleotide similarity when comparing full genomes. GETV, SFV and RRV are closely related

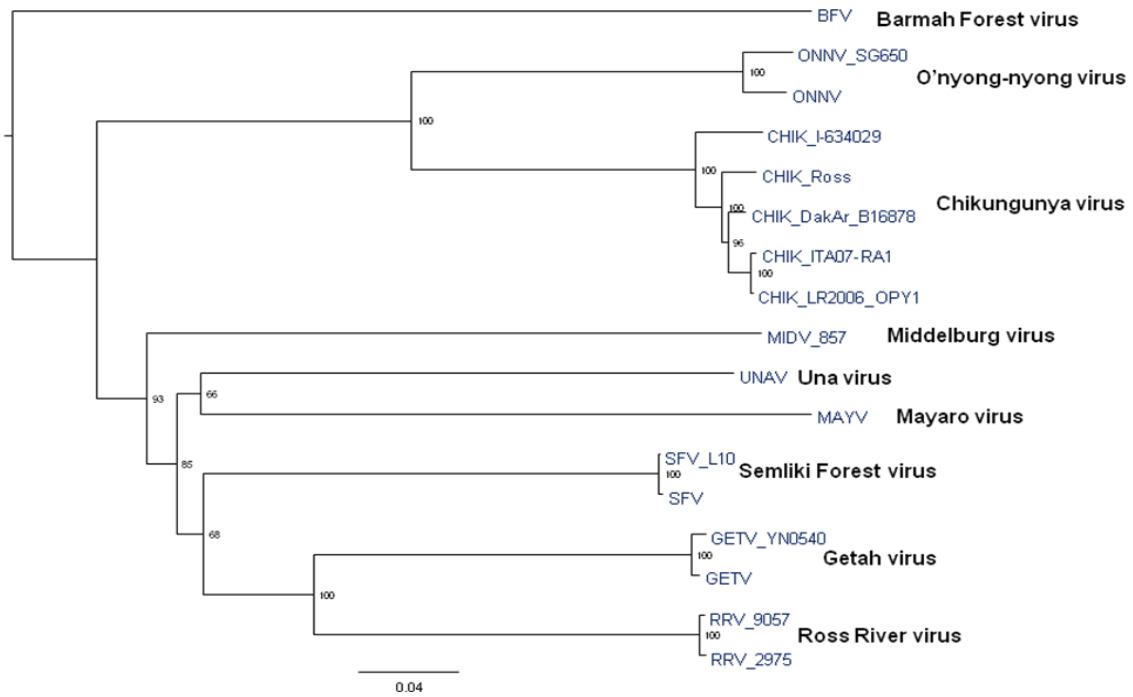


Figure 5: Full genome sequence analysis of Semliki Forest antigenic complex.

A neighbor joining tree with 100 boot-strap replicates. Figure courtesy of Allison McMullen. (Gouy et al. 2010)

phylogenetically. MAYV and UNV also show genetic similarity, which is not surprising since they are both found in South America.

Ross River virus

Ross River virus is an arthralgia-causing *Alphavirus* that occurs in Australia, Papa New Guinea and islands of the Pacific. In the year 2000, more than 4,000 cases of RRV were reported in Australia with a peak during the first half of the year (AIHW 2002). The principal vertebrate reservoirs for RRV are marsupials including, kangaroos and wallabies and the main mosquito vectors are *Aedes vigilax* and *Culex annulirostris* (Tesh 1982, van den Hurk et al. 2010).

RRV disease is almost indistinguishable from CHIKF, and is characterized by a febrile illness, rash and arthralgia with swollen tender joints. Fever and chills are less common with RRV than with CHIKV (Kay et al. 1988). Experimental infection of RRV in mice has led to interesting discoveries regarding the pathogenesis of the virus. Macrophages have been shown to be key mediators of RRV-induced myositis in mice (Lidbury et al. 2008, Lidbury et al. 2000, Rulli et al. 2007, Rulli et al. 2005). Complement receptor-3 has also been shown to be needed for RRV-induced disease (Morrison et al. 2007, Morrison et al. 2008). It has also been shown that in mice deficient in T and B cells that RRV pathogenesis is minimal (Morrison et al. 2006).

Due to the similarities in genome and disease characteristics in humans, parallels can be drawn between the pathogenesis of RRV and that of CHIKV. Early work in mice has also shown that these two viruses produce a very similar disease state in outbred mice.

Mayaro virus

One unique feature of MAYV is that it is the only alphavirus causing significant arthralgia in the New World. MAYV was first isolated in 1954 in Trinidad (Casals et al. 1957). MAYV is the etiological agent of mayaro fever. Mayaro fever, which like CHIKF, presents in humans as an acute febrile illness with arthralgic symptoms that can be recurrent and persistent. Most cases of mayaro fever occur sporadically in individuals that have a close association with the forest. Large outbreaks are usually rare. MAYV is also unique in that the mosquito vector that transmits the virus, *Haemagogus janthinomys,* is an arboreal mosquito found mainly in the canopy (Hoch et al. 1981). It is thought

that the natural reservoirs for MAYV are monkeys in the canopy of the jungle, specifically the white-faced saki (*Pithecia pithecia*) and the red howler monkey (*Alouatta seniculus*) (de Thoisy et al. 2003). Due to the rural nature of the reservoir and vector, outbreaks usually consist of less than 50 people (Torres et al. 2004). The largest reported outbreak of Mayaro fever occurred in Brazil in 1978 and included 55 confirmed cases of MAYV infection (Pinheiro et al. 1981). MAYV still has the potential to cause large outbreaks if it were introduced into a naïve population with a large pool of potential vectors present. Recent studies have shown that some rural communities in Peru have a seroprevalence of 34% of MAYV antibodies (Long unpublished).

Chikungunya virus

CHIKV infection produces an acute febrile illness in people, CHIKF, which most commonly presents as fever, rash and severe arthralgia/myalgia. CHIKF is rarely fatal, but can cause persistent and recurrent symptoms that most commonly are severe arthralgia and myalgia (Powers et al. 2007). The severe pain felt in the joints and muscles of people suffering from CHIKF is how the virus was named. Chikungunya means “that which folds one up” in the native language of the Makonde people where CHIKV was first identified (Lumsden et al. 1953). Since it was first isolated, three genotypes of CHIKV have been identified: Asian, East/Central/South African and the West African genotypes (Powers et al. 2007).

Epidemiology

CHIKV was first isolated in 1952 in south-eastern Tanzania, approximately 60 miles from the ocean, in a region of arid plateaus (Lumsden et al. 1953).

Interestingly, it was during a time of drought that the outbreak occurred and water storage containers were found throughout the village. During this severe dengue-like epidemic, Dr. Marion Robinson at the local hospital made the discovery of the new viral agent, CHIKV (Robinson 1955). This was the first time that the Makonde people remembered an epidemic of this type occurring. It has been theorized that dengue fever and CHIKF have been confused for hundreds of years, with some of the early accounts of dengue-like disease actually being caused by CHIKV (Carey 1971). Recent reviews of some of the original reports suggest that the first use of the word "dengue" actually was during a CHIKV outbreak in Cuba in the 1820's (Kuno 2009). Reviews of previous dengue-like outbreaks have suggested that CHIKV was the causative agent of the outbreak in Cairo in 1779, Zanzibar in 1823, Hong-Kong in 1901 and Calcutta in 1923 (Carey 1971). Carey suggests that there was an outbreak of CHIKF in the southeastern part of the United States in 1827-1828. The physicians described the 1828 epidemic in New Orleans as affecting 99% of the population and many having severe persistent arthralgic symptoms for weeks after recovering from the acute disease (Carey 1971).

In the 21st century, CHIKV is endemic in Central and South Africa, India, Sri Lanka and Southeast Asia with outbreaks occurring sporadically on the islands of the Indian Ocean (Figure 6). Laboratory confirmed outbreaks of CHIKF occurred almost every year from 1952-1990 in countries in South and Central Africa (Jupp et al. 1988, Powers et al. 2007). Countries affected include South Africa, Zimbabwe, Zaire, Senegal, Uganda and Nigeria. While outbreaks of CHIKV were common during this time in Southeast Asia, they were sporadic, sometimes with multiple years of inactivity (Burke et al. 1985, Pavri 1986, Powers

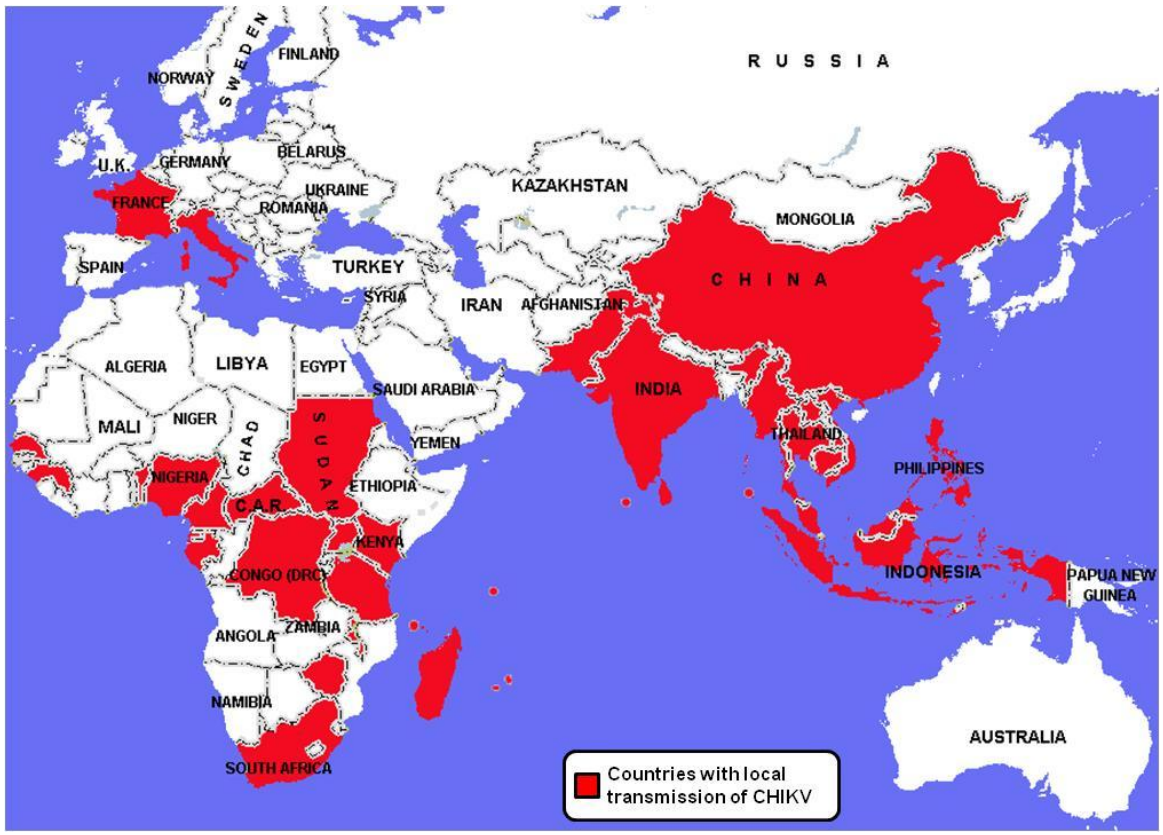


Figure 6: Countries with reported local CHIKV transmission

Countries shown in red have reported local transmission of CHIKV. Map drawn using Microsoft Streets and Trips. Figure adapted from Powers 2007 and updated using www.cdc.gov/chikugnunya.

et al. 2007). A large outbreak of CHIKF occurred in Southeast Asia from 1962-1965 that affected India, Thailand and Vietnam. Descriptions from this outbreak include hemorrhagic and fatal cases of CHIKF (Chaudhuri et al. 1964, Pavri et al. 1964, Sarkar et al. 1966). During this outbreak in Calcutta many studies were done on the hemorrhagic and fatal cases including serology and virus isolation where CHIKV infection was found (Sarkar 1967). This is one of the only outbreaks that describe large numbers of people with severe manifestations, but

it has been speculated that the concurrent dengue outbreak that was ongoing in Calcutta may be attributed to the severity (Myers et al. 1967, Sarkar 1967).

Reports of CHIKF were rare from 1990-1996 and mostly occurred in Thailand. CHIKV outbreaks became more frequent and widespread starting in 2003 in Indonesia. The recent outbreak that has brought CHIKV much attention started in Kenya in 2004 (Kariuki Njenga et al. 2008). In the coastal town of Lamu, a severe drought occurred in the early months of the year and starting in June 2004, there was a large increase in febrile illnesses which was identified as CHIKF (Chretien et al. 2007). This CHIKV outbreak moved from coastal Africa to the Comoros islands in January 2005 (Pialoux et al. 2007). From there it took less than two months to spread to neighboring islands. From March 2005 through 2006, the islands of the Indian Ocean, including Seychelles, Maruitus, Madagascar, and Reunion, were devastated with a widespread outbreak of CHIKF (Powers et al. 2007). On Reunion, approximately one third of the population (266,000) was diagnosed with CHIKF, with an overall attack rate of 35%, and 254 deaths were attributed to CHIKV infections (Pialoux et al. 2007, Renault et al. 2007). These island populations were probably naïve to CHIKV, which helps to explain the large number of affected individuals (Sourisseau et al. 2007). The islands of the Indian Ocean are major attractions for many tourists, and returning travelers were frequently sick in their home countries bringing more attention to the outbreak (Lanciotti et al. 2007, Simon et al. 2007, Taubitz et al. 2007). Between 1995-2009, 109 cases of CHIKF were identified in the United States in returning travelers (Gibney et al. 2011).

The CHIKV epidemic continued in India 2006-2007, where it is estimated that more the 1.3 million people were infected, with some areas experiencing

attack rates of 45% (Arankalle et al. 2007, Mavalankar et al. 2008, Pialoux et al. 2007). In July of 2007, CHIKV emerged from the tropics and caused a localized outbreak in northeastern Italy (Enserink 2007). In Italy, there were 205 confirmed cases of CHIKF with one fatality from July to September of 2007 (Rezza et al. 2007, Seyler et al. 2008). The fatal CHIKF case was a 83-year-old man with underlying health issues, who died of encephalitis (Casolari et al. 2008). The index case was believed to be an Indian man traveling to Italy to visit his relatives who became ill in Italy (Rezza et al. 2007). Since 2007, no further reports of CHIKF have occurred in Italy and surveillance of mosquito pools has not shown any CHIKV activity (Calzolari et al. 2010, Rezza 2010). In 2008, CHIKV infections occurred mostly in Southeast Asia, including Singapore, Thailand, India, Sri Lanka, Indonesia and Malayasia (Hapuarachchi et al. 2010, Ho et al. 2011, Ng et al. 2009, Pongsiri et al. 2010). In 2009, new CHIKV infections were reported in southeast Asia with thousands of confirmed cases in Thailand alone (Rianthavorn et al. 2010). In 2009, new cases of locally transmitted CHIKF cases occurred in Madagascar and Reunion (D'Ortenzio et al. 2009). While there were only 3 confirmed cases, these were the first cases of CHIKV infection on Reunion since December 2006 (D'Ortenzio et al. 2009). In 2010, a CHIKV outbreak continued to affect the east coast of Madagascar and there were sporadic CHIKF cases in Reunion (Dehecq et al. 2010). CHIKV infections continued to be reported throughout southeast Asia in 2010 where China reported for the first time locally acquired CHIKV infections in October of 2010 (ProMED-mail 2010). In September 2010, two locally acquired CHIKV infections were reported in southeastern France (Gould et al. 2010). This was thought to be caused by a febrile traveler returning home and being bitten by an *Ae.*

albopictus mosquito. At the time of this writing, no more cases of locally acquired CHIKF have been reported in France. Early in 2011 CHIKF cases have already been confirmed in the Southern Pacific Island of New Caledonia, which has not experienced a CHIKV outbreak before (ProMED-mail 2011).

The current outbreaks of CHIKV which have occurred on the islands of the Indian Ocean and throughout Southeast Asia have been linked to a new mutation in the E1 protein in a CHIKV strain from the East/Central/South African genotype (Powers et al. 2007, Schuffenecker et al. 2006, Tsetsarkin et al. 2007). The mutation is absent from samples from Kenya in 2004 and was first identified in Reunion samples in late 2005 (Schuffenecker et al. 2006). This mutant strain soon became the most commonly isolated strain from patients in 2006 in Reunion (Schuffenecker et al. 2006). This amino acid mutation, E1-A226V, has been shown to increase the infectivity of *Ae. albopictus* mosquitoes as compared to its parent strain (Tsetsarkin et al. 2007). CHIKV having this amino acid mutation was subsequently identified in Southeast Asia in 2007, being the dominant strain and replacing the endemic Asian genotype of CHIKV (Arankalle et al. 2007, Kumar et al. 2008, Santhosh et al. 2008). This mutant strain was also identified in the Italian outbreak in 2007 (Bordi et al. 2008).

Clinical Features

CHIKF in most healthy individuals is a non-lethal, self-limiting febrile illness. Ross was the first to describe CHIKF, noting the sudden onset of disease being able to incapacitate a person in minutes with the joint pains being “frightening in their severity” (Lumsden 1955). During a CHIKV outbreak, fever and polyarthralgia are the significant diagnostic criteria (Borgherini et al. 2007,

Mohan 2006). Simon et al. 2007 reported that two-thirds of CHIKF patients had to be hospitalized. In most cases of CHIKF, a painful and disabling arthritis is present in the acute stage of the illness that lasts for 7 days and is more likely in a previously injured joint (Simon et al. 2007, Tesh 1982). Usually a person will have more than one joint affected, bilaterally and is more common in the knees, ankles, wrists, hand and feet (Mohan 2006). The fever associated with CHIKV can be biphasic and can reach 104°F in some cases (Powers et al. 2007). Fever and arthralgia are the most common clinical symptoms of CHIKF occuring in approximately 94% of CHIKF cases (Table 2) (Borgherini et al. 2007, Staikowsky et al. 2009, Taubitz et al. 2007, Thiruvengadam et al. 1965, Win et al. 2010). Myalgia and headache occur in approximately 47-53% of the CHIKF cases. Early reports of the CHIKV outbreak on Reunion also documented headache and muscle pain in approximately two- thirds of the cases (Renault et al. 2007). Rash is also associated with CHIKF, but happens only in approximately 40% of documented cases. Conjunctivitis also occurs during CHIKF in 22% of the cases. Nausea, gastrointestinal problems and lymph adenopathy occur in approximately 12 and 16% of CHIKF cases, respectively.

Some common laboratory values that are seen in CHIKF patients include lymphopenia, leucopenia, increased aspartate transaminase (AST) and increased alanine aminotransferase (ALT) (Borgherini et al. 2007, Staikowsky et al. 2009, Taubitz et al. 2007, Win et al. 2010). Decreased lymphocyte numbers have been reported in multiple studies and particularly in 79% of the CHIKF patients seen in Reunion in 2005-2006 with 39% of the patients having severe lymphopenia (Borgherini et al. 2007). Decreased leucocytes are also very common in CHIKF patients in the acute stage but resolves, as does lymphopenia

(Win et al. 2010). Thrombocytopenia can also occur during CHIKF and was seen in 44% of patients on Reunion (Borgherini et al. 2008). Liver enzyme levels have also been shown to be increased in CHIKF patients. AST and ALT are the most

Table 2: Clinical Presentation of CHIKF

Location	Madras, India	Reunion	Infected travelers	Reunion	Singapore	**Combined Data**	
Year	1964[2]	2005-6[5]	2006[3]	2006[4]	2008[1]		
Number of participants	N = 86	N = 157	N = 20	N = 180	N = 97	(N= 540)	
Symptoms	**Number of people with symptom**					**Average**	**Range**
Fever	83	139	20	180	88	94%	89-100%
Arthralgia	74	151	20	180	85	94%	86-100%
Myalgia	27	NA	NA	106	61	53%	31-63%
Headache	7	74	NA	126	39	47%	8-70%
Rash	17	63	15	86	35	40%	20-75%
Conjunctivitis	29	NA	4	41	10	22%	10-34%
Nausea or gastro-intestinal symptoms	9	74	6	67	12	16%	12-37%
Lymph Adenopathy	15	NA	NA	16	NA	12%	9-17%
Hemoragheic manifestations	5	7	1	2	1	3%	1-6%

Symptoms that were reported inconsistently or in low numbers: anorexia, cough, vomiting, sore throat, eye pain, diarrhea, abdominal pain, edema, chills, neurological involvement

NA= study did not record the indicated symptom
1= Win et al. 2010; 2= Thiruvengad et al. 1965; 3= Taubitz et al. 2007; 4= Staikowsky et al. 2009; 5= Borgherini et al. 2007

common, but creatine kinase has also been shown to be increased (Borgherini et al. 2008). In approximately 10% of CHIKF cases, AST and ALT levels can be more than twice the normal range (Borgherini et al. 2008, Win et al. 2010).

Severe Manifestations of CHIKF

During the outbreak in Reunion, with access to modern healthcare, many adverse outcomes were reported due to CHIKV infection, including lethal hepatitis, encephalitis, maternal-fetal transmission and an increased death rate (Sourisseau et al. 2007). This could be due to a large population of naïve individuals, better reporting in the affected areas, and/or the recent mutation in the CHIKV genome (Powers 2008, Tsetsarkin et al. 2007). Other symptoms that occur rarely and are reported inconsistently are anorexia, cough, vomiting, sore throat, eye pain, diarrhea, abdominal pain, edema, chills and neurological involvement. Severe neurological involvement can include seizures, abnormal CT results and altered cerebral spinal fluid chemistry that can lead to permanent sequelae and death (Rampal et al. 2007). Severe cases can also include respiratory failure, cardiovascular decompensation, severe hepatitis and kidney failure (Renault et al. 2007, Tandale et al. 2009, Tournebize et al. 2009).

More than half of the patients that display severe manifestations of CHIKF are over 65 years of age, while up to 1/3 of the severe CHIKF cases can result in death (Renault et al. 2007). On Reunion, a study of 610 patients with atypical manifestations of CHIKF showed that 89% of the cases had underlying heath conditions. Only 15 cases were in people under the age of 24, and 10.7% of the atypical cases resulted in fatality (Economopoulou et al. 2009). This study also showed that atypical, severe and fatal cases of CHIKF increased with age, but

there were only five deaths recorded in people with no underlying health conditions (Economopoulou et al. 2009). While severe CHIKF is usually associated with co-morbidities, a survey of 33 severe CHIKF cases showed that 19 had no underlying health conditions, the age range was from 23-84 (average of 59) and in these cases, with no underlying health conditions, there was a mortality rate of 42% (Lemant et al. 2008). This included 2 patients, ages 23 and 26 years old, who died from CHIKF with no underlying health issues (Lemant et al. 2008). A survey of 86 confirmed CHIKF cases in pediatric patients, aged 10-18 years-old, recorded two deaths with central nervous and cardiac involvement, but few other hospitalizations in children older than six months (Ernould et al. 2008). Many reports of severe bullous skin lesions in infants during CHIKF have shown that although this is not fatal it can be the cause of hospitalization and that CHIKV RNA can be recovered from the blister fluid (Bandyopadhyay et al. 2010, Economopoulou et al. 2009, Inamadar et al. 2008, Robin et al. 2010, Valamparampil et al. 2009). Fatal cases of CHIKF do not follow a predictable pattern with the causes of death ranging from heart failure, multi-organ failure, hepatitis and encephalitis (Casolari et al. 2008, Economopoulou et al. 2009, Ernould et al. 2008, Lemant et al. 2008, Sam et al. 2010). Previously, CHIKV has been associated with hemorrhagic disease; but in looking at four different clinical studies, hemorrhagic manifestations only occurred in 1-6% of the CHIKF cases and was usually described as bleeding from the gums (Borgherini et al. 2007, Staikowsky et al. 2009, Taubitz et al. 2007, Thiruvengadam et al. 1965, Win et al. 2010).

Unfortunately, after the acute phase of CHIKF, polyarthritis can be recurrent and persistent and has been reported for up to several years (Powers

et al. 2007). Persistence and recurrence of arthralgic symptoms associated with CHIKV and RRV are of major concern from a public health standpoint. A previous report stated that over 12% of CHIKV-infected individuals experience long term joint pain (Brighton 1984). It was recently reported that up to 60% of CHIKV-infected individuals during the 2005-2006 outbreak on Reunion had arthralgic symptoms 18 months after infection (Gerardin et al. 2011). It has been shown that the chronic CHIKV-induced arthralgia is more likely in individuals who are greater than 60-years-old (Hoarau et al. 2010). In one case of chronic arthralgia, CHIKV RNA was detected in the synovial macrophages 18 months after CHIKV infection (Hoarau et al. 2010). This was also seen in chronic arthralgic cases of RRV where the synovial fluid had an increase in inflammatory cells and RRV RNA in monocytes and macrophages (Fraser et al. 1981).

Vertical transmission of CHIKV was documented and studied for the first time during the 2005-2006 outbreak on Reunion. The severity of the disease in both mother and fetus/newborn was dependent on the gestational age at which the mother was infected with CHIKV. Early, within the first 16 weeks of pregnancy, CHIKF resulted in at least three fetal deaths (Touret et al. 2006). It is unknown if this was due to the high fever that occurred because of the disease or due to CHIKV infection of the fetus. In these three cases, RT-PCR of the amniotic fluid was positive for CHIKV and no other cause of fetal death was found (Touret et al. 2006). No pregnancy complications were reported due to CHIKF occurring in the second and early third trimesters. Another study looked at 678 CHIKF cases in pregnant women and found no complications if the infection was greater than one week prior to delivery (Gerardin et al. 2008). The most common complications resulted when the pregnant women had CHIKF

within 7 days of birth (Gerardin et al. 2008, Robillard et al. 2006). When the pregnant women had CHIKF within two days of delivery, 50% of newborns developed neonatal CHIKF in an average of 4 days (Gerardin et al. 2008). This severe neonatal CHIKF presented as a febrile illness with severe complications including neurological disorders, seizures and hemorrhagic complications. There was at least one death that was reported due to neonatal CHIKF (Ramful et al. 2007). It is unclear if the transmission occurred across the placenta *in utero* or during birth. Since cesarean births had an equivalent rate of infection, transmission may have occurred during birth, when there is a breakdown of the placental barrier, allowing for the mixing of the maternal and fetal blood supplies (Gerardin et al. 2008). A study of vertical transmission in primates have had similar outcomes as human cases when six pregnant Rhesus macaques were infected with CHIKV 30-40 days before delivery and there was no evidence of intra-uterine infection (Chen et al. 2010). In RRV, vertical transmission has been documented in 3-4% of pregnant women and in laboratory mice (Kay et al. 1988)

Fatalities associated with CHIKF

While fatal cases of CHIKV are rarely documented (Figure 7), there has been renewed interest in looking at crude death rates during outbreaks of CHIKV. The case fatality rate for CHIKF is approximately 1/1000 cases (Manimunda et al. 2010, Mavalankar et al. 2008, Pialoux et al. 2006). A review of original dengue-like epidemics have revealed that 25 fatal cases of CHIKF occurred in India in 1872, but proving cause of death over a hundred years ago was much harder (Kuno 2009). During the Reunion outbreak, fatal cases of CHIKV were reported, mostly in the elderly and CHIKF was reported as a cause

of death on approximately 254 death certificates (Pialoux et al. 2007, Renault et al. 2007). Excess deaths are calculated by calculating the total number of deaths in a certain time and comparing historically to previous years and estimating how many "extra" deaths occurred. In India in 2006, there were reports of a high number of excess deaths during the height of the CHIKV outbreak, but no exact numbers were calculated (Manimunda et al. 2010, Mavalankar et al. 2008). In Mauritius, 743 excess deaths were reported between March and May. If these deaths were directly associated with CHIKF, this would result in a estimated 6% fatality rate (Beesoon et al. 2008). A similarly high case fatality rate could be calculated in Ahmedabad, India with 3,000 excess deaths over a 3 month period

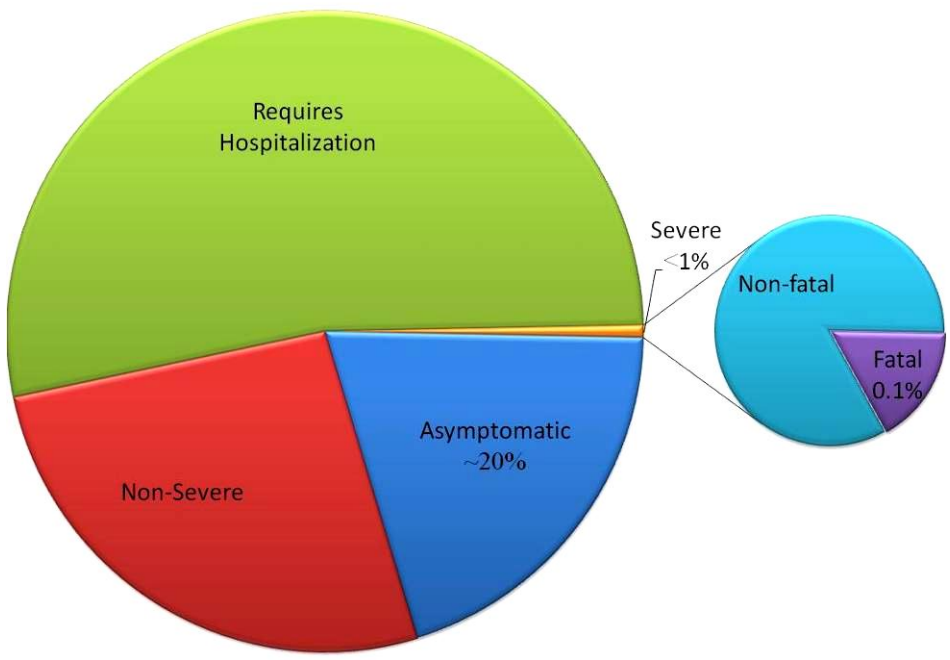


Figure 7: Breakdown of CHIKV infections in people

in 2006 with an estimated 60,000 cases of CHIKF (Mavalankar et al. 2008). More research needs to be done to understand why there is a much higher rate of deaths during CHIKF outbreaks, including the underreporting of non-fatal cases and the inclusion of deaths not resulting from CHIKF.

Seroprevalence and asymptomatic cases

The rate of asymptomatic cases of CHIKV is relatively low when compared to some other viruses. Approximately 75-90% of people who develop CHIKV antibodies have symptomatic disease (Figure 7). Seroprevalence studies for CHIKV antibodies on Grand Comore island in 2005 showed that 69% were seropositive; in 2006 in two Indian districts, 51% were seropositive; and in Lamu, Kenya 75% were seropositive (Dwibedi et al. 2010, Sergon et al. 2007). Prior to the 2004 outbreak less than 1% of people were seropositive for CHIKV in the Coastal province of Kenya, (Morrill et al. 1991). In Mayotte in 2006, 37% of the population was found to be seropositive to CHIKV, but only 72% of these people reported having a CHIKF-like illness (Sissoko et al. 2008). Of seropositive people on Grand Comore, only 14% did not experience a febrile illness with fever and joint pain, while 79% were hospitalized or confined to bed for an average of 6 days (Sergon et al. 2007). Similarly in Malaysia in 2006, 17.5% of seropositive people were asymptomatic (Ayu et al. 2010). In India in 2008, a serosurvey was conducted on 360 people which showed CHIKV antibodies in 62.2% of the people and an overall asymptomatic CHIKV infection of 6.3% (Manimunda et al. 2010).

Treatment of chikungunya fever

No specific treatment is available for CHIKV. Supportive care is usually given in the form of analgesics. On Reunion island a common analgesic given during the acute phase was paracetamol (Michault et al. 2009). Chloroquine has shown promise in inhibiting CHIKV replication *in vitro* (Ozden et al. 2008, Sourisseau et al. 2007). An early study of chloroquine as a treatment for CHIKF showed improvement in chronic cases of arthralgia (Brighton 1984). Unfortunately in recent human trials, chloroquine was not found to have an effect on CHIKF (De Lamballerie et al. 2008).

Chikungunya fever pathogenesis

The pathogenesis of CHIKF in people is still poorly understood. Human viremia has been reported to be in the range of $10^{5.5}$ PFU/mL or 10^{8} viral RNA copies (McIntosh et al. 1963, Sourisseau et al. 2007)). Lanciotti et al. (2007) used PCR to estimate viremia levels in CHIKF cases and showed a range of $10^{3.9}$-$10^{6.8}$ PFU/mL in the first 6 days of reported illness. In chronic cases of CHIKV-induced arthralgia, interferon-alpha (IFN-α) and interleukin-12 (IL-12) are increased months after acute disease (Hoarau et al. 2010). *In vitro*, CHIKV infects most adherent cells and macrophages, but not blood cell lines (Sourisseau et al. 2007). Muscle biopsies of CHIKF patients have shown that the muscle progenitor cells become infected with CHIKV, which was confirmed with *in vitro* work in cell lines (Ozden et al. 2007)

Mouse Models of CHIKF

Many mouse models are in development to study CHIKV infection both to understand the pathogenesis caused by CHIKV and to test different anti-viral and

vaccine candidates. Initial work with CHIKV and mice mainly focused in achieving avirulent strains of the virus or the development of high passage strains to increase the virulence of the virus for mice (Igarashi et al. 1967, Levitt et al. 1986). While intra-nasal and intra-cranal inoculation of CHIKV results in death and neurotropic disease in mice, this is not a good model for human arthralgic disease (Powers et al. 2007, Ryman et al. 2008, Wang et al. 2008). Some of the first work with developing a model of arthralgia induced by an alphavirus is with RRV (Mims et al. 1973). Three basic strategies have been used to replicate human like CHIKF in mice; 1) the use of immune deficient mice, 2) young mice and 3) footpad inoculation of adult mice. Unfortunately, none of these models are a perfect fit for pathogenesis studies as well as vaccine testing. The model that I have developed (described in Chapter 3) is the use of young mice, infected by sub-cutaneous (SC) route in the skin of the back which produces a disease characterized by 3-4 days of viremia and severe myositis in the skeletal muscle which disseminates to all four limbs (Ziegler et al. 2008). These results are similar to that seen in RRV models of disease (Morrison et al. 2006). Adult outbred mice can also be infected by route of footpad inoculation (Gardner et al. 2010, Morrison et al. 2011). For this model, the foot that is inoculated becomes visibly swollen and has severe pathology. Unfortunately, the virus-induced pathology is not disseminated and was localized to the foot that was injected. Interferon deficient mice have been infected with CHIKV producing an illness that is characterized by death in -/- mice and is non-lethal but lacks muscle pathology in +/- mice (Couderc et al. 2008, Partidos et al. 2011). These mice are a good model to study the interaction of CHIKV and interferon *in vivo*.

Primate Models of CHIKF

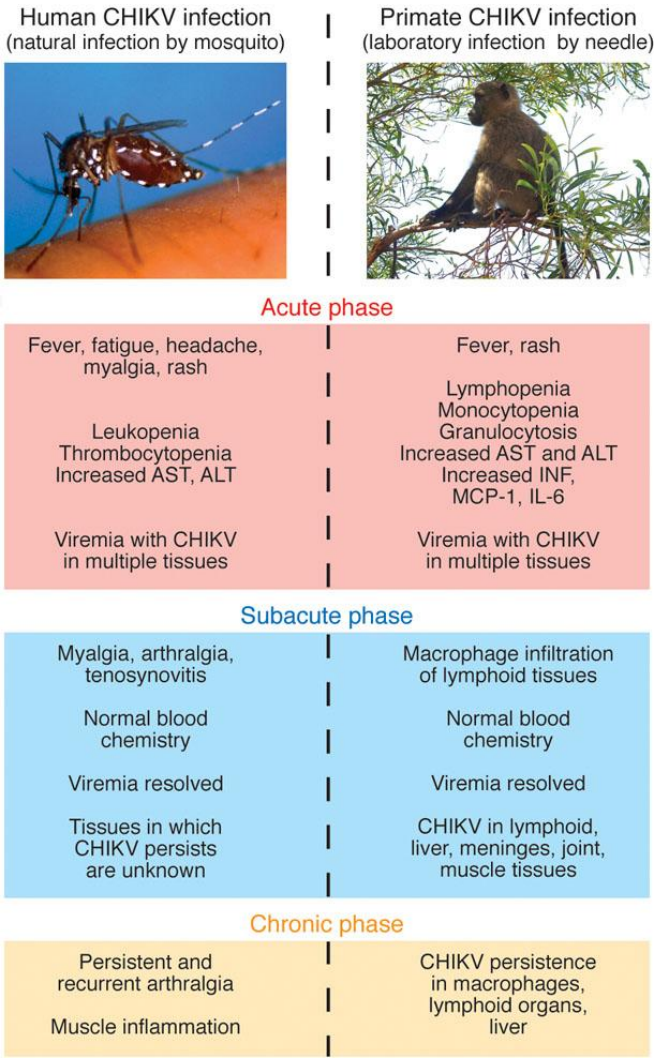


Figure 8: Primate and human phases of CHIKV infection

With more effort being directed towards the development of a CHIKV vaccine, the development of not only a small animal model, but a primate model, is being prioritized. Unfortunately, no good primate model of CHIKF is available at present. Most primate models lack the characteristic severe joint involvement that is seen in humans with CHIKF. Many parallels can be drawn between aspects of primate infection in the laboratory setting and the clinical course of CHIKF in people (Figure 8) (Higgs et al. 2010). CHIKV naturally infects verevet monkeys (*Cercopithecus aethiops)* and baboons (*Papio ursinus*) in Africa (McIntosh 1970). Labadie et al. (2010) infected

cytmongolus macaques with CHIKV and showed long-term persistence of the virus in macrophage cells. Rhesus macaques (*Macaca mulatta*) have also been used to show protection in vaccine studies (Akahata et al. 2010). Studies on pregnant rhesus macaques have shown that these monkeys can have detectable viremias and that virus can be isolated from their tissues (Chen et al. 2010). Rhesus macaques have also been used for studies of efficacy of DNA CHIKV vaccines (Mallilankaraman et al. 2011). In cynomolgus macaques (*Macaca fascicularis*), CHIKV viremias have been detectable by PCR for 6-7 days post infection (dpi) and in rhesus macaques for 5-7 dpi (Chen et al. 2010, Labadie et al. 2010).

Vaccine Development

There is currently a large emphasis on developing and testing CHIKV vaccines (Table 3). The first CHIKV vaccine was a cell culture passaged formalin inactivated vaccine that induced a neutralizing antibody titer in human volunteers after 2 doses (Harrison et al. 1971). This first vaccine was manufactured in limited quantities and it was recommended that a new live vaccine be developed to increase the immunogenicity. An attenuated strain of CHIKV for vaccine development called CHIK 181/clone 25, was derived from serial cell culture passages and plaque purification steps, and was shown to be avirulent in mice while inducing a rigorous anti-body response (Levitt et al. 1986). During Phase II trials of TSI-GSD-218 the live attenuated vaccine derived from CHIK 181/clone 25, 5 of 59 volunteers receiving the vaccine developed transient arthralgic side-effects (Edelman et al. 2000). Because of the incidence of side-

Table 3. CHIKV vaccine candidates of the past and present

Vaccine Candidate	Type of Vaccine	Parent Strain	Testing model(s)	Results	Citation
GMK 10915	Formalin inactivated	CHIKV 15561	Mice, humans	2 doses gave high antibody titers	(Harrison et al. 1971)
Tween-ether inactivated CHIKV	Tween 80 and ether inactivation	African CHIKV 168	Mice	NAb and protection from disease	(Eckels et al. 1970)
TSI-GSD-218	Live attenuated	CHIKV 181/clone 25	Mice, humans	5 of 59 people side effects	(Edelman et al. 2000)
EEE/CHIKV	Live chimeric	LR 2006 OPY1	Mice	Attenuated with NAb	(Wang et al. 2008)
DRDE-06	Formalin inactivated	CHIKV DRDE-06	Mice	NAb and adoptive protection	(Tiwari et al. 2009)
CHIKV-VLP	VLP	LR 2006 OPY1	Mice, primates	NAb and protection from disease	(Akahata et al. 2010)
pMCE321	DNA	Consensus sequence	Mice, primates	NAb	(Mallilankaraman et al. 2011)
CAdVax–CHIK	Adenovirus vector	LR 2006 OPY1	Mice	NAb and protection from disease	(Wang et al. 2011)
IRES-CHIKV	Live attenuated	LR 2006 OPY1	Mice	NAb and protection from disease	(Plante et al. 2011)

NAb = neutralizing antibodies

effects, although well tolerated, this vaccine was not developed for commercial use.

At present, multiple CHIKV vaccine candidates are being developed. A consensus DNA vaccine candidate expressing CHIKV capsid, E1 and E2

proteins has been shown to be immunogenic in mice (Muthumani et al. 2008). Live vaccine candidates with *Alphavirus* chimeric genomes have also been tested in mice and shown to be both immunogenic and attenuated (Wang et al. 2008). A formalin inactivated vaccine grown to high tiers in serum-free conditions has also been shown to produce high neutralizing antibody titers in mice for up to 200 days post-inoculation (Tiwari et al. 2009). A CHIKV-VLP expressing the E1 and E2 glycoproteins from the LR 2006 OPY1 strain of CHKV has been developed as a vaccine candidate. When this VLP was tested in mice and Rhesus macaques it elicited both a neutralizing antibodies and protected against viremia and inflammation in a challenge model in monkeys (Akahata et al. 2010). A DNA vaccine expressing E1, E2 and E3 has also been developed; it conferred protection against CHIKV challenge in mice and induced neutralizing antibodies in rhesus macaques (Mallilankaraman et al. 2011). An adenovirus vector expressing the CHIKV structural proteins E1, E2 and capsid has recently been produced and was able to produce high antibody tiers in inbred and outbred mice as well as protect against viremia and arthralgia with a single dose (Wang et al. 2011).

Each of these vaccines have their advantages and disadvantages, when considering use in humans. While inactivated vaccines are seen as safer than live vaccine candidates, they are less immunogenic then live vaccines, which may have the potential for reversion to a virulent state (Zepp 2010). DNA vaccines are seen as being non-immunogenic due to their failure to replicate inside host cells (Leitner et al. 1999). One factor that continues to hamper CHIKV vaccine development is a good animal model that mimics the disease. Increased knowledge of CHIKV pathogenesis and the role that immunopathology

plays in human disease should help to determine, the type of vaccine that would be most effective in preventing CHIKF may be elucidated (Ryman et al. 2008).

Mosquito vectors of CHIKV

CHIKV has been isolated from a wide variety of mosquito vectors, but has been most commonly associated with *Aedes* species. From the first investigations of CHIKF in Tanzania in 1952, *Ae. aegypti* mosquitoes have been implicated as the main vector of CHIKV to people (Lumsden et al. 1953). Early CHIKV isolations were from *Ae. aegypti* and *Ae. africanus* (Weinbren et al. 1958). But, CHIKV is able to infect many different species of mosquitoes (Table 4). It is thought that the CHIKV reservoir is in the African jungle where the main vectors of CHIKV include mosquitoes that feed primarily on primates in the subgenera *Diceromyia, Stegomyia* and *Aedimorphus* (Reiter et al. 2006). The

Table 4: Mosquitoes associated with CHIKV in nature

Genus	**Species**	**Geographic distribution**
Aedes	*aegypti*	Worldwide (peridomestic)
Aedes	*africanus*	African (sylvatic)
Aedes	*albopictus*	Worldwide (peridomestic)
Aedes	*calceatus*	Africa (sylvatic)
Aedes	*dalzieli*	Africa (sylvatic)
Aedes	*furcifer*	African (sylvatic)
Aedes	*luteoceophalus*	Africa (sylvatic)
Aedes	*neoafricanus*	African (sylvatic)
Aedes	*taylori*	African (sylvatic)
Aedes	*vittatus*	Africa, Mediterranean, Southeast Asia (sylvatic)
Anopheles	*coustani*	Africa, Middle-east (sylvatic)
Anopheles	*rufipes*	Africa (sylvatic)
Anopheles	*gambiae*	Africa (sylvatic)
Culex	*ethiopicus*	Africa (sylvatic)

(Diallo et al. 1999, Gilotra et al. 1967, Knight et al. 1977, Reiter et al. 2006, Weinbren et al. 1958)

two mosquito species that are mostly associated with CHIKV in the African jungle are *Ae. furcifer and Ae. africanus*. In the urban setting, where CHIKV is mainly a human/mosquito transmission cycle, *Ae. aegypti* and *Ae. albopictus* are the main vectors for transmission (Figure 9).

While both sylvatic and urban cycles of CHIKV are found in Africa, in Asia CHIKV appears to exist only in an urban cycle. To date, no animal reservoir has been found in Asia, so CHIKV is presumed to be maintained in a mosquito-human cycle. CHIKV seems able to evolve and adapt to many different mosquito vectors depending on the environment and ecological surroundings. Recent data suggests that a new mutation in the CHIKV has made the virus more fit for the *Ae. albopictus* mosquito (Tsetsarkin et al. 2006).

Mosquito vector-virus biology

Most arboviruses cause little pathology or harm to their mosquito vector. Transmission by the mosquitoes is biological with replication of the virus in multiple tissues of the mosquito. Arboviruses share many obstacles that they must overcome in order to be maintained in nature in an animal-vector cycle. In the mosquito, these obstacles are called “escape barriers” (Hardy et al. 1978, Houk et al. 1979). The female mosquito must first feed on a viremic host with viral titers that are capable of infecting the mosquito. CHIKV causes a viremia in people that has been reported to reach as high as $10^{5.5}$ plaque forming units (PFU) per milliliter (mL); while in macaques, a similar viremia level (10^{5-6} PFU/mL) was detected (Chen et al. 2010, McIntosh et al. 1963). The infectious dose for 50% of *Ae. albopictus* mosquitoes with CHIKV has been shown to range

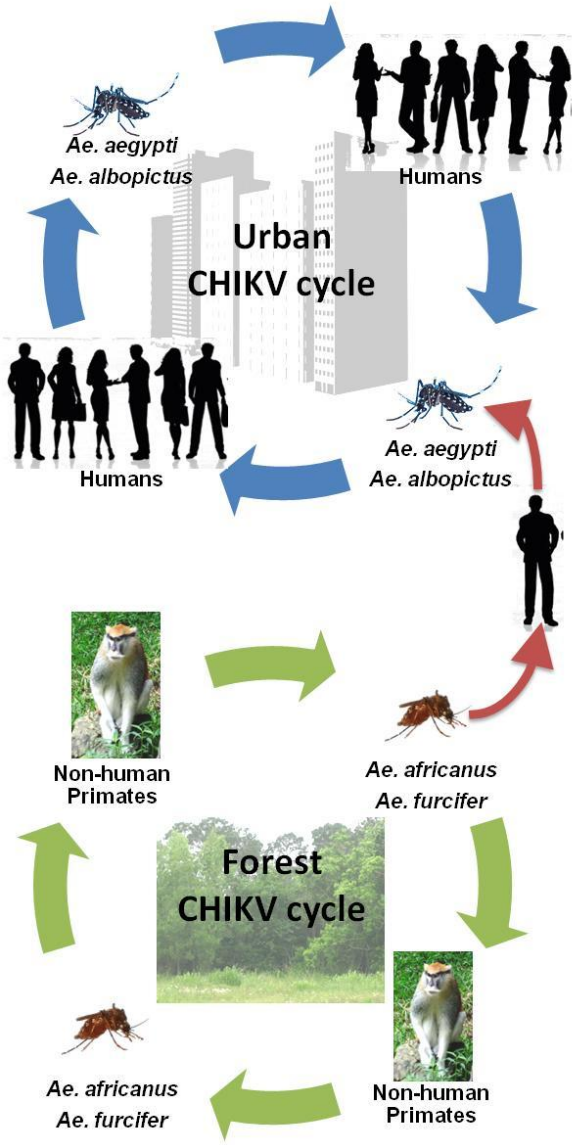


Figure 9: Transmission cycles of CHIKV

from 10^3-$10^{5.5}$ tissue culture infectious dose 50 percent endpoint (TCID50)/mL (Tsetsarkin et al. 2007). In a laboratory setting the amount of virus needed to infect 50% of *Ae. aegypti* mosquitoes is in the range of 10^5 PFU/mL of CHIKV (Pesko et al. 2009, Tsetsarkin et al. 2007). These data illustrate how maintenance of CHIKV in the wild can occur in a cycle consisting of humans or primates with *Ae. albopictus* and *Ae. aegypti* vectors.

The extrinsic incubation period of an arbovirus begins when the female mosquito ingests an infectious bloodmeal (Figure 10) (Higgs 2004). This bloodmeal enters the mosquito midgut lumen. If the mosquito is susceptible and the infectious dose of virus is above the threshold, then the virus will bind and infect the epithelial cells lining the mosquito midgut. The infection usually starts as a limited number of target cells and spreads to larger numbers of epithelial cells (Higgs 2004). Once

infection and replication of the CHIKV in the cells of the midgut have occurred, the virus particles leave the midgut and enter the haemocel. When the virus enters the haemocel, virus can quickly infect multiple organs in the mosquito, including the legs, wings, salivary glands and fat body. CHIKV dissemination has been studied in *Ae. albopictus* mosquitoes, where it has been shown that as soon as 2 days after ingestion of an infected blood meal, the wings and salivary glands are infected with CHIKV (Dubrulle et al. 2009, Vazeille et al. 2007). In the laboratory setting, *Ae. albopictus* have been shown to remain infected for up to 40 days post infection with CHIKV (Tesh et al. 1976). Rates of infection and dissemination are dependent on temperature, with higher temperatures increasing the rate at which a mosquito develops a disseminated infection (Higgs 2004). Once the salivary glands are infected, virus can be secreted into the saliva of the mosquito; as the insect probes and feeds, virus is transmitted to the

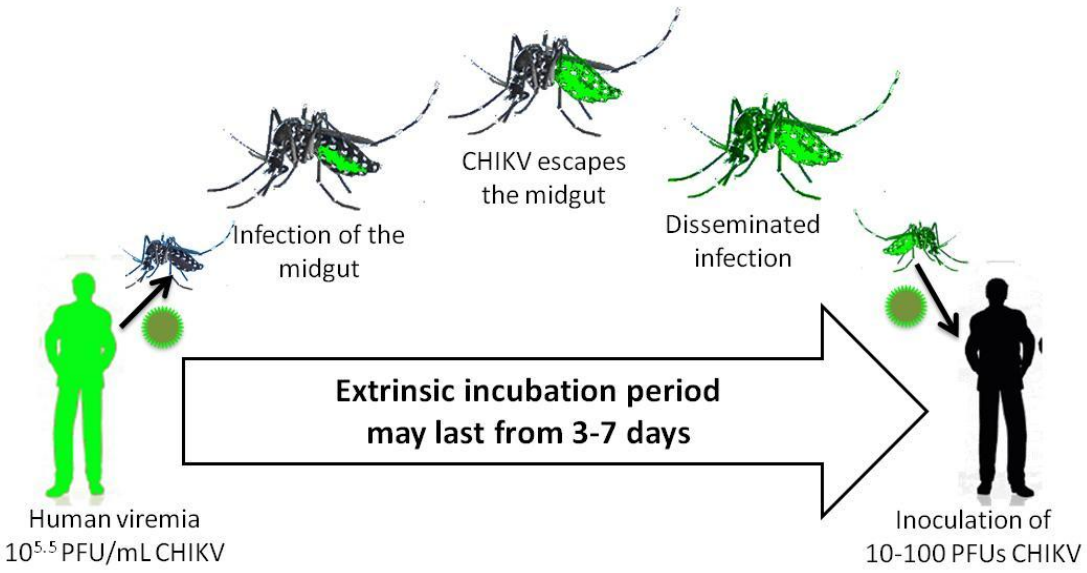


Figure 10: Extrinsic Incubation period of CHIKV in mosquitoes

host (Labuda et al. 1989). Dubrulle et al. (2009) demonstrated that as early as 2 days after oral infection both *Ae. aegypti* and *Ae. albopictus* mosquitoes had infectious virus in their saliva. It is possible for a mosquito to transmit and to infect its host with CHIKV by probing alone. While it is difficult to estimate the exact amount of virus that a mosquito might transmit, excreted saliva has been shown to contain up to $10^{3.3}$ PFU of infectious CHIKV (Dubrulle et al. 2009). This value is comparable to eastern equine encephalitis virus (EEEV) and Venezuelan equine encephalitis virus (VEEV) (Smith et al. 2006, Weaver et al. 1993). It is lower than some flaviviruses which have been reported to be in the range of 10^4 - 10^5 PFU of virus (Vanlandingham et al. 2005).

Mosquito saliva

An important aspect of arbovirus transmission is the role of mosquito saliva during inoculation. There have been numerous studies to identify the proteins and biological components of mosquito saliva. In mosquito saliva there are proteins that prevent blood coagulation as well as proteins that down regulate the host's immune system and cause a change in the type of immune response that is elicited. Each mosquito species delivers a different spectrum of proteins in their saliva. SAAG-4 is one protein that has been identified in *Ae. aegypti* saliva that can enhance the CD4 T cell induction of IL-4 and enhance the Th2 response (Boppana et al. 2009). This has been confirmed in mice infected with West Nile virus, where mosquito saliva enhanced the Th2 response (Schneider et al. 2008).

These immunomodulatory affects may change the course of disease progression in mice as compared to needle inoculation. Mosquito saliva has

been shown to enhance West Nile virus infection in mice, as compared to needle inoculation; mice bitten by infected mosquitoes had higher mortality that those inoculated with virus (Schneider et al. 2007, Styer et al. 2011). Studies with vesicular stomatitis virus have shown that mice infected by mosquito bite had a more pronounced antibody response, as compared to those infected by needle inoculation (Limesand et al. 2000). Mosquito saliva has been shown to recruit immune cells to the bite site including an abundance of eosinophils and some neutrophils, which are absent at needle inoculation sites (Thangamani et al. 2010). These enhanced infections are thought to be the result of a decrease in the anti-viral Th1 immune response due to the mosquito saliva. This effect has been shown to be systemic and can last up to 72 hours after inoculation in mice (Schneider et al. 2004). It is important to understand that these studies have focused on mouse/mosquito saliva interactions where multiple mosquitoes fed on each mouse. While these studies are useful to measure the types of responses induced, they may be different in humans since the ratio of saliva to host body is different and the human response to the components of mosquito saliva may not be the same.

Immunopathology

It has been proposed that CHIKV-pathogenesis in humans is immune-mediated. This theory has much of its base in work with RRV infection in mice and preliminary studies with CHIKV infection in mice and primates. In mice infected with SINDV, cellular damage is caused directly by virus replication, while RRV causes disease through inflammation mediated by macrophages and complement (Ryman et al. 2008). This is an important distinction of the Semliki

Forest complex viruses. In this clade of viruses, removing specific aspects of the innate immune system decreases the pathogenesis of the disease in mice.

Immunopathology implies that an infection results in the immune system causing significant pathology to the host. Alphavirus induced immunopathology is theorized to occur through the cells of the innate immune system. The complete pathway that is involved in this process is still unclear, but in RRV, macrophages and complement have been shown to cause myositis in mice (Lidbury et al. 2008, Morrison et al. 2008, Rulli et al. 2009). Natural killer (NK) cells have been shown to be decreased in number and activity in patients with RRV infection (Aaskov et al. 1987). RRV has also been shown to inhibit the classical activation of the complement pathway (Aaskov et al. 1985).

The generalized immune response to any SC viral infection begins with replication at the site of inoculation, viral dissemination to the regional lymph nodes, viremia and then infection in other tissues including muscle, spleen, pancreas, liver and connective tissues (Ryman et al. 2008). It is thought that in CHIKV infection, viral replication in dendritic and/or macrophage cells causes a dysregulation of the pro-inflammatory cytokine response (Ryman et al. 2008). IFN-α and IFN-β are the hallmark innate immune response to viral infections, released by lymphocytes and monocytes, which cause uninfected cells to become resistant to viral infection (Pier et al. 2004). Type 1 interferon is also released by dendritic cells. NK cells are also important early during viral infection and are capable of inducing apoptosis of virus- infected cells (Abbas et al. 2007). NK cells are also activated by interferon.

Hypersensitivity reactions are immunological responses that cause the body harm. They are common in poison ivy reactions, mosquito bites and

tuberculoses granuloma formation. Delayed type hypersensitivity (DTH) reactions occur through the activation of CD4+ T cells and high levels of cytokine production including IFN-γ, IL-2 and IFN-β (Pier et al. 2004). These cytokines are regulated by Th1 cells of the immune system. It begins with sensitization by dendritic cell presentation of antigen to local T cells. The effector phase of the reaction causes activation of complement, increased tumor necrosis factor – alpha (TNF-α), and recruitment of monocytes, T cells and some basophils. This response causes a large increase in activated macrophages and the release of cytotoxic chemicals including oxygen radicals and nitric oxide. This response is a delayed reaction with the release of lytic enzymes approximately 48 to 72 hours after exposure (Pier et al. 2004). While this response is effective in bacterial killing, it can result in injury to neighboring tissues. DTH responses have also been shown to be critical in herpes virus, poxvirus and HIV infections (Pier et al. 2004).

In some noncytopathic viruses, the tissue injury can be due to cytotoxic T lymphocytes (CTL). One example of this is in lymphocytic choriomeningitis virus (LCMV), where the virus infects the menigeal cells, but actual cell killing is done by activated CTLs. Mice deficient in T-cells will not develop meningitis with LCMV infection, but they become chronic carriers (Abbas et al. 2007).

Dendritic cells have been shown to be important cell targets for some arboviruses including dengue, yellow fever viruses and some neurotropic alphaviruses including VEEV and SINDV (Monath et al. 2003, Rodriguez-Madoz et al. 2010, Ryman et al. 2008). CHIKV pathogenesis has not been shown to be dependent on dendritic cell infection (Sourisseau et al. 2007). It is believed that

alphaviruses target macrophages during the beginning of an infection (Ryman et al. 2008).

Th1 vs Th2

The innate immune system initial response to infection can be classified as either a Th1 or Th2 response by the type of cytokines that are induced (Table 5). For most viral infections, a Th1 response is triggered with the induction of IL-2 (Mosmann et al. 1989). A typical Th2 response is triggered in response to an allergic stimuli. Typically, this involves IL-4. Mixed responses may occur if the signal is not weak or prolonged, but inhibited IgE and DTH responses may result (Mosmann et al. 1989). It is also possible for Th1 and Th2 responses to occur in separate parts of the body as long as each effect is not systemic (Mosmann et al. 1989).

Table 5. Th1 and Th2 responses

Adapted from Mosmann et al. 1989

Th1	**Th2**
IFN-γ	IL-4
IL-2	IL-5
IL-12	IL-6
IL-18	IL-13
IFN α/β	IgE
DTH	
Classical macrophage activation	Alternative macrophage activation

Hypothesis and Specific Aims

The objective of this proposal is to utilize a mouse model of CHIKV infection to study the role of macrophages in the pathogenesis of CHIKV infection. The central hypothesis for this work is that the severe myositis seen in CHIKV-infected mice is due to macrophage involvement during infection and replication of the virus in the cells and organs of the immune system. It is also hypothesized that the immune system responds differently to mosquito bite and

to needle inoculation of CHIKV and that these two routes of infection will show a different disease progression and outcome. To accomplish the objective of this proposal, the following four specific aims were pursued:

Specific Aim 1: Develop a small animal model of CHIKF

Develop a small animal model of CHIKV infection to elucidate the pathogenesis of CHIKV infection in humans. By using this model, parallels could be drawn between the disease in humans and infection in mice. Mice show classic signs of CHIKV infection including myositis in the skeletal muscle and viremia.

Specific Aim 2: Determine the sites of viral replication in CHIKV-infected mice

To understand the role that macrophages play in disease pathogenesis, studies were done to understand the tissues that were infected and showed pathology in mice. This was done with innovative techniques using luciferase and green fluorescent protein (GFP) to visualize CHIKV replication.

Specific Aim 3: Characterize the viral pathogenesis of CHIKV in mice exposed to mosquito feeding

Mosquito bite is the natural route of infection and mosquito saliva modulates the immune system. It is therefore important to understand how mosquito bite-inoculation could affect pathogenesis in mice. It has been shown that mosquito bite decreased the amount of pathology seen in mice infected with CHIKV, while viremia levels were similar. This difference was due to both dose and mosquito saliva.

Specific Aim 4: Evaluate immune modulators as possible therapeutics for CHIKV infection

Since the role of the immune system has been shown to be important for CHIKV pathogenesis and that immunopathology may be involved it was hypothesized that modulating the immune system with pharmaceuticals may decrease the pathogenesis of CHIKV in mice. Genetically engineered mice with decreased interferon signaling were more susceptible to CHIKV infection which was fatal. Chloroquine treatment had no effect on CHIKV infection in mice. Cyclophosphamide, which decreases the lymphocyte response, showed no difference in CHIKV pathogenesis. Dexamethasone showed promising results as a possible CHIKV therapeutic.

Chapter 2: Materials and Methods

Unless otherwise stated in specific chapters, the materials and methods described in this chapter apply to the research described elsewhere in this dissertation. This presentation approach, to assemble all materials in methods in a single dedicated chapter was done in order to reduce unnecessary repetition of procedures in multiple chapters.

Cell culture system

Vero cells (V76) were maintained using common cell culture techniques. Vero cells were grown at 37°C with 5% carbon dioxide in minimum essential medium (MEM), supplemented with 5% fetal bovine serum (FBS), 2% sodium bicarbonate, 2mM L-Glutamine and 1% Penicillin-Streptomycin (Invitrogen, Carlsbad, CA). C6/36 cells (*Ae. albopictus*) were grown at 27°C in L15 media (Invitrogen, Carlsbad, CA) supplemented with 10% Tryptose Phosphate Broth solution (Sigma-Aldrich, St. Louis, MO), 10% FBS, 1% Penicillin-Streptomycin and 2mM L-Glutamine (Invitrogen, Carlsbad, CA).

Virus

CHIKV strain LR 2006-OPY1 was used for mouse infections. The virus isolate was obtained from a CHIKF patient during an outbreak on La Reunion Island in 2006. The virus was kindly provided to us by Dr. Remi Charrel, Emerging Virus Unit, Faculty of Medicine, University of the Mediterranean, Marseilles, France. A single virus stock was made from the supernatant of infected Vero cells and 1 mL aliquots were generated. This stock was used for all experiments involving CHIKV. The passage history of this virus includes 1

passage in sucking mice, 1 passage in C6/36 cells and 5 passages in Vero cells. Before use in experiments, virus stock was not frozen and thawed more than two times. Virus stock was at a concentration of $10^{7.17}$ PFU/mL and for mouse inoculations, virus stock was diluted 1:50 in diluent (phosphate-buffered saline (PBS) with 15% FBS).

Animals

The model used for most studies were young (13-15 days old) CD-1 mice obtained from Charles River Laboratories (Wilmington, MA) which has previously published (Ziegler et al. 2008). The Charles River CD-1 mice are closely related to the Institute for Cancer Research (ICR) strain. The CD-1 mice were obtained by Charles River in 1959 from the Roswell Park Memorial Institute where they were designated as HaM/ICR. The strain originated from Swiss mice and was imported into the US in 1926 to the Rockefeller Institute.

ICR mice used for experiments were obtained from Harlan Sprague-Dawley and treated in the same way as the CD-1 mice (Indianapolis, IN). Adult A129 mice were obtained from the laboratories of Nigel Bourne, Ph.D. and Greg Milligan, Ph.D. and were deficient in IFN-α and IFN-β. Syrian Golden Hamsters were obtained as 6 week old females from Harlan Sprague Dawley (Indianaoplis, IN). Mongolian gerbils were obtained from Charles River Laboratory (Wilmington, MA).

The newborn mice used for these studies were 3-5 days old. The young mice used were 13-15 days old. These mice were obtained as pregnant females and allowed to give birth in our facility. If more than one litter was used, at approximately 3-days old all the pups from multiple mothers were randomized

and an even distribution of the pups were put with each mother. In newborn mice, this was done at the time of inoculation. By doing this, variations between different litter sizes and mothers was minimized and the exact age of all mice was known.

All animals were cared for in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources National Research Council, Washington, DC). All experiments with CHIKV were conducted in an Animal Biosafety level 3 (ABSL-3) or Arthropod Containment level 3 (ACL-3) facility under a protocol approved by the University of Texas Medical Branch (UTMB) Institutional for Animal Care and Use Committee (IACUC).

Animal inoculations

Unless otherwise noted, mice inoculations were SC in the skin of the back. SC injections were with a volume of 0.1 mL in the skin of the lower region of the back. The mouse was immobilized with forceps, while the skin was tented and the inoculum was injected without the use of anesthesia. Footpad inoculations were used in some experiments. For footpad inoculations, a total volume of 0.04 mL was injected into a single rear footpad. Mice were lightly anesthetized with isoflurane and immobilized, while the inoculum was injected subcutaneously into the footpad area. Unless otherwise noted, all mice were inoculated at a dose of $10^{4.5}$ PFU/mL with the CHIKV LR 2006-OPY1.

Weight gain in newborn mice

The daily weight gain and development were compared by taking newborn mice that were randomized and distributed equally between three respective

mothers. Two litters were used for the infected group, and the third litter served as the control group. The animals were weighed each day, using a Scott ProBalance (OHAUS, Pinebrook, NJ). Mean weight gain was calculated by subtracting the weight of the pups on the day of inoculation. For young mice weight gain studies, 5 mice per group, per day were weighed and the average was reported.

Plaque assay

Samples of mouse blood, brain, liver, spleen and hind limb muscle were titrated by plaque assay in monolayer cultures of Vero cells. Blood samples were diluted 1:10 in diluent (PBS with 15% FBS). Tissues were first homogenized in individual sterile 2-mL glass TenBroeck tissue grinders (Kimble/Kontes, Vineland, NJ) in 1.0 mL of diluent to prepare ~10% (wt/vol) suspensions. After centrifugation, serial 10-fold dilutions from 10^{-1} to 10^{-6} of the tissue supernatants and of the blood samples were prepared in diluent. Duplicate wells of 24-well microplate cultures of Vero cells were inoculated with each dilution. After virus absorption for 1 hour and addition of an overlay, the cultures were incubated at 37°C; a second overlay was added 3 days later and plaques were counted 5 days after inoculation of the virus. Overlays were composed of 2X MEM, 2% sodium bicarbonate, 2mM L-Glutamine, 1% Penicillin-Streptomycin, 2% neutral red and 1% agar. Virus titers were calculated as the number of plaque-forming units (PFU) per milliliter of blood or tissue suspension. The limit of detection of the plaque assays was 100 PFU/mL or PFU/gm. If a single plaque was present in one well the sample was scored as 50 PFU/mL. If no plaques were seen, the sample was scored as 10 PFU/mL.

Mice were killed and necropsied each day. Before death, a blood sample was collected for virus assay and serology. At necropsy, samples of selected organs (brain, leg muscle, liver and spleen) were obtained for histopathologic examination and culture. The hind legs were severed above the hip joint and the skin removed; one leg was used for virus assay and the other for histopathology. For virus assay, the foot was also removed and the quadriceps muscle of the upper thigh with the bone was homogenized.

Serial passage of CHIKV in mice

CHIKV was serially passaged from mouse leg samples. Virus was inoculated into a litter of 3-day old ICR mice SC in the back in a volume of 0.1 mL. Five dpi mice were killed and the hind limb was taken and homogenized. Hind limb samples from five mice were pooled together, centrifuged and filtered. The filtrate was then re-inoculated into a new litter of 3- day old mice. Virus was passaged 19 times.

Mosquitoes

Ae. aegypti (Rexville D strain Higgs white-eye) and *Ae. albopictus* (Galveston) mosquitoes were reared at 27°C with a relative humidity of 80% under a 16-hour light: 8-hour dark photo period, as previously described (Higgs 2004). Four to six days post-eclosion, female mosquitoes were intra-thoracic (IT) inoculated with virus or fed an artificial bloodmeal using the Hemotek feeding system (Discovery Workshops, Accrington, UK) in an isolation glove box located in an ACL- 3 insectary. For artificial feeding, a 1:1 mixture of defibrillated sheep's blood (Colorado Serum Company, Boulder, CO) and virus stock was heated to 37ºC and feeders were placed on the mosquito cartons. After feeding,

mosquitoes were sorted and fully engorged mosquitoes were transferred to a container. For IT inoculation, approximately 5 µL of viral stock was inoculated into the abdomen of the anaesthetized mosquitoes on a cold plate. After infection, all mosquitoes were transferred to an environmental chamber at 27°C and supplied with 10% sucrose *ad libitum* (Higgs 2004).

Animal Inoculation as per mosquito bite

For mouse inoculation per mosquito bite, IT infected mosquitoes were sorted into groups of five and placed in separate containers. Sugar was removed from the cartons 48 hours prior to feeding and the mosquitoes were allowed water *ad libitum*. Each mouse was anaesthetized with an intra-peritoneal (IP) injection of 0.1 mL of 2.5mg/mL Nembutal in PBS. Once mice were sedated, a single foot was placed through a slotted index card and the mouse was placed on the top of the carton to allow the mosquitoes to feed only on the selected foot. For a few of the experiments, an ear was placed through the slot of the index card instead. If location of mosquito bites was not controlled for, the anesthetized mouse would be placed on top of the screen of the mosquito carton with no barriers. Mice were not removed until at least three of the mosquitoes were fully engorged.

Production of antibody to mosquito saliva

For production of mosquito saliva antibodies four adult mice was used each for *Ae. aegypti* or *Ae. albopictus* mosquitoes. Greater than 25 mosquitoes were allowed to feed on the mouse each week for a 4 week period. One week after the last feeding, each mouse was injected with sarcoma cells. Hyperimmune ascitic fluid (HIAF) was collected from each mouse, centrifuged

and the supernatant was frozen as a stock of HIAF against each mosquito species.

CHIKV-GFP and CHIKV-LUC clones

CHIKV-GFP has been described earlier (Tsetsarkin et al. 2006). The creation of the infectious clone of CHIKV was using a double sub-genomic promoter system, with the enhanced GFP under the control of the first promoter (Figure 11). CHIKV-LUC clone was generated from the same backbone as the CHIKV-GFP which has been described in more detail previously (Ziegler et al. 2011). This infectious clone takes advantage of the humanized *Renilla* luciferase gene (Promega, Madison, WI).

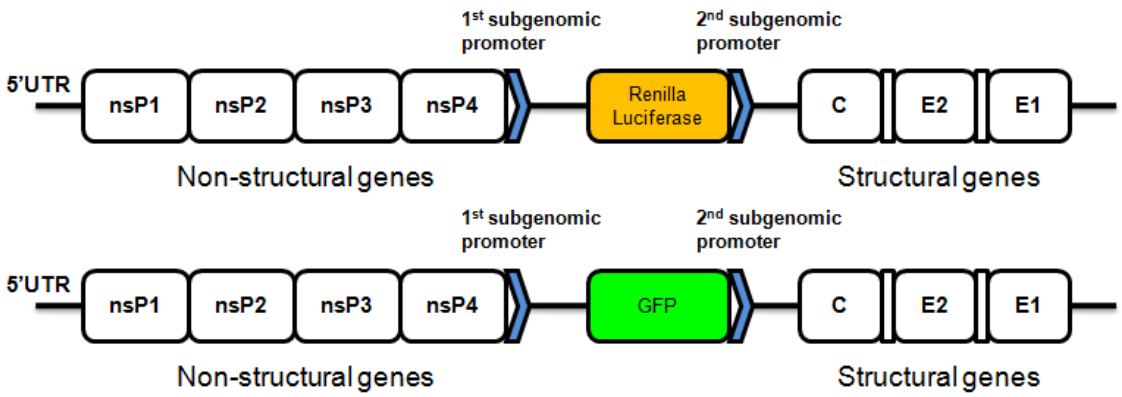


Figure 11: Genome map of CHIKV-GFP and CHIKV-LUC infectious clones

GFP visualization of mouse tissues

Mouse tissues were sampled at necropsy and placed on microscope slide to be visualized by fluorescent microscopy. Later studies utilized a cryostat to section tissues at 10 nm and then placed on microscope slides with a glycerol

overlay with microscope coverslips. GFP infected tissues were visualized by viewing through an Olympus IX51 epiflourescence microscope.

Preparation of ViviRen substrate

ViviRen substrate was purchased from Promega Corporation (Madison, WI). The substrate was first dissolved in DMSO (0.37mg in 10µL of DMSO) and diluted in PBS with 10% FBS to a final concentration of 0.236mM. Prior to imaging, 50µL of the stock solution was injected in each mouse at a dose of approximately 1mg/kg (Otto-Duessel et al. 2006).

***In vitro* infections and luciferase imaging**

For *in vitro* studies of CHIKV-LUC a 6-well tissue culture plate was seeded with C6/36 cells or Vero cells and 48 hours later, when cells were confluent, they were infected with CHIKV-LUC at a concentration of $10^{4.5}$ $TCID_{50}$. Briefly, 100uL of viral stock was added to each well and was allowed to incubate for one hour at 27°C (C6/36) or 37°C (Vero cells). After incubation, the appropriate medium was added to each well. Cells were imaged in the 6-well plate 24 hours post-infection (hpi). The cell culture plate was placed in the Xenogen *in vivo* imaging system (IVIS®) 200 Series (Caliper Life Sciences, Hopkinton, MA)) and images were recorded immediately before and after the addition of the ViviRen substrate. Prior to substrate addition, cell medium was removed and fresh medium was added. ViviRen was dissolved in dimethyl sulfoxide (DMSO) and diluted in phosphate buffered saline (PBS) with 10% FBS and added to the tissue culture plate wells to a final concentration of approximately 1-0.1 nM. An exposure time of 1 second was used to image the luciferase expression in cell culture.

Mosquito IVIS with CHIKV-LUC

At 3 and 7 dpi, mosquitoes were chilled and legs and wings were removed. A stock solution of the substrate ViviRen (0.24mM) was IT inoculated to visualize the luciferase expression from the CHIKV-LUC infection. Mosquitoes were placed into a 6-well plate and imaged using the Xenogen IVIS instrument located in a Biosafety level 3 (BSL3) laboratory. An exposure time of 5 seconds was used and images were taken 30-40 minutes following injection of substrate.

CHIKV-LUC imaging in mice

Mice were anesthetized and inoculated in the right rear footpad with $10^{5.5}$ $TCID_{50}$ of CHIKV-LUC in a total volume of 0.04mL in PBS. At set time points, mice were anesthetized and injected with the ViviRen substrate. Mice were imaged immediately prior to substrate injection. Immediately after substrate addition, mice were returned to the IVIS chamber and were imaged every minute for time course studies. For later experiments, substrate was allowed to diffuse in the mice for 20 minutes before images were taken. CHIKV 5' GFP was used as a negative control and injected at the same concentration and location as the CHIKV-LUC. GFP signal can only be seen with the IVIS using the fluorescent lamp, which is not used during luciferase imaging. The exposure time for the images was 30 seconds or 2 minutes (as noted) and region of interest (ROI) calculations were made using the IVIS Living Image Software (Caliper Life Sciences, Hopkinton, MA).

Immune response.

The humoral immune response of the mice to CHIKV infection was measured by hemagglutination inhibition (HI) test. A standard HI technique was

used. Antigens for the HI test were prepared by the sucrose acetone extraction method from brains of newborn mice infected with CHIKV and treated with propiolacetone. Mouse sera were tested at serial 2-fold dilutions from 1:20 to 1:1,280 at pH 6.6 using four units of antigen and a 1:200 dilution of goose erythrocytes.

Histologic and immunohistochemical examination.

At necropsy, selected tissues and limbs from each mouse were fixed in 10% neutral-buffered formalin before being processed for routine paraffin embedding. Hind limbs were decalcified with formic acid prior to embedding in paraffin. Several 4- to 5-m sections were made from each tissue; one section was stained by the hematoxylin and eosin method (H&E), and the others were used for immunohistochemical (IHC) studies to localize viral antigens. A CHIKV HIAF was used as the primary antibody that was biotinalated; this was detected by streptavidin-peroxidase conjugate, followed by substrate, as described before (Xiao et al. 2001).

Histopathological analysis was initially done by Judith Aronson, M.D. who then trained myself. Pathology samples were blindly analyzed. Initial screening was done at 4x magnification with further confirmation of necrosis and neutrophils at 40x magnification.

Dexamethasone, cyclophosphamide and chloroquine

Dexamethasone was given as a single injection IP at a dose of 0.2 mg per mouse. Dexamethasone was obtained as a 4 mg/mL solution and 0.05mL was injected into each mouse. Cyclophosphamide treatment was given every fourth day IP. Cyclophosphamide was reconstituted with PBS to a concentration of 20

mg/mL and 0.06 mL was given to each mouse. This resulted in a dose of 100mg/kg body weight. Chloroquine phosphate was dissolved in sterile PBS to a concentration of 12 mg/mL. Each mouse received a single dose of 0.1 mL or 0.05 mL IP for a final dose of approximately 80mg/kg or 40 mg/kg respectively.

Hematology and chemistry analysis of blood samples

Enzyme levels were analyzed in the ABSL-3 with an Abaxis VetScan VS2 (Union City, CA). The comprehensive profile analysis rotors were used which analyze albumin, alkaline phosphatase, alanine transaminase, amylase, blood urea nitrogen, calcium, creatine, globulin, potassium, sodium, phosphorous, total bilirubin and total protein. A blood sample was collected from mice prior to euthanization in a lithium heparin coated tube and 100uL of whole blood was placed into the rotor. The rotor was immediately placed into the VetScan machine. The Drew scientific HEMAVET 950 was used for analysis of complete blood chemistries (Dallas, TX). Blood samples were placed in an EDTA coated tube and the tube was placed within the HEMAVET machine for automatic sampling of approximately 20 µL of whole blood.

Graphing and statistical analysis

GraphPad Prism 5.0 was used for all graphing as well as arithmetic calculations. Points on graph were of arithmetic mean and standard error measurement unless otherwise noted on graph. Geometric averages were calculated by Graph Pad. Statistical analysis was done using GraphPad InStat 3.10 with the Mann-Whitney test between individual values. Two values were determined to be statistically significant if the p value < 0.05.

Chapter 3: Small animal model of CHIKV infection

Abstract

Animal models are important for many aspects of viral disease research including investigations of disease pathogenesis, anti-viral treatments and vaccine safety and efficacy. To aid in all aspects of CHIKV research, development of a small animal model is fundamental. While modeling of RRV has been successful in mice, CHIKV has less pathogenesicity in mice as compared to RRV. The aim of these studies was to develop a small animal model of CHIKV infection. Presented here are the details of infection in different rodent species and age groups. A mouse model of CHIKV was developed and characterized in young CD-1 mice. This mouse model was characterized by an acute febrile illness with severe myositis in the skeletal muscle.

Introduction

The efforts to produce a small animal model for CHIKV have been renewed with the recent large outbreaks of CHIKV and the large number of possible vaccine candidates that need a platform for testing. Presently, an animal model that truly represents human disease still has not been found. Ross was one of the first scientists to inoculate CHIKV into mice (Ross 1956). He inoculated human sera from sick patients intracranally into young mice, which resulted in sporadic mortality in the mice. More recently, different inoculation strategies, including footpad inoculation have been used, not to produce mortality, but to produce human-like CHIKF illness. Different strains of immune deficient mice were also tested, but severe muscle and joint pathology is lacking in these models (Couderc et al. 2008).

The consequences of CHIKV inoculation of mice is dependent on strain, age, route, dose and viral strain of the inoculum. The basis of the young mouse model presented in these studies is in previous work with RRV. Early work with RRV determined that there was a strong correlation to age and disease severity in mice, whereas immune suppression was not a determinant of disease severity in older mice (Seay et al. 1981). Young CD-1 and C57BL/6 mice were infected with RRV in the footpad which showed severe inflammation and established a mouse model of RRV (Morrison et al. 2006). Further work with this model of RRV infection showed that T and B lymphocytes were not essential for myositis, while macrophages and complement play an important role in RRV-induced pathology and that RRV-induced pathology is immune-mediated (Lidbury et al. 2000, Lidbury et al. 2008, Morrison et al. 2007, Rulli et al. 2009)

The objective of this aim was to develop a small animal model for the study of the pathogenesis of CHIKV infection and to relate that to human disease. Many different rodent species were analyzed initially for their potential as a model. It was hypothesized that young (13-15 day old) CD-1 mice would be good model of CHIKV infection because of previous work with RRV. The results of these studies have been previously published (Ziegler et al. 2008).

Results

Viremia and viral loads in selected in CHIKV-infected mice

During the course of these studies, it was observed that CHIKV infection was not lethal in adult or young outbred mice inoculated SC. Using high-passage strains of CHIKV (Ross strain) or using intranasal or intracranial inoculation, higher rates of mortality would be expected. CHIKV had limited

lethality in newborn mice inoculated SC (Figure 12). In 3-day old ICR mice, mortality was 10%, while in CD-1 mice mortality was 18%. Newborn mice inoculated SC in the back experienced hind limb dragging and lethargy approximately 10 dpi but recovered completely by 21 dpi. Newborn mice inoculated with CHIKV also developed patches of alopecia on their back near the site of inoculation. This seemed to be due to an inhibition of hair growth, not a loss of existing hair. This also seemed to be transient; because by 28 dpi the hair growth appeared normal over the back.

While mortality was uncommon in newborn mice infected with CHIKV, these animals did develop disease. CHIKV-infected newborn mice did not gain weight as rapidly as uninfected mice. This could be seen in ICR mice infected SC with CHIKV. As early as the second dpi, there was a significant difference in the weight gain as compared to uninfected mice (Figure 13). Newborn ICR mice

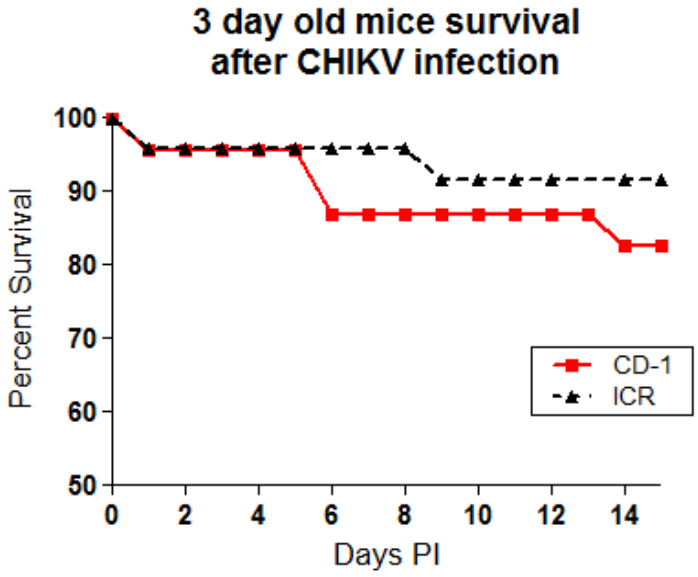


Figure 12: Survival of newborn mice infected with CHIKV

seemed to recover rapidly and by 14 dpi there was almost no difference between the infected mice and the control mice. In CD-1 mice, there was a larger difference and it was significant from 5 dpi and lasted at least until 14 dpi when the mice had cleared the infection (Figure 14). This difference illustrated that early in CHIKV infection, the disease affected the overall health of the newborn mice.

Even though newborn mice infected with CHIKV had high levels of viremia and cleared the virus, their HI antibody response was minimal (Table 6). In newborn ICR mice infected with CHIKV, HI antibody was not detected until 9 dpi and then only in one of five mice tested. Three weeks after CHIKV inoculation in newborn mice only 60% of the mice had detectable HI antibody titers. It was assumed that this was an age dependent phenomenon and older mice were investigated

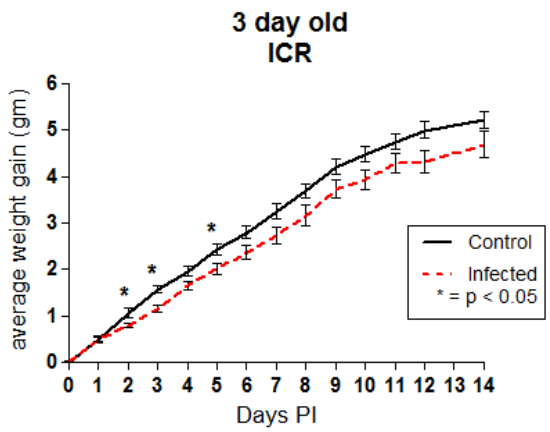


Figure 13: Weight gain of newborn ICR mice infected with CHIKV

3-5 day old ICR mice inoculated SC in the back with either CHIKV or PBS were weighed daily.

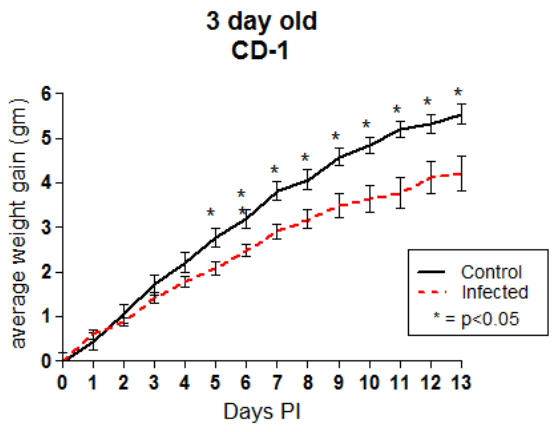


Figure 14: Weight gain in newborn CD-1 mice infected with CHIKV

3-5 day old CD-1 mice inoculated SC in the back with either CHIKV or PBS were weighed daily.

Table 6: Antibody Production in 3 day old ICR mice

Days PI	Mice with positive HI antibody ≥1:20
7	0/4
8	0/4
9	1/5
10	1/3
11	1/3
12	3/5
15	3/7
17	7/7
22	6/10

. To characterize viral loads in both young and newborn mice, multiple studies were undertaken. All mice were inoculated with CHIKV at a dose of $10^{4.5}$ PFU SC in the skin of the back. Newborn mice were inoculated at 3-5 days old, and young mice were inoculated at 13-15 days old. Mice were necropsied and samples taken every 24 hours and viral titers were determined by plague forming assays. At each time point at least 3 mice were sampled. Viremia levels in CD-1 mice were higher in newborn mice than in young mice (Figure 15). The same pattern was seen in ICR infected mice (Figure 16). Both in ICR and CD-1 newborn mice viremia levels started at approximately 10^6 PFU/mL, and peak levels were more than 10^7 PFU/mL. Virus was isolated from all newborn mice for 5 dpi and occasionally up to 8 dpi. In young mice, virus was isolated 3 to 4 dpi

Table 7: Antibody levels in 14-day old CD-1 mice

HI antibody levels were assessed in serum of young mice daily. Initial serum dilution was 1:20.

Days PI	1	2	3	4	5	6	7	8	9	10	11
M1	<1:20	<1:20	<1:20	<1:20	**1:40**	**1:80**	**1:320**	**1:80**	**1:320**	**1:320**	**1:160**
M2	<1:20	<1:20	<1:20	<1:20	**1:20**	**1:20**	**1:320**	**1:80**	**1:160**	**1:160**	**1:320**
M3	<1:20	<1:20	<1:20	<1:20	**1:80**	**1:80**	**1:80**	**1:80**	**1:80**	**1:20**	**1:320**
M4	-	-	<1:20	<1:20	**1:40**	**1:40**	**1:160**	**1:160**	**1:320**	-	-
M5	-	-	-	<1:20	**1:40**	**1:20**	**1:320**	**1:40**	**1:320**	-	-

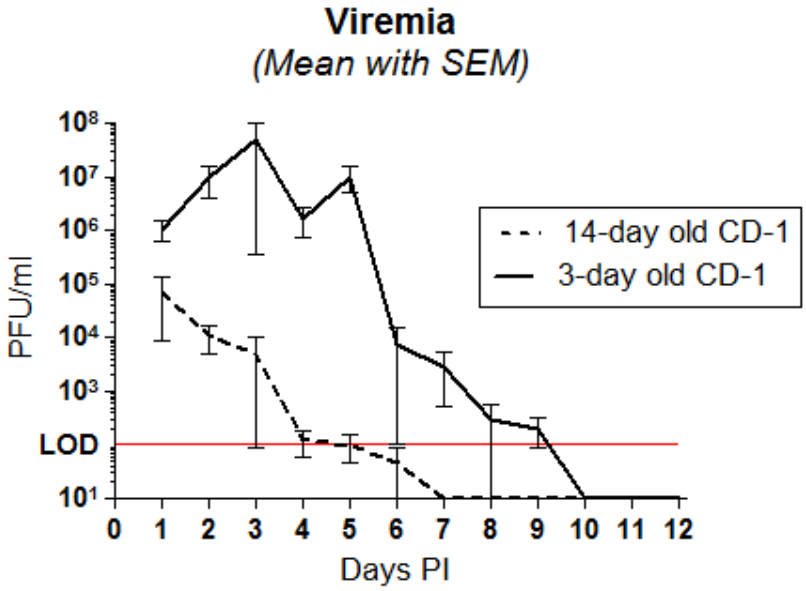


Figure 15: Viremia levels in CHIKV-infected CD-1 mice

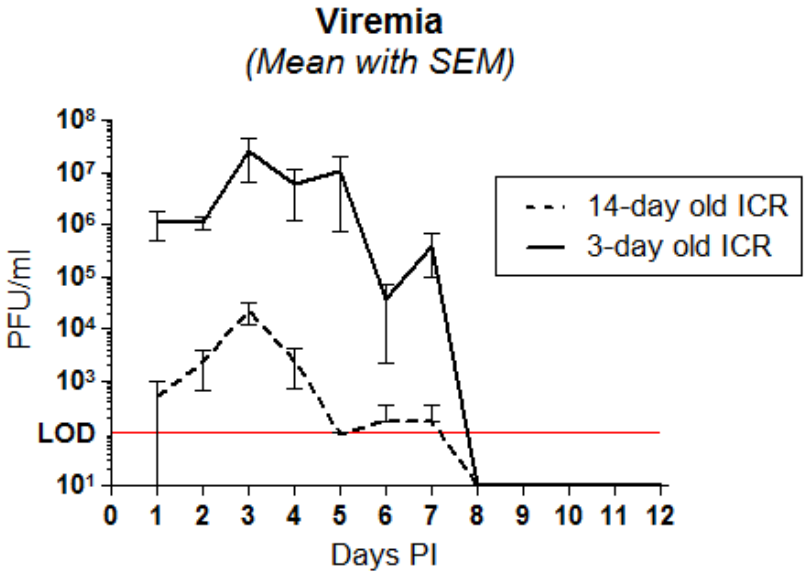


Figure 16: Viremia levels in CHIKV-infected ICR mice

and reached a peak viremia level of $10^{4.8}$ PFU/mL. The inability to isolate virus from serum corresponded to the appearance of HI antibody at 5 dpi (Table 7). Unlike newborn mice, all of the CHIKV-infected young mice developed detectable HI antibody. These titers usually reached levels ≥1:160.

Viral loads in the skeletal muscle of the leg were also investigated in both newborn and young mice. In CD-1 mice, peak viral loads were $10^{8.5}$ PFU/gm of tissue in newborn mice and $10^{6.4}$ PFU/gm of leg tissue in young mice (Figure 17). Titers were approximately 10-fold lower in ICR mice, as compared to CD-1 mice with peak viral loads of $10^{7.8}$ PFU/gm of leg tissue in newborn mice and $10^{5.6}$ PFU/gm of leg tissue in young mice (Figure 18). In both groups of mice tested, the duration of time that CHIKV could be isolated was longer in leg tissue, than in blood. In newborn mice, this ranged from 11-12 dpi and in young mice it was 6-7 dpi. This suggested that there was virus replication in the leg tissue and was not dependent on viremia levels.

Neurovirulence

Neurovirulence is very common with some alphaviruses including western equine encephalitis virus (WEEV), VEEV and EEEV. Among the Semliki Forest group viruses, neurovirulence is uncommon in human infection. In mice infected with CHIKV SC, virus could be isolated from the brains of newborn mice, but was rare in young mice (Figure 19 and Figure 20). In both CD-1 and ICR newborn mice, virus can be isolated from the brains of the mice 8-9 dpi. Peak viral loads were between 10^{7-8} PFU/gm of tissue. Unlike in newborn mice, CHIKV in young

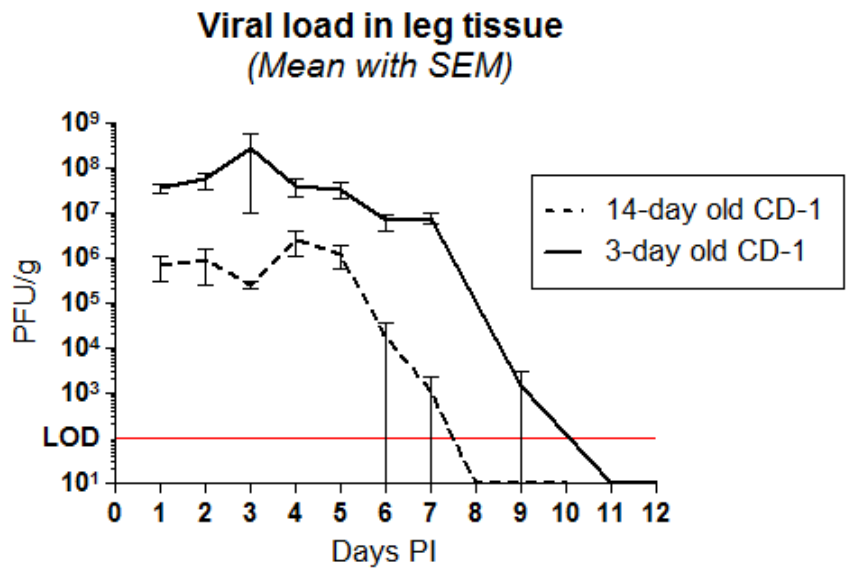


Figure 17: Viral load in leg tissue in CHIKV-infected CD-1 mice

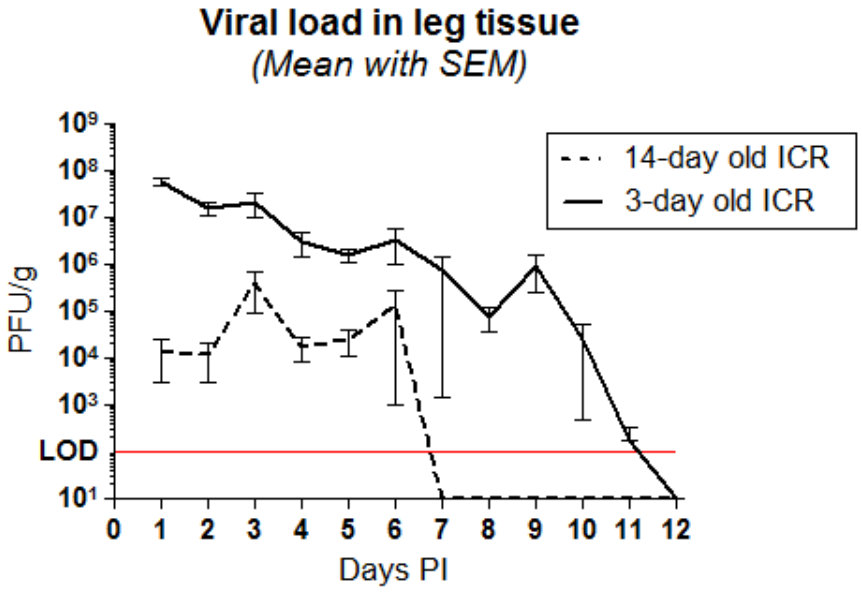


Figure 18: Viral load in leg tissue in CHIKV-infected ICR mice

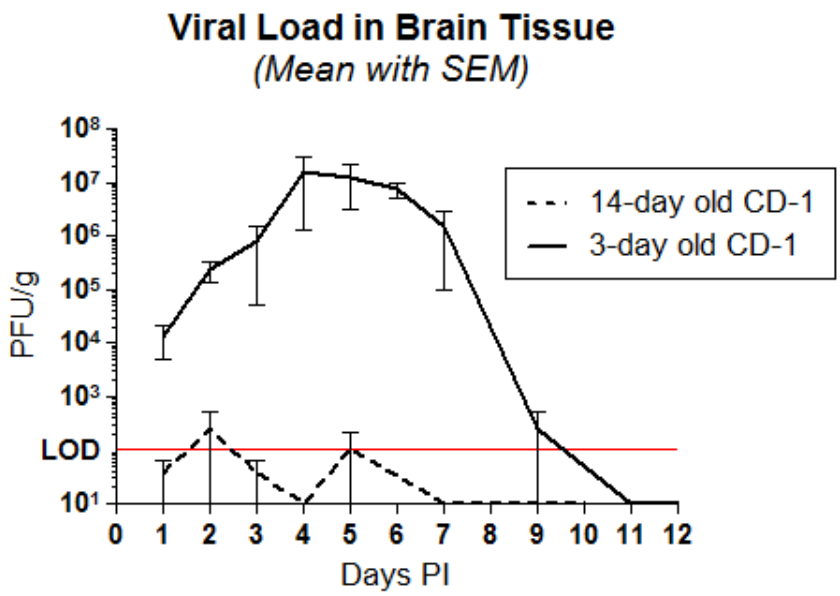


Figure 19: Viral load in brain tissue in CD-1 mice infected with CHIKV

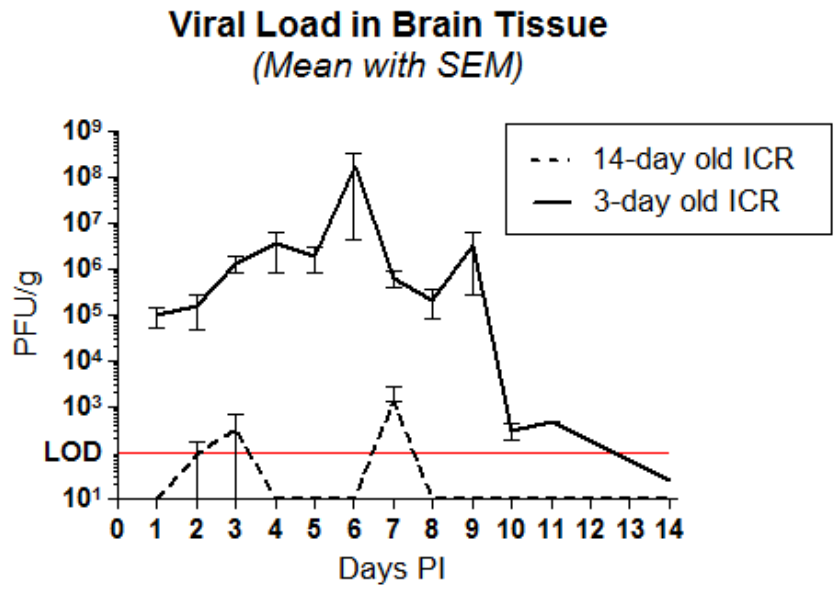


Figure 20: Viral load in brain tissue in CHIKV-infected ICR mice

mice was rarely isolated from the brains with peak titers less than 10^3 PFU/gm of tissue. Interestingly, there was very little pathology seen in the brains of newborn mice even despite the high titers of virus present. When the pathology of the brains of CHIKV-infected newborn mice was studied, occasionally neurons showed degenerative changes; but this was more likely a postmortem artifact than true pathological changes. Immunohistochemistry studies of the brain of newborn mice showed some positive staining for CHIKV antigen in the endothelial cells of the vessels of the brain but not in the neurons (Figure 21). In young mice, no pathology was observed in the brain nor was CHIKV antigen.

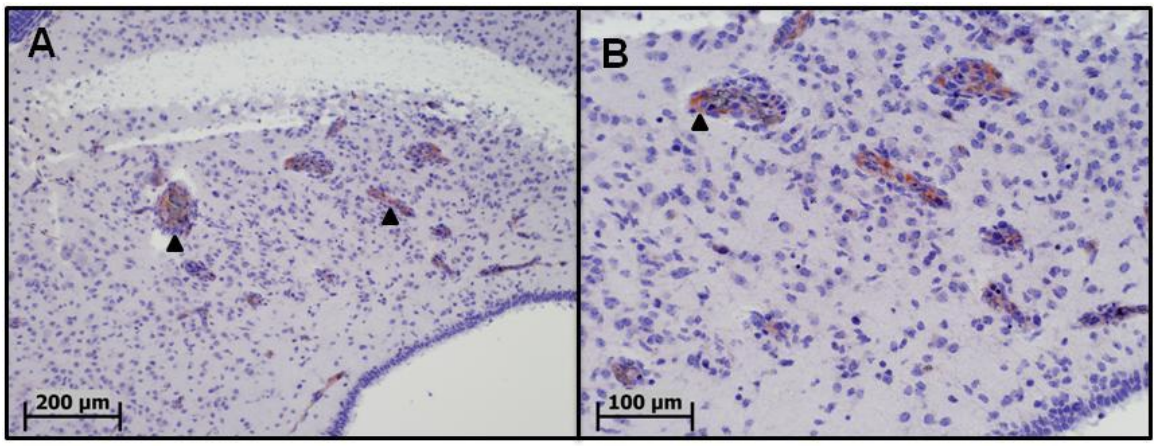


Figure 21: Immunohistochemistry of neuronal tissue in CHIKV-infected newborn mice

Newborn ICR mice neuronal tissue showing CHIKV antigen in the endothelial tissue, but not the neurons. Arrow heads show locations of CHIKV antigen. Figure B is of the same image at a higher magnification.

Pathology in CHIKV-infected mice

The most significant pathology observed in CHIKV-infected mice was severe focal necrosis of the skeletal muscle and inflammation in the connective tissue. Prior to 4 dpi, the pathological changes in the skeletal muscle were minimal with a slight increase in inflammatory infiltrates. From 5 to 7 dpi, there was increased muscle necrosis, with minimal mononuclear inflammatory cellular infiltration. The peri- and intramuscular brown fat began to show focal necrosis, with dystrophic calcification. These changes became more severe during the course of infection, yet the lesions remained focal and were usually adjacent to the joint. Two animals were examined on 17 dpi and showed residual foci of prominent muscle necrosis with calcification and prominent fibrosis (scar formation).

Full pathological studies were done in newborn mice (Figure 22). The small intestine, colon, pancreas, kidneys and adrenals showed little to no pathology. Regarding the heart, there were no abnormalities involving the endocardium and the pericardium in CHIKV-infected mice. In the liver, features of extramedullary hematopoiesis were present, including nucleated red blood cells and megakaryocytes in sinusoidal spaces. Starting from day 3 of infection, rare foci of spotty hepatocytic necrosis were found scattered in the parenchyma, without a specific zonal distribution, or changes in severity. In the spleen, there was a mild increase in macrophages containing cellular nuclear debris (tangible body macrophages), as well as a mild increase in lymphocytic necrosis during the acute stage of the infection.

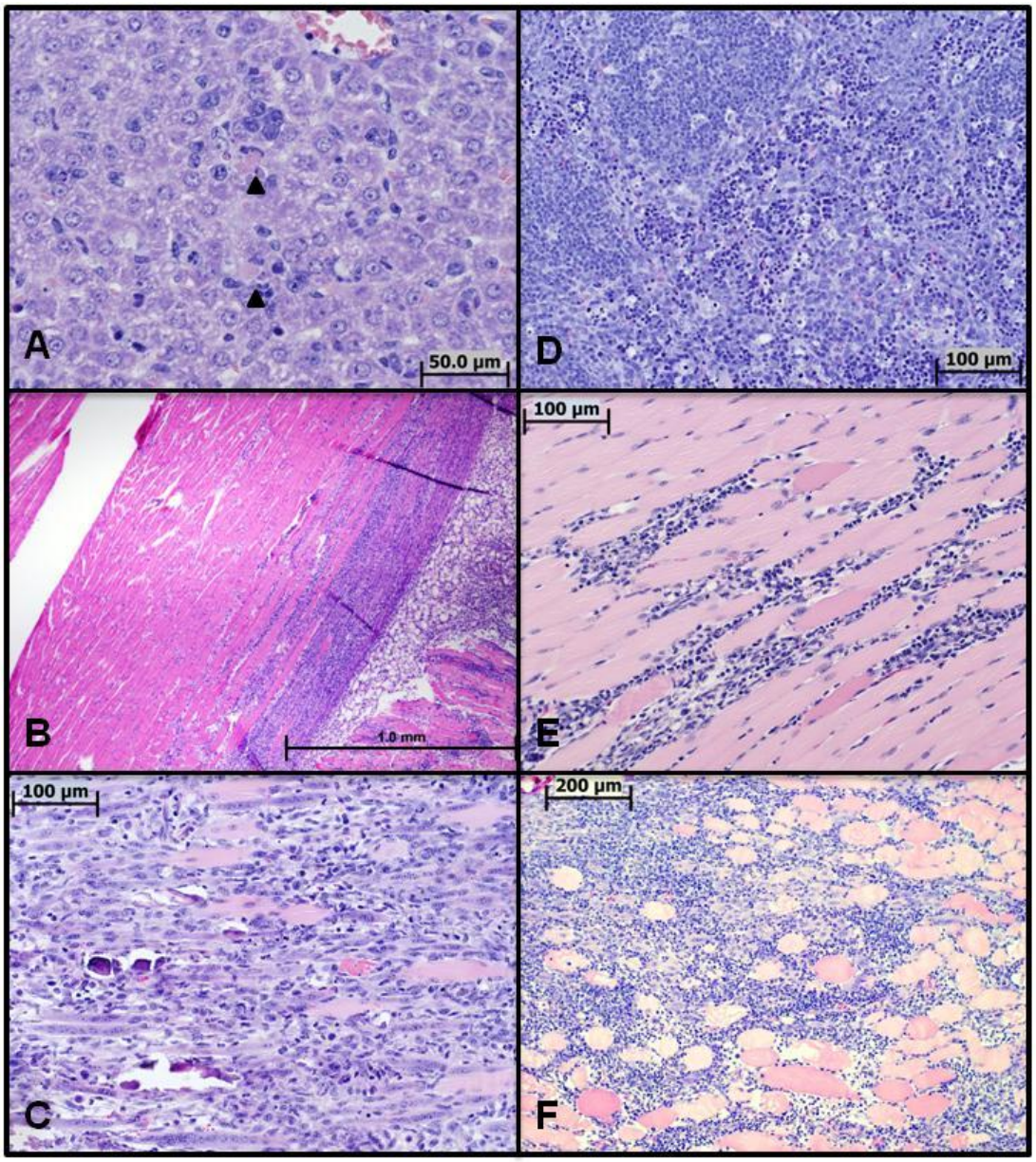


Figure 22: Histopathological changes in liver, spleen and skeletal muscle in CHIKV-infected mice

A: Liver from newborn mouse, 3 dpi showing spotty necrosis (arrowhead). B: Myositis in a young mouse, 5 dpi. C: Skeletal muscle from a young mouse, 10 dpi, showing inflammatory infiltrates and necrotic muscle fibers. D: Spleen from newborn mouse 3 dpi, showing increased number of the macrophages. E: Myositis in a young mouse, 8 dpi. F: Myositis in a 25-day old mouse, 8 dpi.

To quantify the severity of the myositis seen in CHIKV-infected mice, a grading system was developed by Judith Aronson, M.D. (who also trained me to grade histopathology)(Table 8). The grading criteria involved assessing the distribution of the lesions in muscle, bone, synovial membrane and fat tissue. Distribution was assessed by determining what percentage of the muscle fibers had inflammation or lesions. It is also determined if there was any inflammation or lesions present in the synovial membrane or synovium, on the bone itself, or in the soft tissue (fat) surrounding the muscle. Next, the area with the most severe

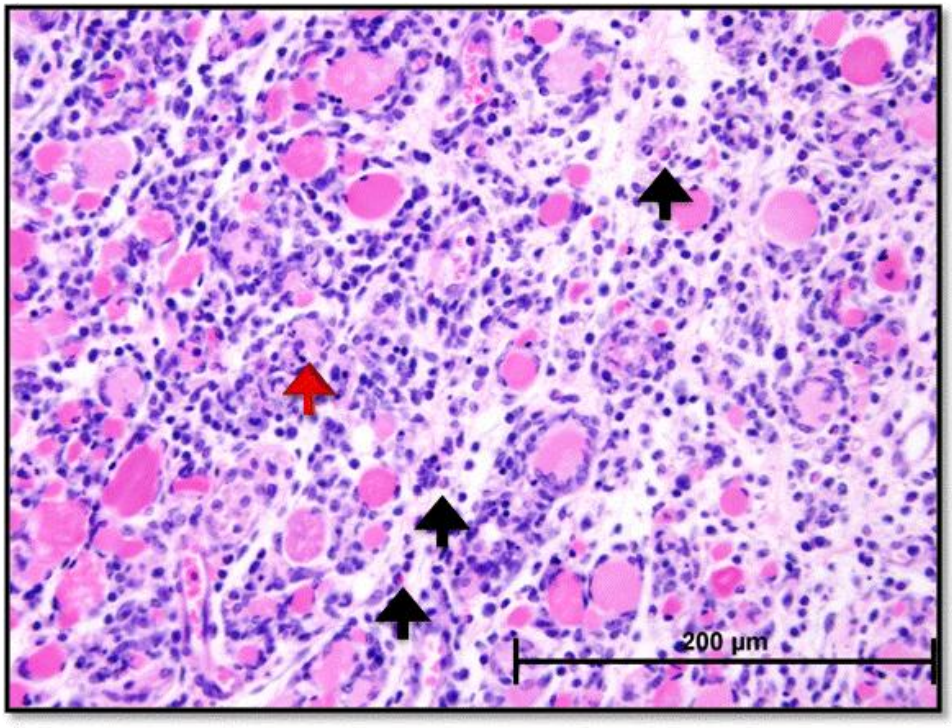


Figure 23: Skeletal muscle lesion in CHIKV-infected young mouse

Black arrowheads show representative neutrophils throughout the lesion. Red arrowhead shows representative necrotic muscle fiber. A large number of inflammatory infiltrates can be seen throughout the field mainly consisting of lymphocytes, but monocytes and neutrophils are also present. The inflammation has caused the widening of the interstitial space in this legion.

Table 8: Muscle pathology grading criteria

Category	Possible Scores
Lesion distribution	0 = none 1 = <10% 2 = 10-25% 3 = 25-50% 4 = 50-75% 5 = >75%
Inflammation	0 = none 1 = Perivascular only 2 = Interstitial only 3 = Interstitial with widening less than a muscle fiber width 4 = Interstitial with widening greater than a muscle fiber width
Necrosis	0 = absent 1 = present
Neutrophils	0 = absent 1 = present
Bone lesion	0 = absent 1 = present
Synovial inflammation	0 = absent 1 = present
Soft tissue lesion	0 = absent 1 = present
Scoring Range	**0-14**

pathology was graded on the severity of inflammation (perivascular or interstitial with or without expansion of the fibers) and the presence or absence of necrosis and neutrophils (Figure 23). The total pathology score was between 0-14. For the first 4 dpi in 14-day old CD-1 mice, the average pathology score was less than 5. Between 5-14 dpi, scores ranged between 5 and 11 and rarely were greater than 11. After 14 dpi, myositis was less severe, which led to the conclusion that the mice recovered from the severe myositis. Bone lesions and inflammation in the synovial membrane were rare findings in CHIKV-infected mice. Most common findings were large focal regions within the skeletal muscle of severe inflammation, necrotic muscle fibers and neutrophils. It was also common to see with severe myositis, that the adjoing fatty tissue was also inflamed.

Immunohistochemistry analysis of samples of CHIKV-infected mice revealed that most CHIKV antigen was located in the skeletal muscle fibers (Figure 24). CHIKV antigen was also present frequently in the dermis of the skin.

Rarely was antigen seen in other tissues including the endothelial tissue in the brain. In some regional lymph nodes, CHIKV antigen was also seen. In newborn mice specifically, the heart, intestines, kidneys, adrenals, pancreas, and spleen were all negative for antigen while the liver showed some scattered staining in hematopoetic cells. In newborn mice, CHIKV antigen was present starting on 3 dpi and remained positive until 17 dpi.

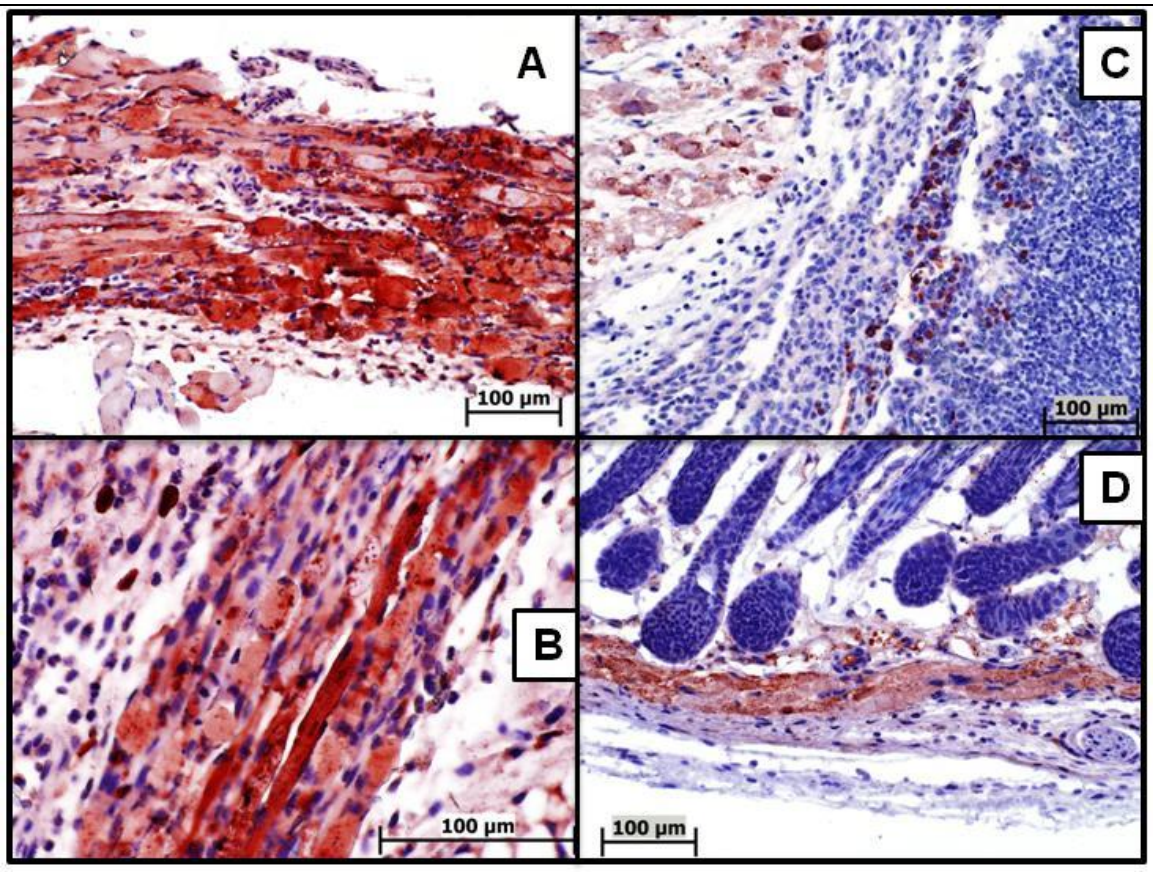


Figure 24: Immunohistochemistry of CHIKV-infected newborn mice

Skeletal muscle showing positive antigen staining for CHIKV 7 (A) and 8 dpi (B). C: Newborn mouse 9 dpi, positive antigen in the necrotic muscle tissue and in the large lymphocytes in the lymph node. D: CHIKV antigen was found in the dermis in newborn mice

Other rodent models of CHIKV infection

In older ICR mice, 25-day old and 6-weeks old, CHIKV did not cause any signs of disease. Also, in these older age groups of mice, viremia was very transient and peak viremias were less than 10^4 PFU/ml in 25-day old mice and less than 10^3 PFU/ml in 6-week old mice (Figure 25 and Figure 26). In 25-day old mice, CHIKV could be isolated from the leg muscle for up to 4 dpi.

In adult (6-8 weeks old) C57BL/6 mice CHIKV was transient and viral loads in leg, brain and liver tissue were undetectable in most mice (Figure 27).

In adult hamsters, viremia levels were also transient

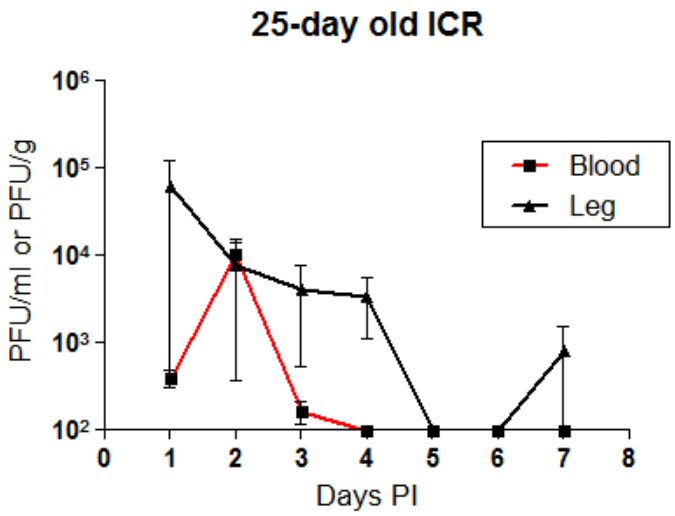


Figure 25: Viral loads in CHIKV-infected 25-day old ICR mice

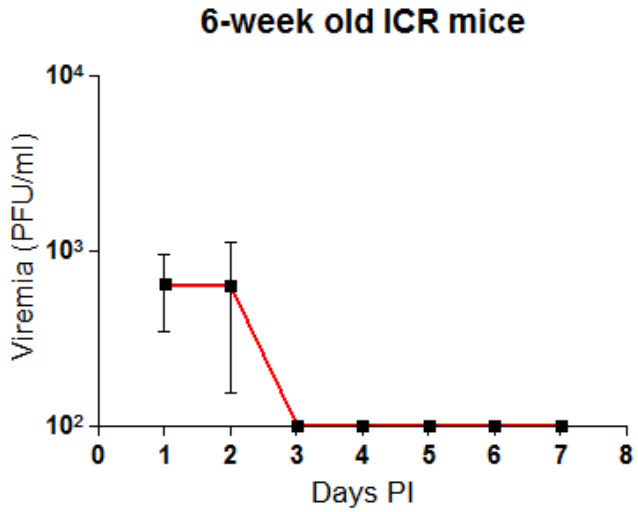


Figure 26: Viremia levels in CHIKV-infected 6-week old ICR mice

and no clinical signs of disease were present in infected hamsters (Figure 28). Gerbils were also investigated as possible models. Gerbils did not show any outward signs of infection or disease.

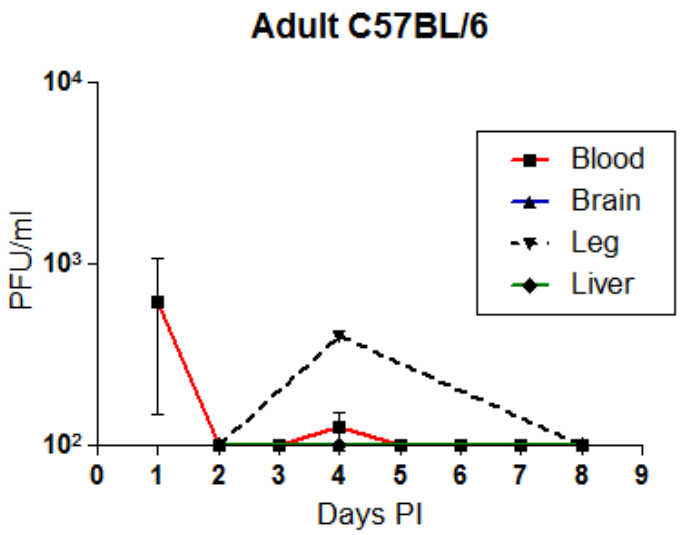


Figure 27: Viral loads in CHIKV-infected C57BL/6 mice

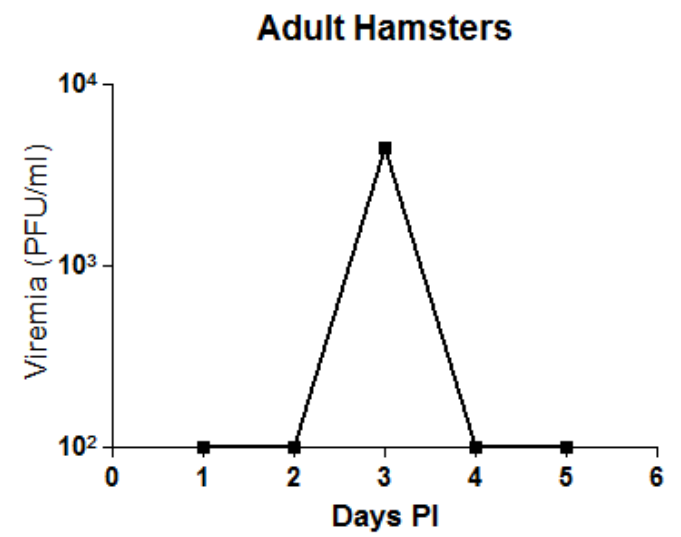


Figure 28: Viremia levels in CHIKV-infected hamsters

Serial Passage of CHIKV in mice

In order to obtain a more virulent virus in mice, serial passage of virus was undertaken. This technique has sometimes yielded a virus that has a higher virulence, such as the serially passaged yellow fever virus (Tesh et al. 2001). The Reunion CHIKV strain was inoculated into newborn

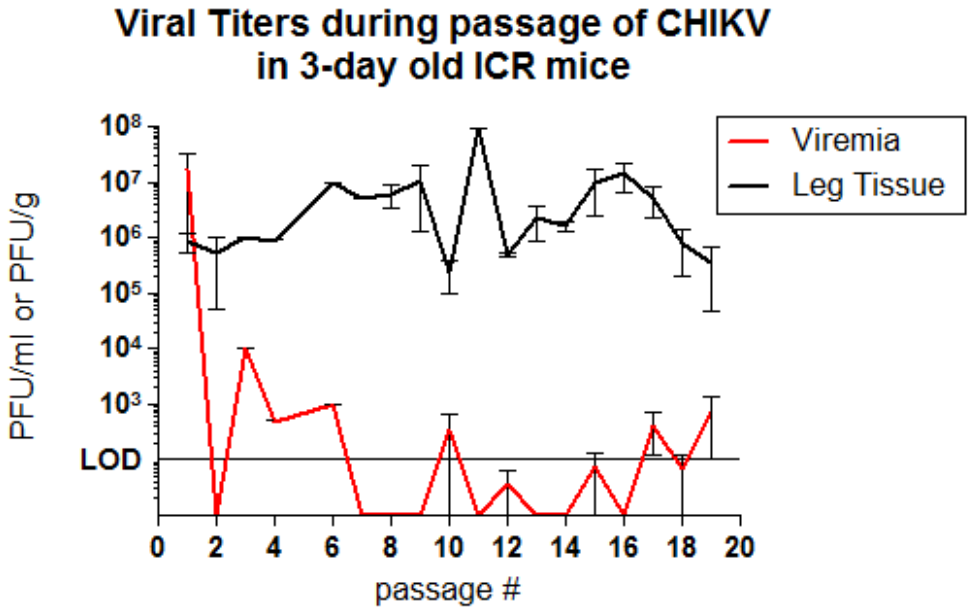


Figure 29: Serial passage of CHIKV in leg tissue

3-day old ICR mice were inoculated SC and 5 dpi, legs were harvested, homogenized, pooled and inoculated into 3-day old mice.

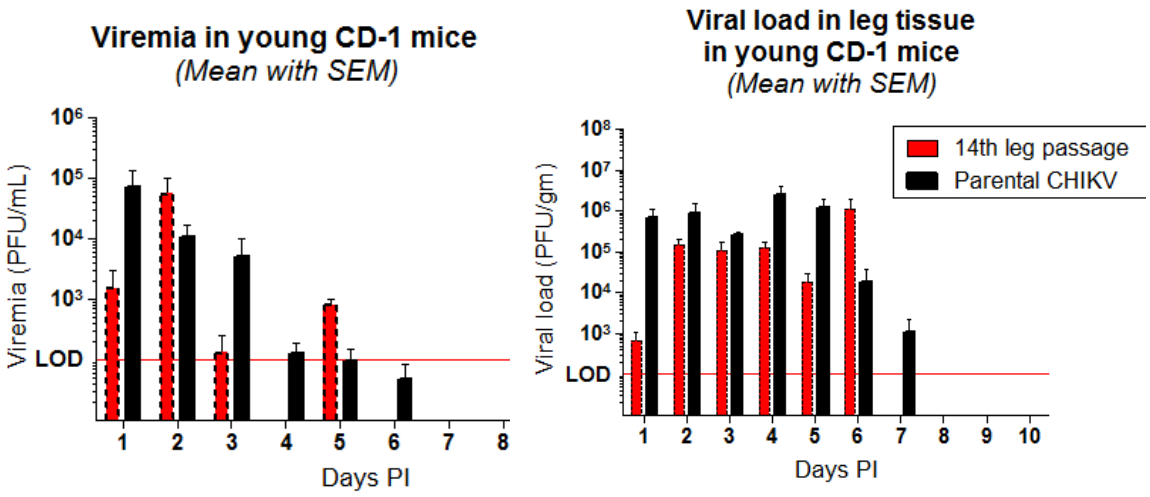


Figure 30: Comparison of serially passaged and parental CHIKV strains

Viremia and viral load in the leg tissue of 14-day old CD-1 mice inoculated with parental or 14th passage of CHIKV. Mice were inoculated SC in the back.

ICR mice and on 5 dpi mice were sacrificed and the muscle tissue was homogenized, filter and re-inoculated into a new litter of newborn mice SC in the back. This was done 19 times in newborn mice to try and achieve a strain of CHIKV that would be more muscle tropic and increase the disease seen in mice. Interestingly, this did not occur. The passaged strains of the virus produced lower viremia titers in newborn mice 5 dpi, but leg titers increased approximately tenfold (Figure 29). When the 14^{th} passage of the virus was inoculated into young mice and compared to the parent strain, there was no significant difference in the viral load in the leg tissue and no significant signs of disease were present (Figure 30). Viremia levels again were lower in the high passage strain of the virus with only approximately 2 days of detectable viremia.

Focus on young CD-1 mice

Young CD-1 mice were developed as a model of CHIKF and further studies were done to understand the pathogenesis of the virus in mice. Not only were leg and brain tissues examined for viral load, but also liver and spleen tissues. Viral load in the liver in CHIKV-infected young CD-1 mice followed the same trend as viremia levels, peak levels were slightly higher at greater than 10^5 PFU/gm but became undetectable by 4 to 5 dpi (Figure 31). CHIKV in the spleen was isolated for a longer period of time with virus isolated on 6 dpi. Also as shown in Figure 31, viremia levels became undetectable as antibody levels increased, although virus persisted in the leg tissue after CHIKV antibodies were present.

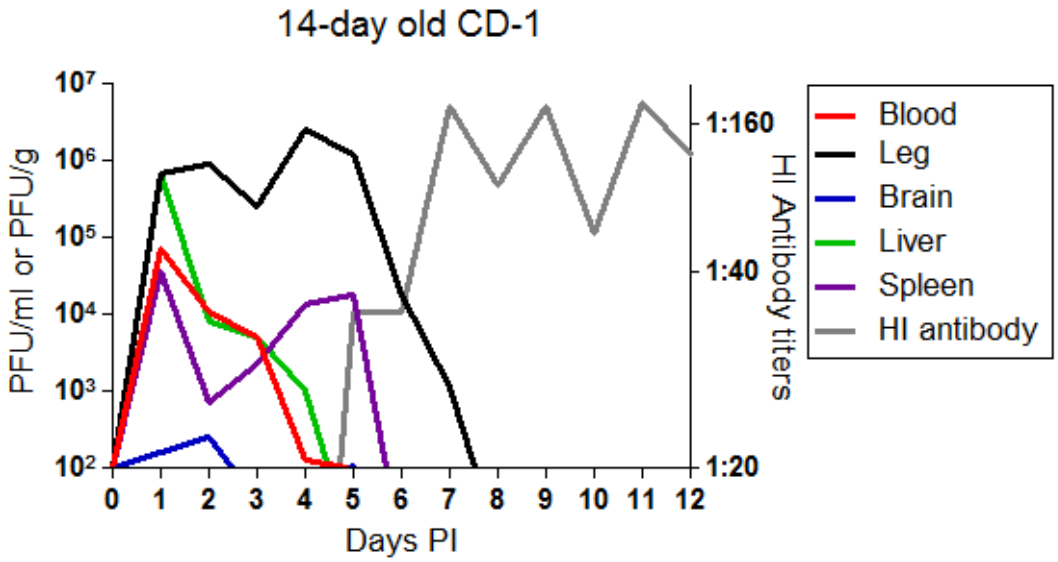


Figure 31: Viral load in tissues and antibody response in CHIKV-infected young CD-1 mice

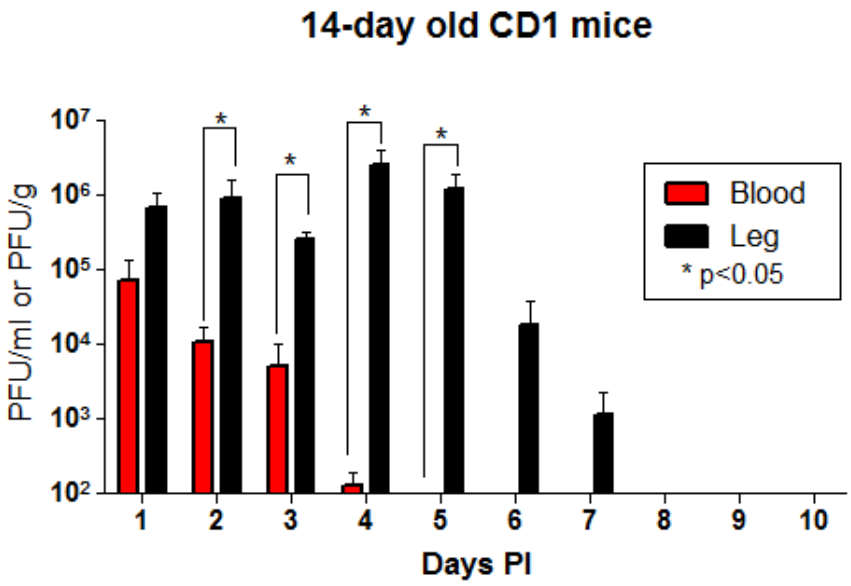


Figure 32: Viremia and viral load in leg tissue in CHIKV-infected young CD-1 mice

Discussion

Multiple rodent species, mouse strains and age groups were looked as a model of CHIKV infection (Table 9). Young CD-1 mice are a good model of CHIKV infection to study the pathogenesis as it relates to human cases of CHIKF. CHIKF in people most commonly is associated with an acute febrile illness with arthralgia/myalgia, fever and rash. Muscle pathology is rarely looked at in people with CHIKF, but myositis and CHIKV has been seen in the few biopsies of human muscles (Ozden et al. 2007). The young CD-1 mice infected with CHIKV presented here had severe myositis in the skeletal muscle and CHIKV antigen present, also. The cutaneous manifestations of CHIKF in humans, specifically rash, may also be described by the presence of pathology and CHIKV antigen in the dermis of the mouse skin (Riyaz et al. 2010). The lack of neuroinvasiveness of CHIKV in older mice as compared to newborn mice, is also correlative to human CHIKF cases. Neurological manifestations of CHIKF are more common in newborns than in healthy adults.

This mouse model presented here is similar to those presented of RRV (Lidbury et al. 2000, Morrison et al. 2006, Rulli et al. 2005). In these models, RRV presents with a more severe arthralgic disease in mice. RRV infected mice routinely are characterized by hind limb dragging, limited mortality and ruffled fur. The young mice infected with CHIKV did not routinely present with this type of disease. Curiously enough, the histopathology images of RRV myositis are very comparable to those presented here even though RRV-infected mice have a more severe presentation clinically. It is also curious as to why the CHIKV-infected mice do not exhibit more signs of disease with the amount of severe pathology in the muscle that is observed, such as hind limb dragging or a

decrease in weight gain. With further research in the CHIKV-infected mouse model, more can be learned about the pathogenesis of the virus.

These studies have presented a mouse model of CHIKF that is representative of some aspects of CHIKF in people. With limited knowledge of CHIKF pathogenesis, this mouse model may be used to answer questions on how the virus causes disease in people. This model is very similar to other models that have been published and any differences that are apparent can be explained by differences in inoculation site or mouse strain. These studies have also emphasized the similarities in RRV and CHIKV-induced disease in mice. This is not surprising due to the similarity of the clinical presentation of these viral diseases in humans. These studies also lead to unanswered questions as to the places of viral replication within the mice. One might hypothesis that there is viral replication in the skeletal muscle and spleen of CHIKV-infected mice.

Table 9: Summary of work with CHIKV in various rodent species

Rodent Strain	Age	Mortality	Clinical or pathological disease
CD-1 mice	3-day	20%	6-8 days of viremia, severe myositis, hind limb dragging
	14-day	None	2-3 days of viremia, 4-6 days of virus in the leg, severe myositis
ICR mice	3-day	10%	6-8 days of viremia, severe myositis, hind limb dragging
	14-day	None	2-3 days of viremia, 4-6 days of virus in the leg, severe myositis
	6-week	None	1-2 days of viremia, no severe disease
C57BL/6	6-week	None	Transient viremia, no severe disease
Syrian Golden Hamsters	6-week	None	Transient viremia, no severe disease
Mongolian Gerbils	6-week	None	Transient viremia, no severe disease

Chapter 4: Tissue tropism of chikungunya virus in mice

Abstract

Viral tropism is an important feature to help determine specific viral treatments. The tropism of CHIKV is unknown in humans and other animals. *In vitro* CHIKV infects a wide range of cells, but in animals seems to target the skeletal muscle and causes an immune-mediated disease. Heparin-sulfate has been shown to be a receptor of CHIKV *in vitro*, but is ubiquitously expressed on vertebrate cells so it may not the key factor determining virus tropism (Ryman et al. 2007). To determine the tissue and cells of CHIKV replication in mice, GFP and luciferase infectious clones of CHIKV were used with novel imaging technology. This work has shown that CHIKV replicates in the myocytes in the skeletal muscle and in the lymph nodes. GFP and luciferase associated with CHIKV replication can be seen for up to 5 dpi. *In vivo* imaging technology was a powerful tool to be used not only for animal imaging, but for imaging of mosquitoes.

Introduction

CHIKV-GFP clones have been used previously to determine virus infectivity in mosquitoes and in cell culture (Tsetsarkin et al. 2006, Vanlandingham et al. 2005). The CHIKV-GFP is an infectious clone of the LR 2006-OPY1 strain of CHIKV. It has an enhanced GFP gene under the control of its own subgenomic promoter on the 3' side of the nonstructural protein genes and before the structural protein genes (Figure 11). Although the GFP expression from the CHIKV infectious clone has been shown to be very stable in cell culture and in infected mosquitoes (Tsetsarkin et al. 2006, Vanlandingham et

al. 2005). With these previous successes, the use of this infectious clone was investigated in mice.

A new CHIKV infectious clone with a luciferase gene, CHIKV-LUC' was designed and constructed by Charles McGee, Ph.D. and used for these experiments (Ziegler et al. 2011). This clone is based on the CHIKV-GFP infectious clone in which the GFP was substituted with a humanized *Renilla* luciferase gene (Promega). *Renilla reniformis*, the organism from which the luciferase gene was cloned from, is a sea coral known for its' luminescent properties. The advantage of using the *Renilla* luciferase molecule as compared to firefly luciferase is that it does not need ATP or magnesium to catalyze the luminescence reaction and only requires oxygen as a co-factor. Coelenterazine and its analogs are the natural substrates for many types of luciferase enzymes found in marine organisms including the *Renilla* luciferase (Inouye et al. 1997). The *Renilla* luciferase catalyzed reaction emits a blue light with a peak emission at 480 nm (Inouye et al. 1997).

There are many different commercially available substrates for use with the *Renilla* luciferase reaction. For our experiments, the ViviRen substrate (Promega) was used (Figure 33). Its use in mice and in IVIS imaging has previously been published with good results (Kimura et al. 2010, Otto-Duessel et al. 2006). It has recently been shown to have a better signal to noise ratio and a higher bio-availability as compared to other substrates.

In using both GFP and luciferase infectious clones, the visualization of the signal is restricted to the cells that have active viral replication and is not expressed extracellularly. By using these technologies visualization of the

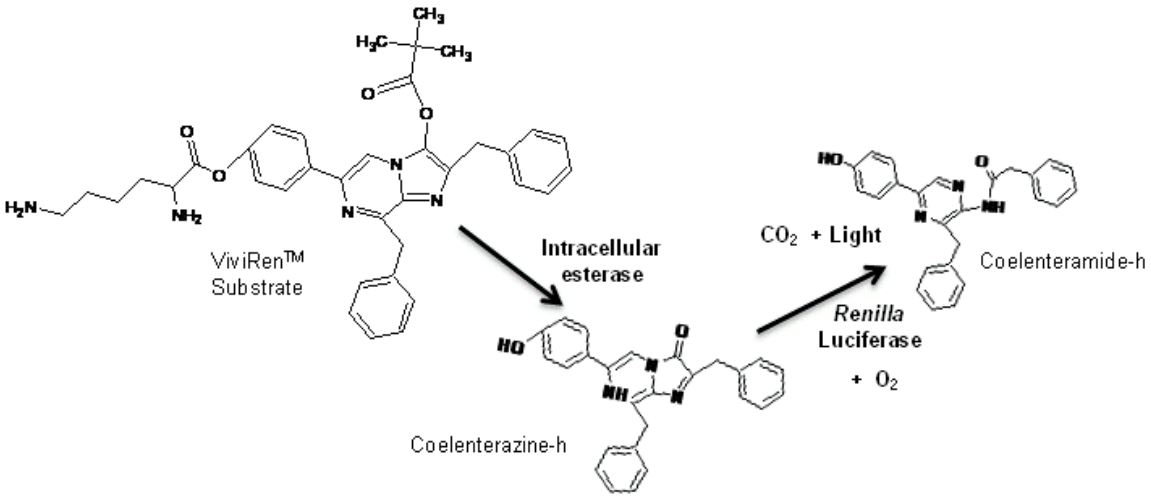


Figure 33: Chemical equation for the conversion of ViviRen with *Renilla* luciferase

location(s) of viral replication was possible. Isolation of virus from mouse tissue was not necessarily indicative of viral replication. Viral loads within highly vascularized tissues may be attributed to high viremia levels, and may not be indicative of viral replication. Also, using whole animal imaging allowed for a complete examination of the mouse's physiology without overlooking any area that may be of interest and requires further examination.

The objective of this aim was to better understand the tissue tropism and sites of replication of CHIKV in mice. The hypothesis was that CHIKV replicates in the myocytes, macrophages, lymph nodes and the spleen in CHIKV-infected mice. To pursue this objective infectious clones of CHIKV with either a GFP or a luciferase reporter gene inserted into the genome were used. This permitted fluorescent microscopy of mouse tissue and the use of IVIS imaging technology

Results

CHIKV-GFP in infected mice

The CHIKV-GFP virus was infectious in both cell culture (Figure 34) and mosquitoes, and grew to similar titers as the parental virus (Tsetsarkin et al. 2006). In early studies in young CD-1 mice infected with CHIKV-GFP, peak viremia was approximately 10^5 PFU/mL at 24 hpi, and the viremia lasted for 2 to 3 dpi. This was comparable to previous infections with the parental CHIKV in young mice. Early work with the CHIKV-GFP infectious clone exhibited a high stability of the GFP. This stability was apparent in an experiment in which CHIKV-GFP was inoculated into mosquitoes incubated for 7 days. The insects were then allowed to feed on mice. Viremic blood samples from these mice, 2 days post-exposure to the mosquitoes and assayed on Vero cells using a plaque forming assay, still exhibited GFP in 99% of the plaques.

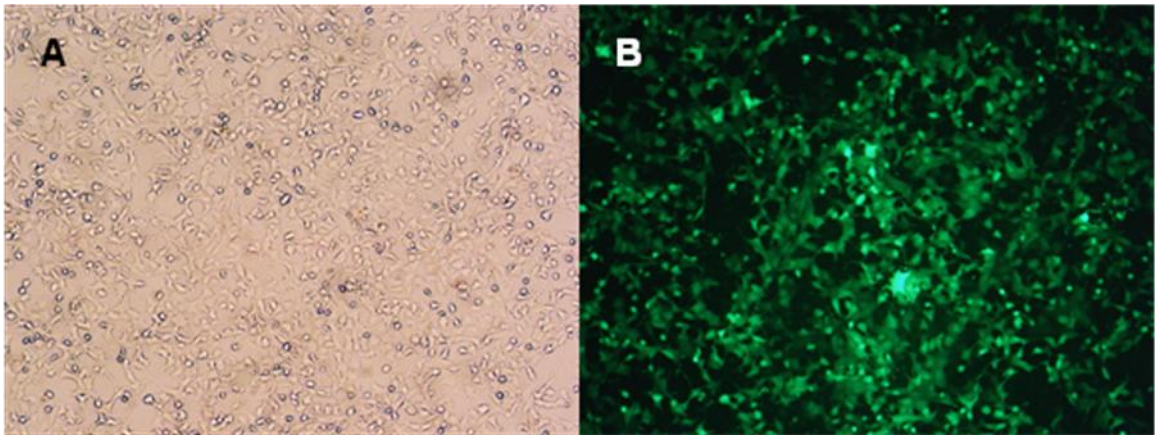


Figure 34: Vero cells infected with CHIKV-GFP

Vero cells 48 hours after infection with CHIKV-GFP. A: Bright field and B: flourescent images of infected Vero cells.

In an attempt to identify the locations of CHIKV replication in mice, microscopy studies of CHIKV-GFP were initiated. Mosquitoes were inoculated with CHIKV-GFP and were allowed to bite one ear of a young CD-1 mouse. Viremia levels assayed in these mice were similar to earlier results with 2 days of detectable viremia that reached a peak of 10^6 PFU/mL. GFP was detected as early as 12 hpi in the bitten ear; it started as focal areas of fluorescence followed by dissemination to most of the bitten ear within 48 hpi (Figure 35). GFP was localized only to the bitten ear for 48 hpi. After which the GFP activity began to diffuse. At 48 hpi, the bitten ear had a diffuse pattern of GFP expression; by 5 dpi the GFP expression had spread to both ears with limited amounts of GFP activity. This pattern of GFP activity suggested local virus replication at the site of the mosquito bite. Unfortunately, this technique was limited; visualization of GFP in the organs of the infected mice was problematic, since fixation of the infected organs with either ethanol or buffered formalin resulted in loss of GFP activity or a high background signal. Histopathologic exam of frozen sections also resulted in very little GFP signal and a high amount of damage to the sectioned tissue. Blood smears, as well as, heart, brain, kidney and lung tissues never had GFP expression in CHIKV-GFP infected young mice. Since there is some evidence that CHIKV may replicate in the dermis, skin samples were also examined (Figure 24). Unfortunately, skin (keratin) and hair had intrinsic fluorescent properties that interfered with the identification of CHIKV-GFP specific activity in the dermis.

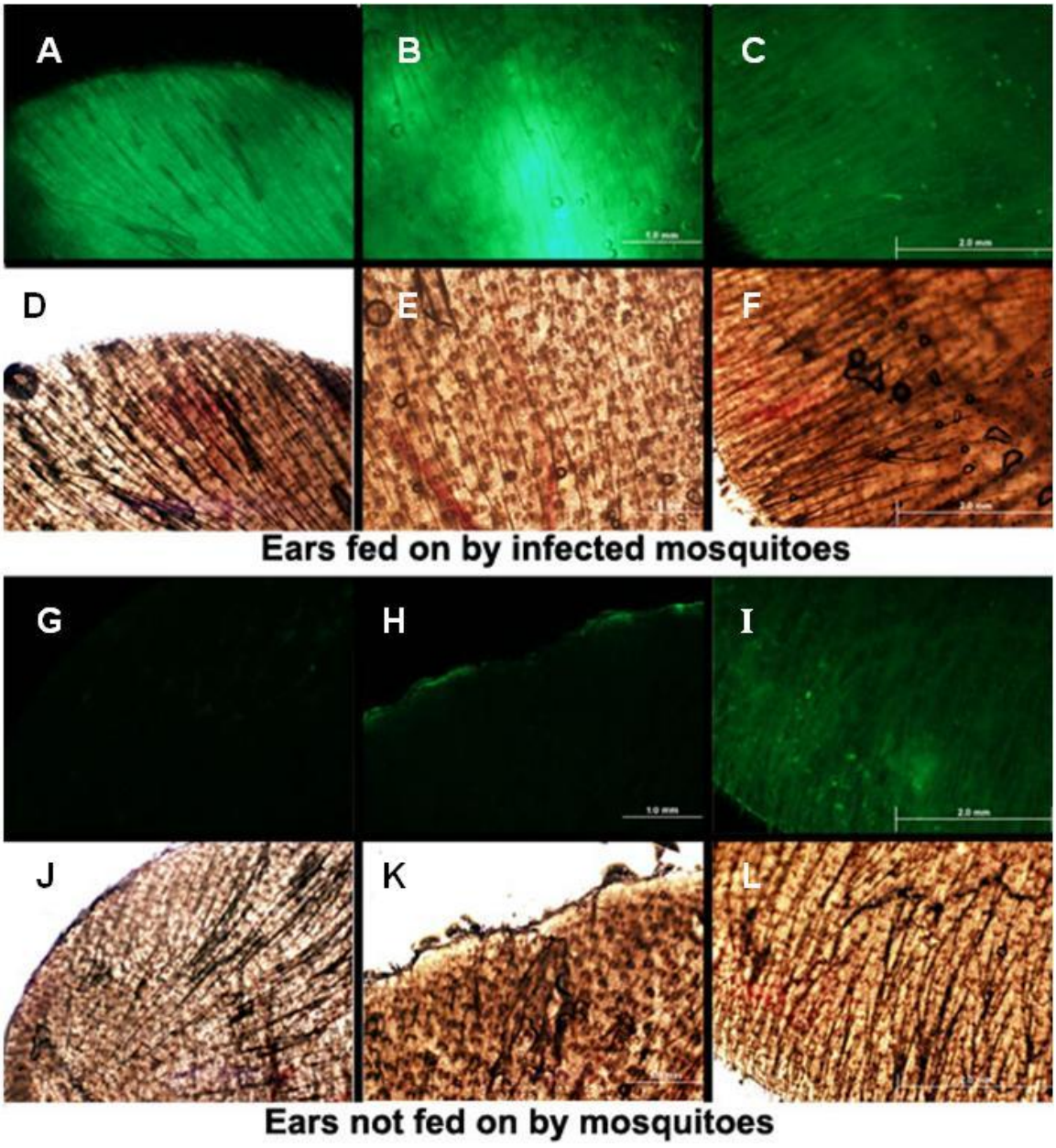


Figure 35: Mouse ears after bites of CHIKV-GFP infected mosquitoes

Mouse ears after being bitten by CHIKV-GFP infected mosquitoes and imaged at 24 hpi, 48 hpi, and 5 dpi. A-F: Ears bitten by infected mosquitoes. G-L: Opposite ear from the same mouse, but not bitten by mosquitoes. Each ear was imaged both in bright field (D-F, J-L) and fluorescent images (A-C, G-I). A,D,G,J: 24 hpi, B,E,H,K: 48hpi and C,F,I,L: 5 dpi.

The earliest CHIKV-GFP activity in tissues aside from the bite site was found 2 dpi in hind limb muscle tissue, and could be found up to 8 dpi (Figure 37). GFP activity was in focal areas and involved groups of muscle fibers, but was never observed in the whole leg tissue sample. It was hard to distinguish the exact cells in which virus replication occurred, but it was apparent that muscle fibers in the area were positive for fluorescent activity.

Other tissues examined for GFP expression from 4 to 8 dpi included liver, spleen and cervical lymph node tissue. Lymph nodes had positive GFP activity at 4 and 6 dpi (Figure 37), primarily in the cortex area of the node. At no time was the whole lymph node positive for GFP activity. The spleen also showed positive GFP activity over the same time frame. This was a spotty pattern (Figure 37, Panel E) which may have correlated to endothelial tissue within the spleen (Figure 37).

Due to the intrinsic problems with formalin fixation in microscopy studies of CHIKV-GFP infected mice, a new technology, IVIS, was investigated. At the initiation of this project, the IVIS was located in a BSL-2 area, so both ONNV-GFP and MAYV-GFP were used initially, instead of CHIKV-GFP, as the latter is a BSL-3 agent and the three viruses have similar clinical symptoms (Tesh 1982). Previous studies within the laboratory have shown that MAYV causes a similar disease in mice with severe myositis (data not shown). I had limited success in visualizing ONNV-GFP expression in the IVIS (Figure 38). A diffuse pattern of GFP activity could be seen over the flanks of the mice. The signal was low, and it was hard to distinguish between the GFP and non-specific intrinsic fluorescence. In an attempt to find the location of the GFP signal, a young mouse was necropsied and then imaged.

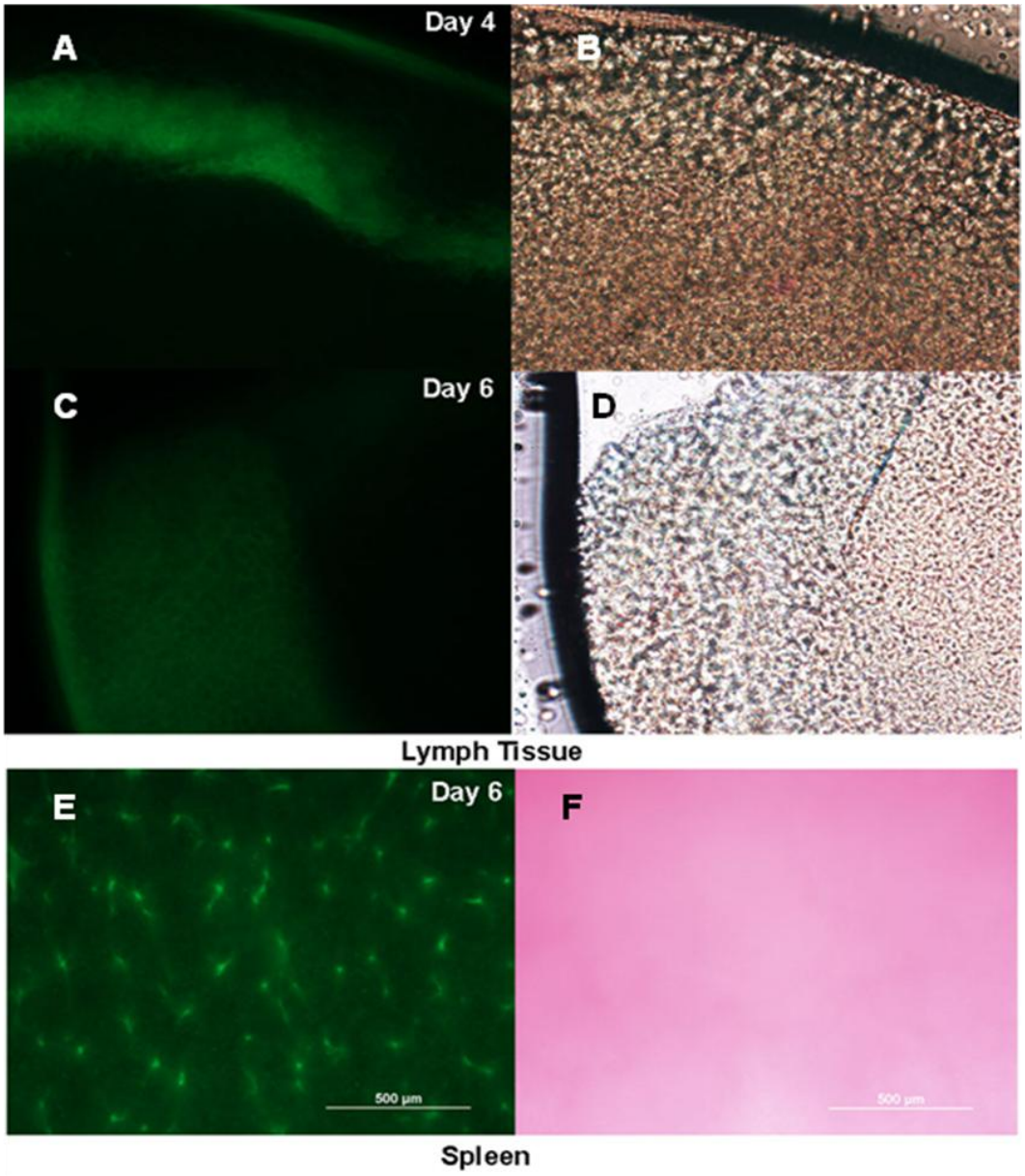


Figure 36: Lymph nodes and spleen tissue from CHIKV-GFP infected young mice.

A-D: Histologic sections of lymph nodes from CHIKV-GFP infected mice. Fluorescence was detected on 4 dpi (A-B) and 6 dpi (C-D). Spleen tissue at 6 dpi of CHIKV-GFP infected mice. Fluorescent images (A,C,E) with corresponding bright field images (B,D,F).

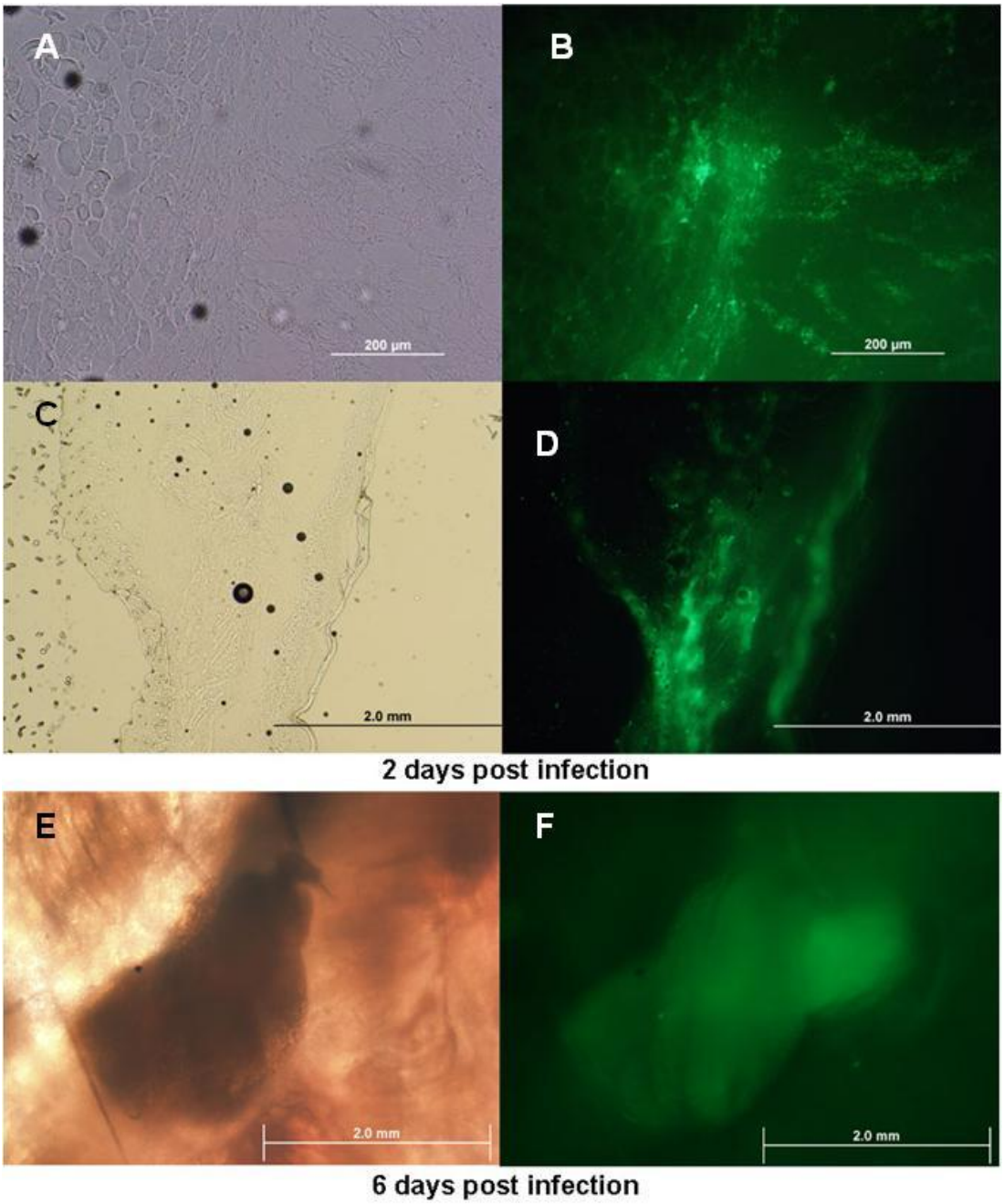


Figure 37: Leg muscles from CHIKV-GFP infected mice

Hind limb muscles from individual CHIKV-GFP infected mice. A-D Hind limb muscles from different CHIKV-GFP infected mice 2 and 6 dpi. Bright field images (A,C,E) and corresponding fluorescent images (B,D,F).

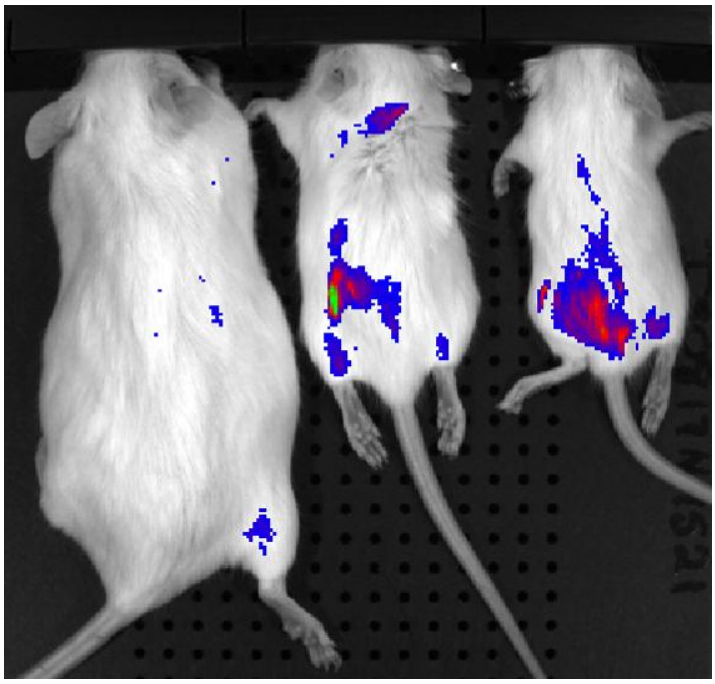


Figure 38: ONNV-GFP infected mice using IVIS

Mouse on the left was uninfected and mice on the right were infected with ONNV-GFP and imaged 48 hpi.

During this study, it was seen that all GFP signal was in the skin of the mouse. These studies were repeated with MAYV-GFP and in the ABSL-3 using CHIKV-GFP, both of which gave similar negative results. For this reason a luciferase CHIKV clone was developed for use in the IVIS system.

CHIKV-LUC in vitro and in vivo

The new CHIKV-LUC clone was designed in the same format as the CHIKV-GFP clone and therefore was theorized to exhibit the same properties as the parental virus. Titers of CHIKV-LUC in cell culture were similar to parental virus 48 hpi (10^6 $TCID_{50}$/mL). Initial studies to optimize the system were conducted in cell culture. In both Vero and C6/36 CHIKV-LUC infected cells, high levels of luminescence were seen with the addition of the ViviRen substrate and visualized with the IVIS (Figure 39). Titrations of the ViviRen substrate on both C6/36 and Vero cells showed that the intensity of luminescence was directly dependent on the concentration of the ViviRen substrate (Figure 39 and Figure 40). Both in uninfected cells with ViviRen and

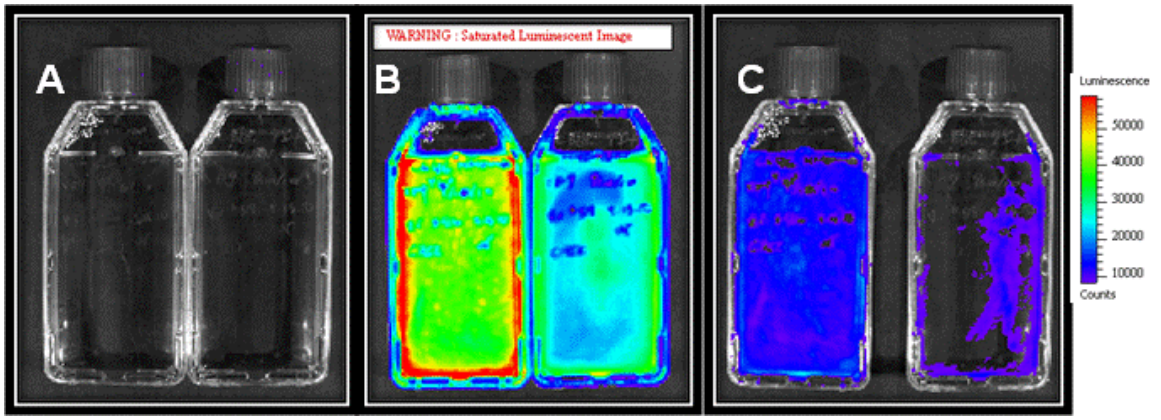


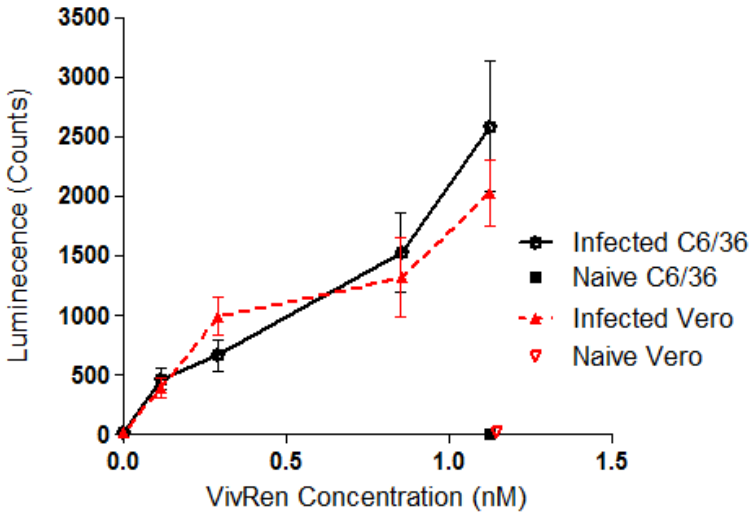
Figure 39: CHIKV-LUC infected cell culture

CHIKV-LUC infected Vero cells (left) and C6/36 cells (right) 48 hpi. A. Infected cells immediately prior to substrate addition. B. 1 minute after ViviRen addition. C. Twenty minutes after ViviRen addition.

CHIKV-infected cells without the substrate, no signal was detected, as was expected. With increasing concentrations of ViviRen from 0 to 1 nM of substrate, the luminescence signal was directly proportional to the concentration (R^2: 0.9617 and 0.9197).

Autoluminescence of luciferase substrates was a problem when using the IVIS technology and live animals. ViviRen had autoluminescence properties in young mice and so the location and concentration of the substrate were optimized. It was possible to see the location of the inoculation of the substrate IP both on the CHIKV-GFP infected mouse (control) and the CHIKV-LUC infected mouse (Figure 42).

To minimize the interference of substrate autoluminesce, the ViviRen substrate was injected SC in the scruff of the neck on the back. In Figure 43,



Linear Regression Results

	Infected C6/36	Infected Vero
R square	0.9617	0.9197

Figure 40: ViviRen titration on cell culture

Titrations of ViviRen *in vitro* on both Vero and C6/36 cells. The R^2 value is indicative of a direct correlation between the luminescence and concentration of ViviRen.

Panel A shows the dorsal view of the mice and the large amount of autoluminescence of the substrate as early as 5 minutes after injection of ViviRen, while in Panel B, the autoluminescence is not seen on the ventral side 20 minutes after ViviRen injection.

In order to optimize the time and the inoculation of the ViviRen substrate a series of measurements were done on infected 3- week old CD-1 mice. Footpad inoculations of CHIKV-LUC were used for the IVIS experiments to help track the spread of the virus and to maximize the signal. CHIKV-LUC infected mice were inoculated with ViviRen 2 dpi either IP or SC in the scruff of the neck and images were taken every 90 seconds for 30 minutes. On each image the maximal luminescence was recorded in the foot that was inoculated with CHIKV-LUC. Both IP and SC inoculations resulted in a high amount of signal which reached its maximum at approximately 25 minutes after

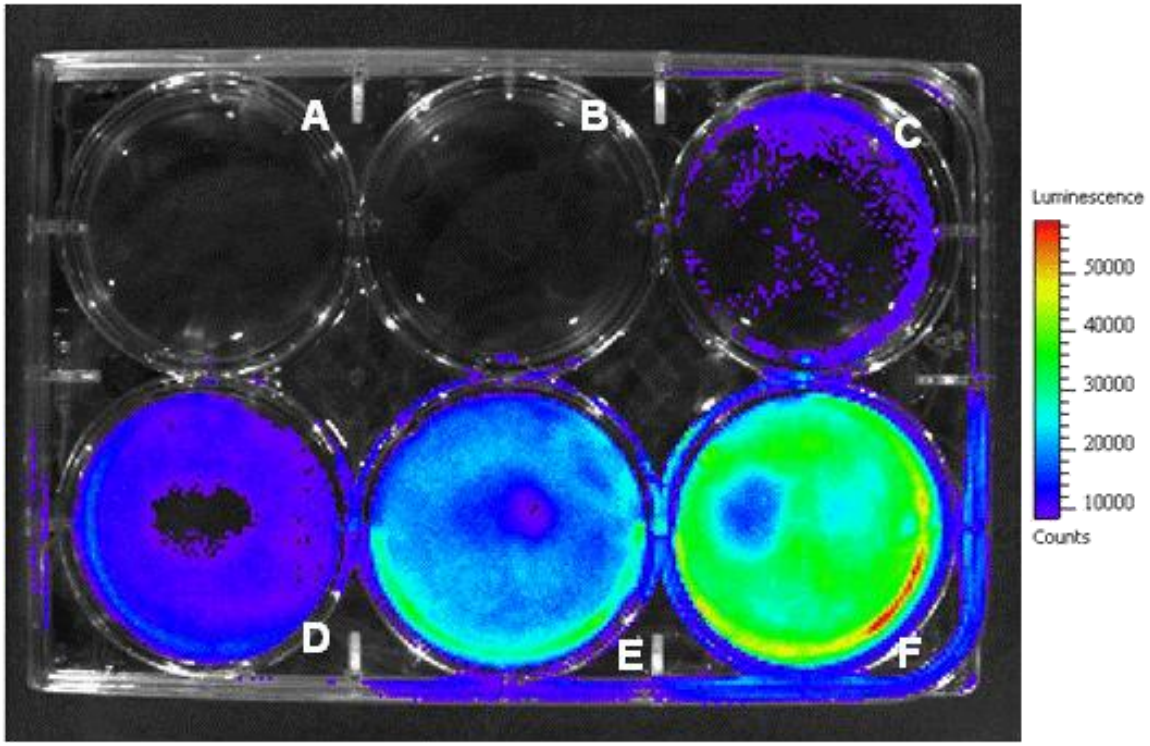


Figure 41: ViviRen titration on infected C6/36 cells

CHIKV-LUC infected C6/36 cells with a titration of ViviRen substrate. Negative controls were wells A and B. A: Uninfected C6/36 cells with 1 nM ViviRen. B-F: C6/36 cells infected with CHIKV-LUC at a concentration of $10^{4.5}$ $TCID_{50}$, 24 hpi. B: no ViviRen substrate, C: 0.1 nM ViviRen substrate, D: 0.25 nM ViviRen, E: 0.075 nM ViviRen and F: 1 nM ViviRen.

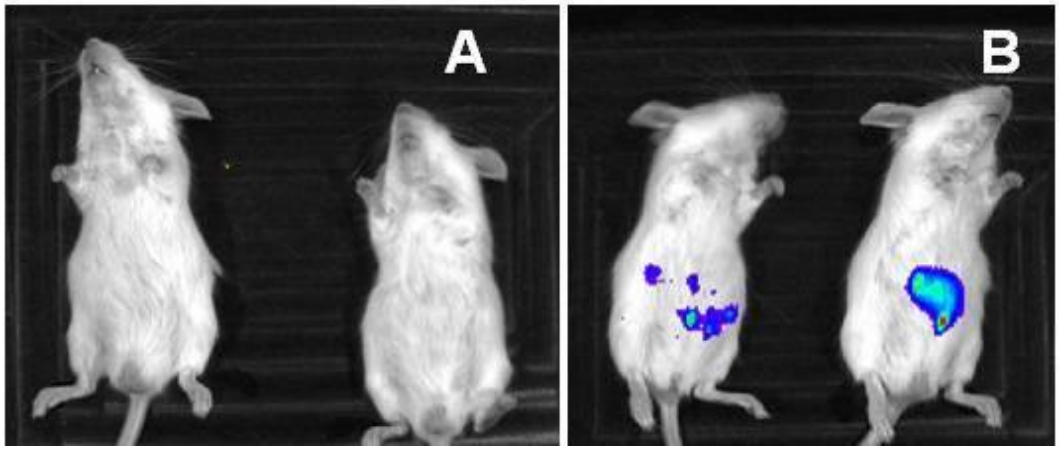


Figure 42: Autoluminescence after IP inoculation of ViviRen in CHIKV-LUC infected mice

Mouse on the left (control) in both images is infected with CHIKV-GFP, but also received ViviRen. Prior to ViviRen (A) and 5 minutes after ViviRen injection(B) imaging in the IVIS.

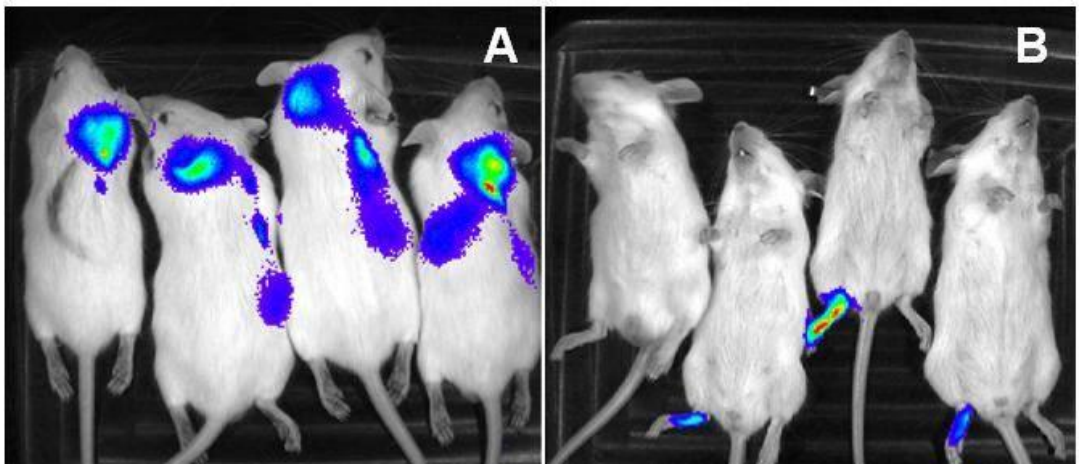


Figure 43: Autoluminescence of SC inoculation of ViviRen in CHIKV-LUC infected mice over time.

Mouse on the left (control) in each panel is uninfected, but received ViviRen inoculation. Five minutes (A) and twenty minutes(B) after inoculation of ViviRen in mice infected with CHIKV-LUC 48 hpi. Luminescence in the scruff of the neck and back is due to autoluminescence of ViviRen.

substrate addition (Figure 44). Luminescence was seen for up to 60 minutes after inoculation of ViviRen, but was significantly less than the signal at 25 minutes. At 12 hours after ViviRen inoculation, the substrate had been cleared from the mice and no luminescence was seen. With the optimization of the IVIS machine and the substrate timing and location we were able to commence pathogenesis experiments on young mice.

The first study of CHIKV-LUC in mice with IVIS imaging was with 3- week old CD-1 mice. These mice received an inoculation of CHIKV-LUC in the right hind footpad. As quickly as 12 hpi, luciferase activity could be seen in all mice studied (Figure 45). The luciferase activity could be seen for up to 6 dpi when it was minimal and was completely gone by 7 dpi. In one mouse, some dissemination was seen in the upper hind limb muscle starting on 4 dpi. Unfortunately, very little dissemination was seen outside of the hind limb muscle.

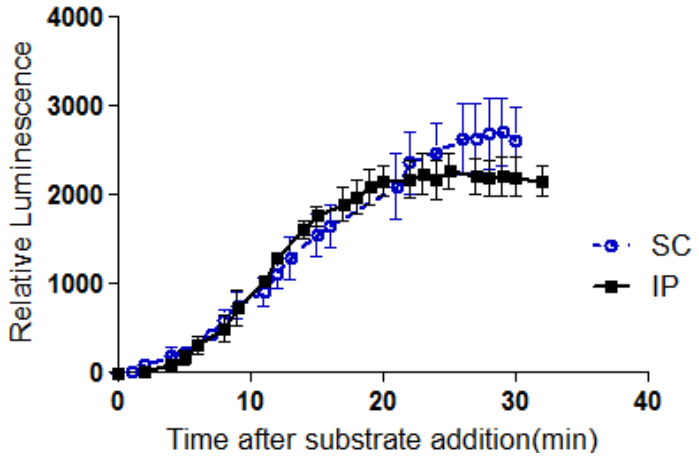


Figure 44: Comparison of inoculations of ViviRen

A comparison of subcutaneous and IP inoculation of ViviRen. Maximal luminescence was reached at approximately 25 minutes after ViviRen inoculation.

A second study was performed in 14-day old CD-1 mice, using the same inoculation strategies (Figure 46). This experiment was repeated with similar results. In these mice, luciferase activity was greatly increased. This was probably due to both the younger age of mice

(which we know increases the level of viremia) and the optimization of the IVIS imaging procedure including a longer imaging time. From 1 to 2 dpi there was a high amount of luciferase activity in the foot that was inoculated and in the surrounding tissue including the tail and the upper hind limb muscle. This activity decreased each day and as it was observed in previous experiments, by 7 dpi there was little to no luciferase signal. In all three mice, there was dissemination to the upper right leg and in one case, to the upper left leg. It was unknown which tissues had luminescence, but it was assumed that both the lymph nodes and the myocytes had viral replication (Gardner et al. 2008). There was no activity seen in the front limbs after 4 dpi. In the smallest mouse, there was luciferase activity seen in the mouth at each time point. It was assumed that the increased amount of viral replication in this mouse may be due to its smaller size and dilution effect. It was curious as to which tissue may be infected that resulted in the localized signal from the mouth and could possibly be lymphatic tissue in the cervical lymph node. Also, in the first 3 dpi there were patchy, inconsistent areas of the upper abdomen that had positive luciferase activity. It was unclear as to what organs this may correspond to, but it may be the liver or spleen.

CHIKV-LUC imaging of mosquitoes using IVIS

It was decided to apply the IVIS technology to infected mosquitoes and attempt to visualize CHIKV-LUC within the infected mosquitoes. Both *Ae. aegypti* and *Ae. albopictus* mosquitoes were used as they are the main urban vectors of CHIKV. The mosquitoes were fed an artificial blood meal mixed with

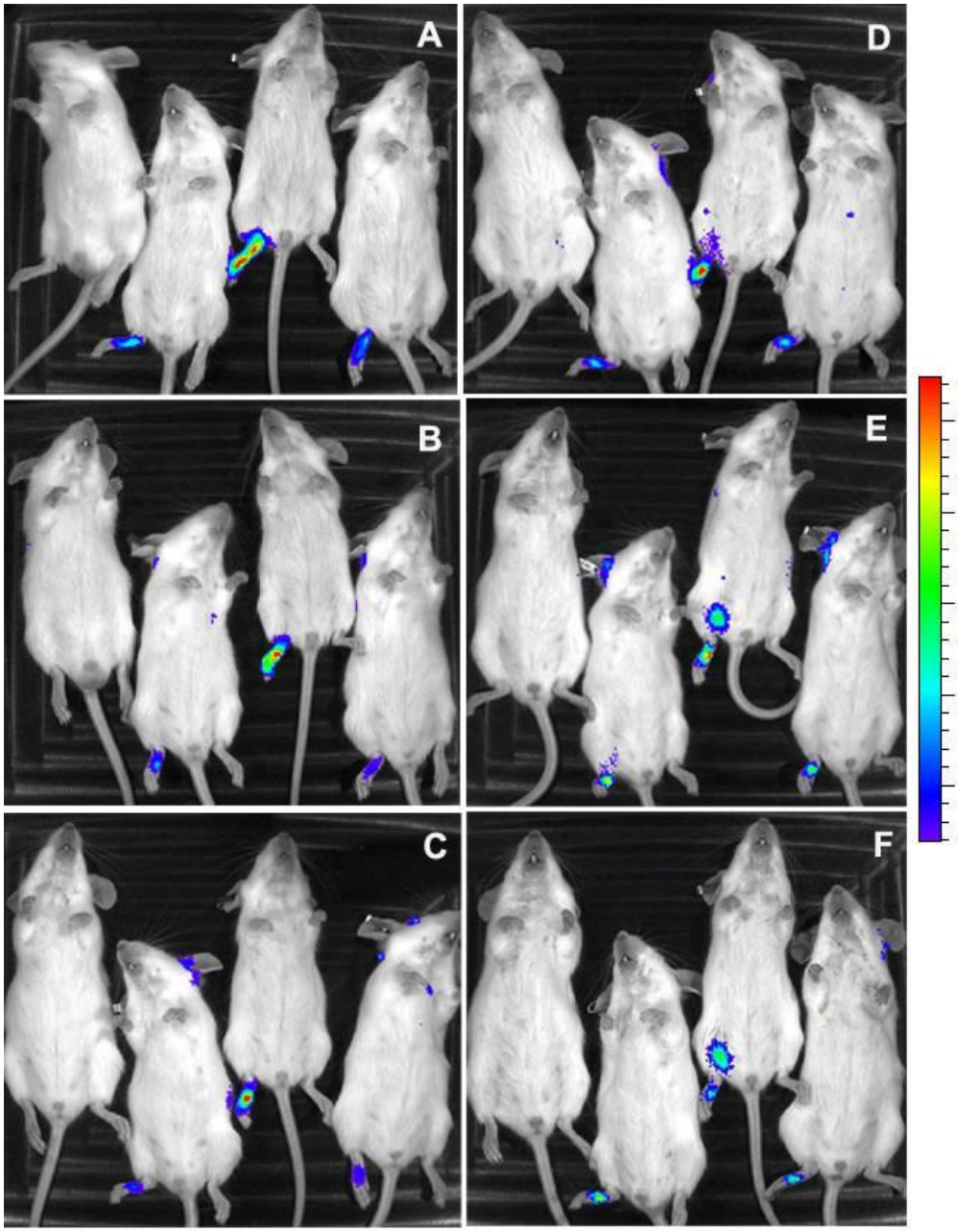


Figure 45: CHIKV-LUC infected 3-week old mice with IVIS imaging

The mouse pictured on the left on each panel was infected with CHIKV-GFP, but also received ViviRen (control). Mice were imaged at 12 h (A), 24h (B), 48h (C), 72h (D), 96h (E), and 5 days (F) after inoculation of CHIKV-LUC in the right rear foot pad.

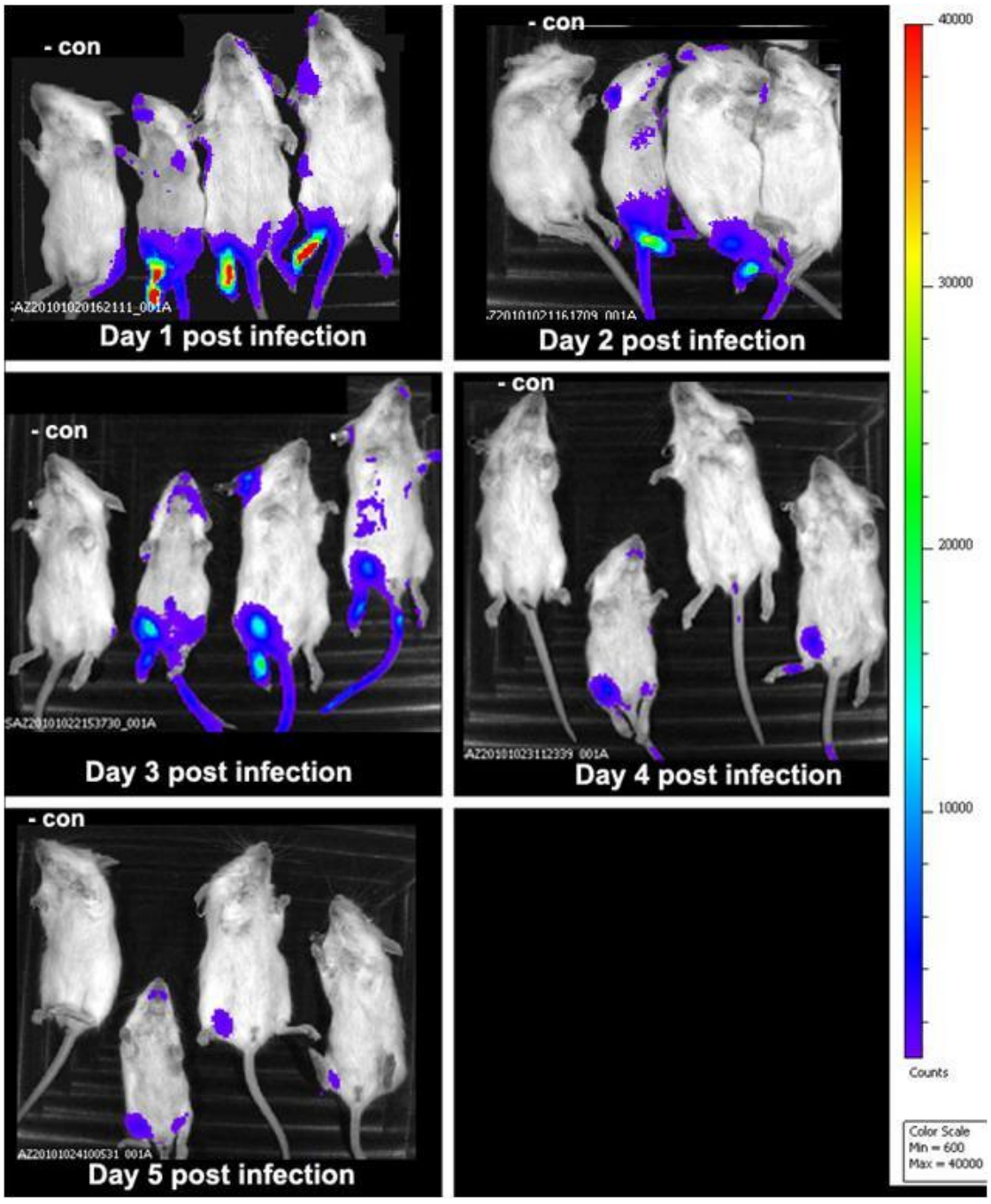


Figure 46: CHIKV-LUC infected 14-day old mice imaged with IVIS

The mouse pictured on the left on each panel was uninfected, but received ViviRen. Mice were imaged at 1, 2, 3, 4 and 5 dpi of CHIKV-LUC in the right rear foot pad.

CHIKV-LUC at a titer of $10^{4.5}$ $TCID_{50}$/mL. Mosquitoes were held for 7 days in environmental chambers. They were visualized using the IVIS technology by immobilizing live mosquitoes, with the legs and wings removed, and placed into 6-well dishes and held in place using double sided tape. The ViviRen substrate was injected IT into each mosquito. The mosquitoes were visualized approximately 40 minutes after substrate injection (Figure 47). There are multiple fields of view available on the IVIS, ranging from 23 cm across to 4 cm across. Images of the whole plate were first taken and then individual wells were analyzed.

We were able to detect positive luciferase activity in individual mosquitoes (Figure 47). Uninfected mosquitoes that were injected with ViviRen were used as a negative control. These uninfected mosquitoes did not have any luciferase activity (Figure 47; panel D). Infected mosquitoes were analyzed at both 3 and 7 dpi. There was a range of luciferase activity in both *Ae. albopictus* and *Ae. aegypti* mosquitoes (Figure 48). Using the IVIS images we were able to quantify both infection and dissemination of both *Ae. aegypti* and *Ae. albopictus* mosquitoes following infection by artificial blood meal (Table 10).

To determine a limit of detection of the IVIS on infected mosquitoes, individual mosquitoes were imaged, and then dissected and the head and body were subsequently separately titrated. Images of these mosquitoes were evaluated for being positive or negative luciferase activity. The luciferase intensity data was compared to the viral titers for the equivalent mosquito(Figure 49). There was an association between higher viral titers and luciferase activity. An approximate limit of detection for the IVIS in the mosquitoes was 2500 $TCID_{50}$/mL. Unfortunately, using the living image software to quantify the amount

of signal and comparing that to the titers in the mosquitoes was not directly correlative. It is unknown why the signal strength was not directly correlative to the virus titer, but the appearance of any luminescence signal was associated with higher titers of CHIKV-LUC in the mosquitoes.

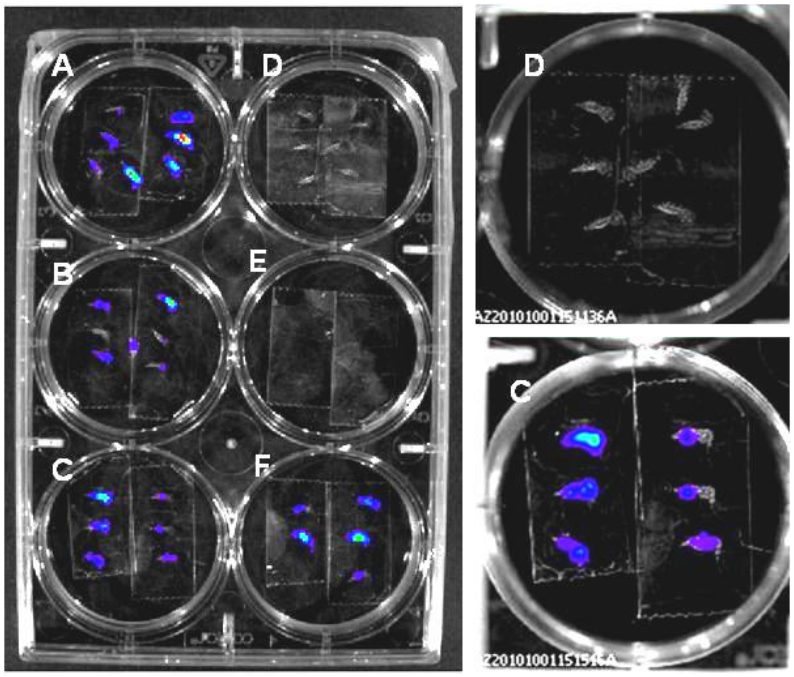


Figure 47: Mosquitoes infected with CHIKV-LUC and imaged with IVIS

Ae. aegypti mosquitoes were infected with CHIKV-LUC by artificial bloodmeal and 7 dpi were visualized for luciferase activity by placing them in 6-well plates. The whole plate (left panel) and a higher magnification picture of individual wells (right panels). Letters correspond to the individual wells found on the whole plate. Uninfected mosquitoes (D) and infected mosquitoes (C) with IT inoculation of ViviRen.

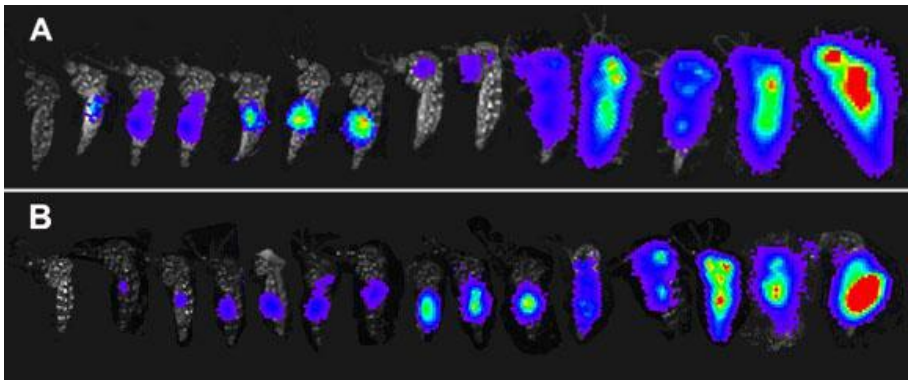


Figure 48: Ae. aegypti and Ae. albopictus infected with CHIKV-LUC

Ae. albopictus (A) and *Ae. aegypti* (B) infected with CHIKV-LUC at 3 and at 7 dpi visualized with the IVIS. Pictures are composites made up of individual pictures to show a range of infection seen in mosquito species.

Table 10. Mosquito infection and dissemination

Mosquito species	**3 dpi**	**7 dpi**
Ae. aegypti		
Midgut only	5/5 (100%)	6/23 (26.1%)
Disseminated	0/5	17/23 (73.9%)
Total	5/9 (55.6%)	23/24 (95.8%)
Ae. albopictus		
Midgut only	5/8 (62.5%)	8/10 (80%)
Disseminated	3/8 (37.5%)	2/10 (20%)
Total	8/9 (88.9%)	10/15 (66.7%)

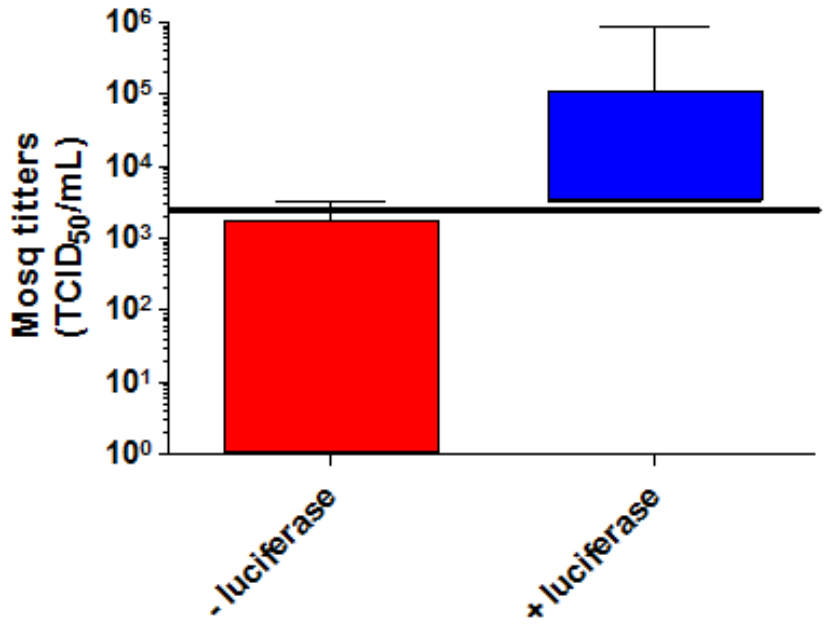


Figure 49: Limit of detection for IVIS luciferase activity in individual mosquitoes

Individual mosquitoes were categorized as either infected or disseminated and individual heads and abdomens were titrated. The line at 2500 $TCID_{50}$/ml represents an estimate of the cutoff for visualization with the IVIS machine

Discussion

The use of CHIKV-GFP in mosquitoes has lead to easy identification of infected mosquitoes and visualization of viral replication at the tissue level. But unfortunately, this technology has not yielded good results for use with mice. Fixation of GFP was problematic because both ethanol and formalin lead to intrinsic fluorescence in negative tissues. Frozen sections of mouse tissues with GFP worked much better, but the disruption of the tissue by freezing hampered the identification of the specific cells that were involved in viral replication. IVIS

imaging with GFP infectious clones is not useful, as the intrinsic fluorescence of the mouse skin and hair hamper the ability to visualize the signal. Even shaving the mice before visualization does not decrease the background to a point where signal can be seen to a significant level.

One limitation of these studies is that the CHIKV-LUC infectious clone was not rigorously compared to the wild type CHIKV. It is unknown if the CHIKV-LUC had the same pathogenic properties as the wild type virus and if replication of the infectious clone was altered by the addition of the luciferase gene. It is also currently unknown if the CHIKV-LUC clone excises the luciferase gene during replication, but this was not observed in these studies. While direct comparisons were not conducted, the results obtained imply that the CHIKV-LUC clone behaved similarly to wild type CHIKV. In previous studies (Figure 17), CHIKV was isolated for 5-6 days post infection from leg tissue in 14-day old mice, while in the IVIS studies luciferase activity was seen for 5 days in the leg muscle. Also in previous studies, virus was rarely isolated from the brains of 14-day old mice and in the IVIS work, no luciferase activity was seen in any of the brains of the young mice inoculated with CHIKV-LUC.

Recent studies with SINDV-LUC in mice have utilized the IVIS technology to understand the neuroinvasiveness of this virus (Cook et al. 2003, Ryman et al. 2007, Ryman et al. 2007). It is not surprising that the CHIKV-LUC infected mice did not have this same tissue association since earlier published work have shown that SC and footpad inoculation of CHIKV does not result in neuroinvasive disease (Gardner et al. 2010, Ziegler et al. 2008). Infection with CHIKV-LUC in the footpad of young mice is similar to results seen in with VEEV and EEEV (Gardner et al. 2008). In comparison with this study, CHIKV behaved most like

VEEV with dissemination to the upper leg, which would then give more proof of CHIKV ability to infect macrophages and lymph nodes similar to VEEV and in contrast to EEEV. These mice exhibited areas of luciferase activity in the neurons and in the brains, which, is to be expected due to the nature of the virus tropism.

Mosquito imaging using IVIS technology is a novel technique that promises to be useful in the screening of live mosquitoes infected with luciferase infectious clones. By using the IVIS technology infected mosquitoes were rapidly detected, that could be used to express saliva or to dissect for titrations. In these experiments the legs and wings of the mosquitoes were removed, but in future experiments could be done with whole mosquitoes. By doing this, mosquitoes with disseminated infections could be utilized for transmission experiments. This is one of the only systems to image whole live mosquitoes without causing them permanent damage.

The definition of dissemination in infected mosquitoes may need to be better defined when using IVIS. When examining the mosquitoes, there are mosquitoes in which the infection involves the whole body of the insect, but there are more mosquitoes that only parts of the mosquito have luciferase activity. Using IVIS, one may be able to better define the extrinsic incubation period and categorize to viral replication to the abdomen, thorax and or head. This could also help to better understand the interaction of the mosquito and CHIKV. It is still unclear which factors play a role in a viruses refractory nature in some mosquitoes, or why infection rates vary so widely between different mosquito species and strains. Having a technology where one can see viral replication in

real time, in whole mosquitoes may have great potential for the future of vector biology.

Overall, I feel that IVIS imaging with the CHIKV-LUC clone is a useful technique in understanding CHIKV pathogenesis. There is continued work that needs to be completed to understand the limitations of the CHIKV-LUC clone. The use of IVIS greatly decreases the number of animals needed for specific studies. This technology could be used as a screening tool for different therapeutics and modeling systems. The IVIS technology with further validation could be a very quick and inexpensive tool to analyze CHIKV pathogenesis.

Chapter 5: The role of mosquito saliva in CHIKV pathogenesis in mice

Abstract

Over the past 15 years there has been mounting evidence demonstrating a distinct difference between natural transmission of an arbovirus via vector feeding and laboratory needle transmission. This is thought to be due to substances in vector saliva that facilitate feeding and have immune modulating functions in vertebrates (Almeras et al. 2010, Boppana et al. 2009, Chen et al. 1998, Kramer et al. 2011). Recently, some arboviruses have been shown to have a different disease outcome due to vector transmission as compared to needle inoculation (Limesand et al. 2000, Schneider et al. 2008, Schneider et al. 2007, Schneider et al. 2006, Styer et al. 2011, Thangamani et al. 2010). The aim of these studies was to determine the effects of vector transmission of CHIKV to mice. Infected *Ae. aegypti* transmission was used as a comparison to needle inoculation. Mice were also passively immunized to mosquito saliva prior to mosquito feeding. In addition, needle inoculation at an uninfected mosquito bite area was compared to needle inoculation in unbitten mice to determine dose effects. These studies have shown that mosquito bite-inoculation of CHIKV results in a decrease in myositis as compared to needle inoculation. This was determined to be in part due to both a mosquito bite effect as well as dose. With the knowledge from this work, it is determined that while mosquito saliva does cause a less severe disease in mice, dose is also a key factor in disease severity.

Introduction

There is a growing body of literature that suggests that arthropod saliva modulates viral disease pathogenesis (Schneider et al. 2008, Schneider et al. 2007, Styer et al. 2011). Tick saliva has been shown to have a number of factors that affect coagulation and the innate immune system in humans (Francischetti et al. 2009, Kern et al. 2011, Kramer et al. 2011, Oliveira et al. 2011). This is also true, to a lesser extent, of mosquitoes. The SAAG-4 protein has been identified in *Ae. aegypti* saliva and has been shown to further enhance the Th2 response of the host immune system by down regulating CD4 T cells and IFN-γ while increasing IL-4 (Boppana et al. 2009). Recent investigations of the early markers of host immune response during CHIKV infection in mice has shown that mosquito bite-inoculation of virus causes a Th2 immune response (Thangamani et al. 2010). Previous work has also shown that mice develop antibodies to mosquito saliva and that passive immunization with these antibodies can further enhance the Th2 response to mosquito bites (Chen et al. 1998, Schneider et al. 2007).

The factors that distinguish the mosquito bite from needle inoculation include: location of the bite, exact tissue that is inoculated, dose of the inoculum, the volume of the inoculum, components of mosquito saliva and differences in the virus once it replicates within the mosquito. Mosquitoes transmit viruses in their saliva through probing and feeding. On mice, mosquitoes tend to bite those areas without hair, including the feet, nose and tail. During mosquito probing, the saliva is deposited in the intradermal space until a suitable pool of blood is found for feeding resulting in virus both outside the vasculature and directly into the vasculature (Ribeiro 2000, Styer et al. 2007, Turell et al. 1992). The amount of

saliva deposited while probing is very small (1-13 µg) (Hurlbut 1966), and in the case of CHIKV in *Ae aegypti* and *Ae. albopictus* the viral titer in saliva peaks at $10^{3.3}$ PFU/saliva for *Ae. albopictus* and $10^{2.5}$ PFU/saliva for *Ae. aegypti* (Dubrulle 2009). Virus replicating in mosquito cells has a different pattern of glycosylation and cholesterol incorporation within the lipid membrane as compared with virus replicated within vertebrate cells (Burge et al. 1970, Hafer et al. 2009, He et al. 2010, Knight et al. 2009). In these studies, a comparison of needle-inoculation and mosquito bite-inoculation of CHIKV was undertaken and specifically the role of the viral dose and mosquito saliva in the pathogenesis of CHIKV in mice was studied.

The objective of this aim was to characterize the viral pathogenesis of CHIKV in mice exposed to mosquito feeding. We hypothesized that mosquito saliva would decrease the disease severity in CHIKV-infected mice. It was also hypothesized that by passively immunizing the mice to mosquito saliva prior to feeding, the immune modulating effects of the mosquito saliva would be increased. This decrease in disease severity would also be seen when needle inoculated virus was delivered at the site of mosquito feeding.

Results

Mosquito bite-inoculation of CHIKV as compared with needle-inoculation

To determine the role of mosquito bite-inoculation with CHIKV pathogenesis, *Ae. aegypti* served as the vector to inoculate mice. To further enhance the saliva induced Th2 response, one group of mice received passive immunization against mosquito saliva (Chen et al. 1998, Schneider et al. 2007). This was achieved by allowing *Ae. aegypti* mosquitoes (n=25) to feed on 4 adult

female mice four times, one week apart. Two weeks after the last feeding, the mice were given sarcoma cells and HIAF was collected. This was centrifuged and frozen for later use. In order to passively immunize the mice, the mice received 100µL of the HIAF IP, 1 hour prior to *Ae. aegypti* feeding. All infected mosquitoes used for these studies were inoculated IT with the same virus stock. The mosquitoes were held for 7 to 10 days at 28°C before feeding on mice.

For initial studies, mice were infected by either the bite of 3-5 *Ae. aegypti* or by needle inoculation SC in the skin of the back with $10^{4.5}$ PFU of CHIKV. This dose was used as it was comparable to that of West Nile virus titers from mosquitoes (Vanlandingham et al. 2004). Mice infected by mosquito bite with and without passive immunization did not show any signs of overt disease, including hind limb paralysis or mortality. Randomly selected mice were necropsied daily to assay viral titers and histopathologic changes. Viremia levels in mice inoculated by mosquito bite were higher on the second dpi and this difference was amplified by prior passive immunization to mosquito saliva (Figure 50). There was approximately a ten-fold difference between the geometric mean of the needle inoculated group and the mosquito bite group with passive immunization. This is a transient effect and by the fourth dpi viremia levels were below the limit of detection in all groups.

The contrasting effect was observed when investigating the viral loads within the leg tissues of the infected mice (Figure 51). On days 1, 3 and 5 pi the viral load in the mosquito bite inoculated groups were significantly less than those seen in the needle inoculated group ($p < 0.05$, Mann-Whitney statistical analysis). On every day tested, the needle inoculated group had higher titers of CHIKV in the leg muscle tissue. When comparing the viral load in the leg tissue

of the immunized and non-immunized mosquito bitten group, the immunized group tended to have lower titers in the leg tissue, but there were no statistically significant differences ($p > 0.05$).

Viral load in the liver and spleen were also determined for the mosquito bitten and needle inoculated groups. Previous results have suggested that viral titers in the liver are similar in trend to that of viremia, while titers in the spleen are correlative to that of leg muscle (Chapter 3). For the viral loads in the liver, this trend held true, with the higher viral loads found in the mosquito bitten groups (Figure 52). Two dpi the mosquito bitten groups had higher viral loads than the needle inoculated groups, while virus was cleared quickly from the liver and was undetectable by 4 dpi. Viral loads in the spleen were more sporadic with no definite trends seen (Figure 53). The mosquito bitten group that had been immunized had higher viral titers throughout and by the sixth dpi the viral titer had fallen below the limit of detection for all groups .

Pathology samples were also taken and analyzed to determine the severity of the myositis seen in all groups using a histopathological grading scale described previously (Chapter 3). It was apparent that the mosquito bitten groups had significantly less pathological changes in the skeletal muscle. This difference can be seen specifically in the amount of inflammation and lesions seen within the skeletal muscle (Figure 54). The lesion distribution was graded by approximating the percentage of the muscle that is affected by inflammatory lesions. In needle-inoculated mice, lesion distribution reached 38% on 6 dpi and remained high until 10 dpi, with a peak of 76% on 7 dpi. For the mosquito bitten group, lesion distribution peaked at 38% on 8 dpi, and the mice with prior

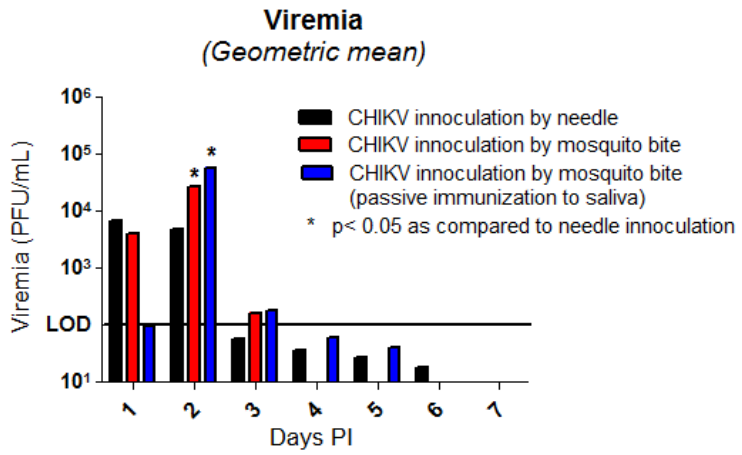


Figure 50: Viremia levels in needle inoculated mice as compared to mosquito bite-inoculation

Viremia levels in young CD-1 mice after inoculation of CHIKV either by needle or *Ae. aegypti* bite. The third group received passive immunization of antibodies to *Ae. aegypti* saliva 1 hour prior to mosquito inoculation of CHIKV. Bars represent the geometric mean of individual mouse samples.

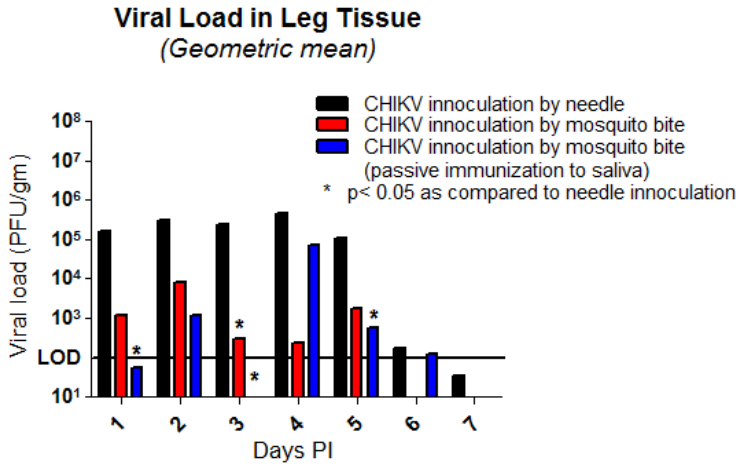


Figure 51: Viral load in leg tissue of needle inoculated mice as compared to mosquito bite-inoculation of CHIKV

Viral loads in the leg tissue of young CD-1 mice after inoculation of CHIKV either by needle or *Ae. aegypti* bite. The third group received passive immunization of antibodies to *Ae. aegypti* 1 hour prior to inoculation of CHIKV by *Ae. aegypti*. Bars represent the geometric mean of individual mice samples.

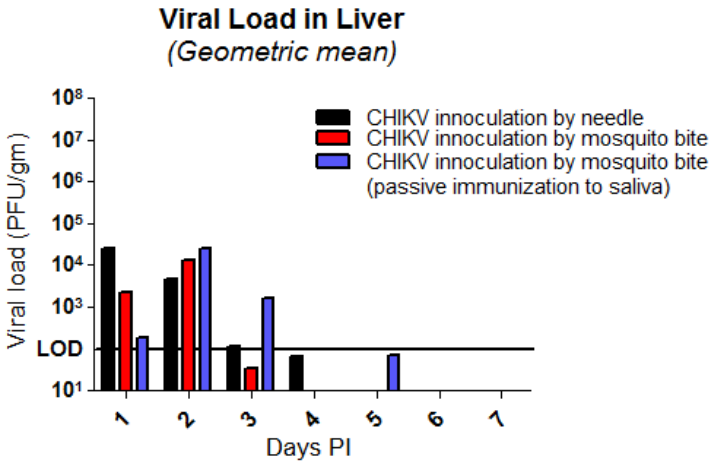


Figure 52: Viral loads in liver of needle inoculated mice as compared to mosquito bite-inoculation

Viral loads in liver of young CD-1 mice after inoculation of CHIKV either by needle or *Ae. aegypti* bite. The third group received passive immunization of antibodies to *Ae. aegypti* 1 hour prior to inoculation of CHIKV by *Ae. aegypti*. Bars represent the geometric mean of individual mice samples.

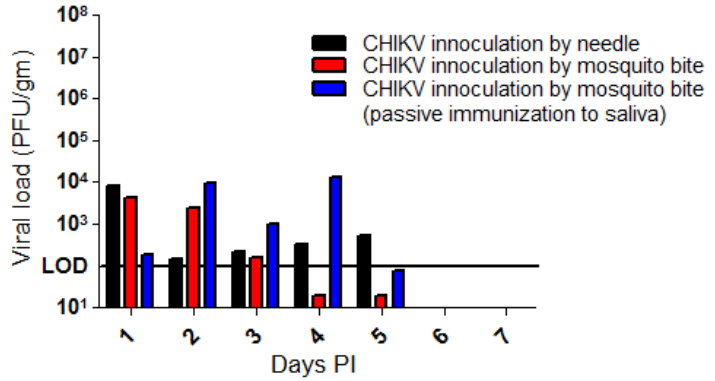


Figure 53: Viral load in spleen of needle inoculated mice as compared to mosquito bite-inoculation

Viral loads in the spleen of young CD-1 mice after inoculation of CHIKV either by needle or *Ae. aegypti* bite. The third group received passive immunization of antibodies to *Ae. aegypti* 1 hour prior to inoculation of CHIKV by *Ae. aegypti*. Bars represent the geometric mean of individual mice samples.

immunization had peak distribution at 28% on 7 dpi. These data were not statistically significant ($p>0.05$), but shows that there was a difference seen in the amount of pathology in the leg tissue.

The severity of inflammation was also measured in the leg tissue. The inflammation was scored from 1-4, with 3-4 being severe inflammation with expansion of the muscle fibers. The differences in the severity of the inflammation were not as notable between the 3 groups (Figure 55). In needle inoculated mice, the average score was 4 on both 6 and 7 dpi and was severe (score >3) from 6-10 dpi. In the mosquito bitten group, without immunization, it peaked with a score of 4 on 8 dpi and was severe on 4 dpi and 8-11 dpi. The mosquito bitten group with prior immunization peaked at a score of 3 on 7 dpi

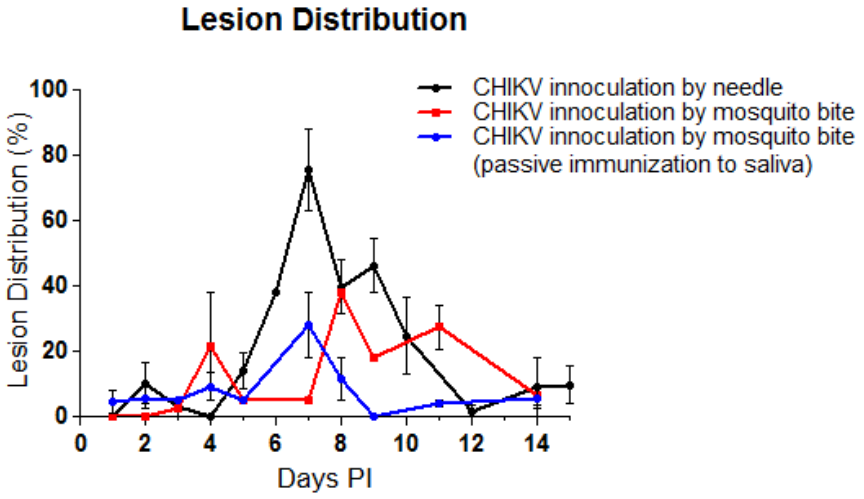


Figure 54: Distribution of lesions in leg tissues of needle inoculated mice as compared to mosquito bite-inoculation

Lesion distribution in leg tissue samples of young CD-1 mice after inoculation of CHIKV either by needle or *Ae. aegypti* bite. The third group received passive immunization of antibodies to *Ae. aegypti* 1 hour prior to inoculation of CHIKV by *Ae. aegypti*. Pathology samples were fixed, embedded and stained with H&E. Samples were graded as previously described (Chapter 3).

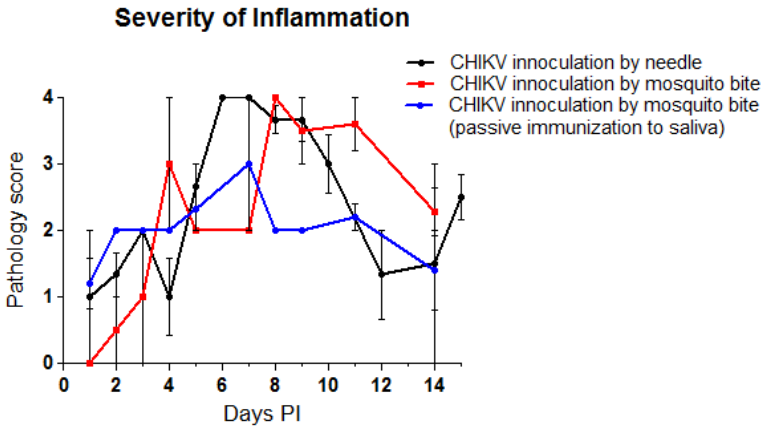


Figure 55: Inflammation severity in leg tissues of needle inoculated mice as compared to mosquito bite-inoculation

Severity of inflammation in leg tissue samples of young CD-1 mice after inoculation of CHIKV either by needle or *Ae. aegypti*. The third group received passive immunization of antibodies to *Ae. aegypti* 1 hour prior to inoculation of CHIKV by *Ae. aegypti*. Pathology samples were fixed, embedded and stained with H&E.

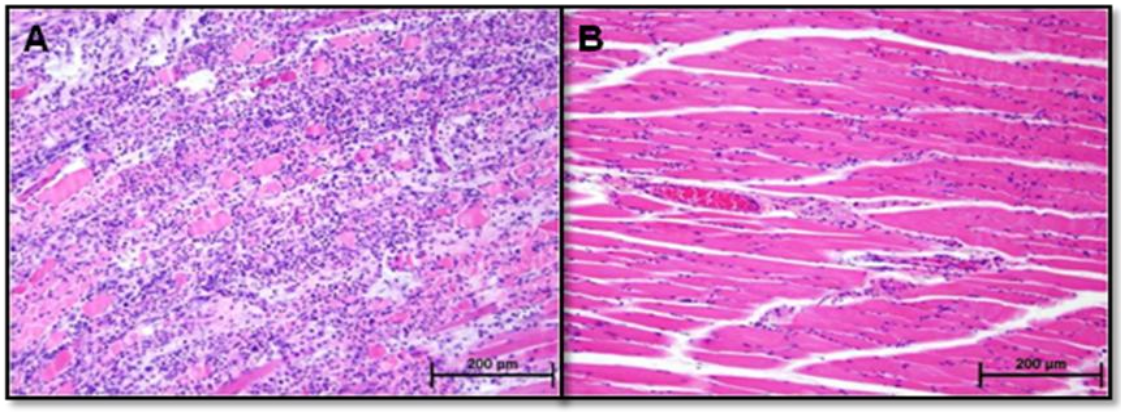


Figure 56: Pathological changes seen in CHIKV-infected mice by needle innoculation and mosquito bite

Skeletal muscle from young mice infected with CHIKV, 7 dpi. Samples were fixed, embedded and stained with H&E. A: Needle inoculated CHIKV at $10^{4.5}$ PFU SC in the skin of the back. B: Mosquito bite inoculated CHIKV, by the bite of 3-5 infected *Ae. aegypti* mosquitoes.

and this was the only day that it was severe. Representative pictures show the severe inflammation, necrosis and neutrophils within the skeletal muscle 7 dpi in the needle-inoculated mice, while the mosquito bite inoculated mouse had only minor inflammation (Figure 56).

Neutrophils were also identified on the pathology slides of the CHIKV-infected mice because neutrophils are usually an indicator of a more severe inflammatory response. The presence of neutrophils was analyzed by looking at the percentage of tissues that had neutrophils present in the areas of inflammation (Figure 57). Needle inoculated mice were more likely to have neutrophils present in the areas of inflammation, while prior immunization with mosquito bite-inoculation had less neutrophils present. In needle-inoculated mice, more than 50% of the tissues had neutrophils present from 5 to 9 dpi and specifically on dpi 6 and 7, 100% of the tissues were positive for neutrophils. Mosquito bite inoculated mouse tissues were more than 50% positive for neutrophils from 6 to 8 dpi and specifically, 100% positive for neutrophils on 7 dpi. Mosquito bitten mice with prior immunization peaked at 50% positive for neutrophils on 7 dpi.

Myositis in the skeletal muscle is a hallmark of CHIKV infection in mice, with severe myocyte destruction being present. In needle inoculated mice, necrosis could be seen in over half of the tissue samples from 5-10 dpi (Figure 58). On 6 to 9 dpi, all needle inoculated mouse samples had myocyte destruction present in the skeletal muscle. In mosquito bitten mice, the amount of necrosis seen was less. In mosquito bite inoculated mice, without prior immunization, 50% or more of tissues had necrosis on 4, 8, 9 and 11 dpi with 100% of the tissue samples having some necrosis on 8 dpi. In the mosquito bite

inoculated group receiving immunization, necrosis was even less likely with the only time point above 50% was on dpi 7, when 100% of the tissues had some necrosis.

The last measure of pathology used to grade the leg tissue samples was the presence or absence of inflammation of the soft tissue, mainly fat or brown fat, surrounding the skeletal muscle. In the needle inoculated group, the presence of inflammation in the soft tissue was much higher than either of the mosquito bitten groups (Figure 59). On both 7 and 8 dpi, 100% of the tissue samples displayed inflammation in the soft tissue. Neither of the mosquito bite inoculated groups had more than 50% of the legs sampled with inflammation in the soft tissue. There was very little difference in the amount of soft tissue inflammation between both of the mosquito bitten groups.

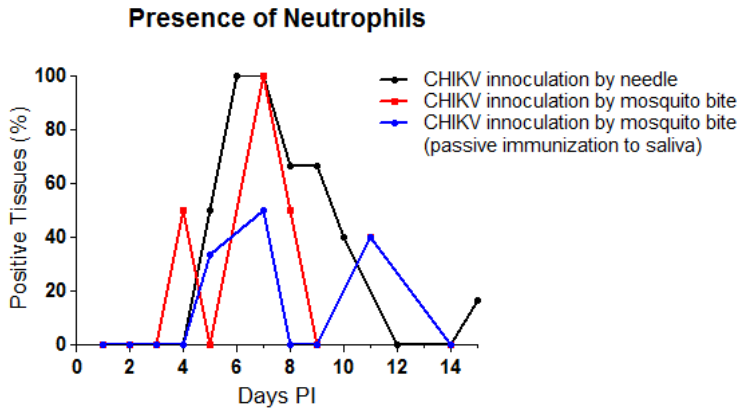


Figure 57: Neutrophil presence in leg tissues of needle inoculated mice as compared to mosquito bite-inoculation

Neutrophil presence in leg tissue samples of young CD-1 mice after inoculation of CHIKV either by needle or *Ae. aegypti* bite. The third group received passive immunization of antibodies to *Ae. aegypti* 1 hour prior to inoculation of CHIKV by *Ae. aegypti*. Pathology samples were fixed, embedded and stained with H&E. Graph represents the percent of tissues that neutrophils were present upon examination.

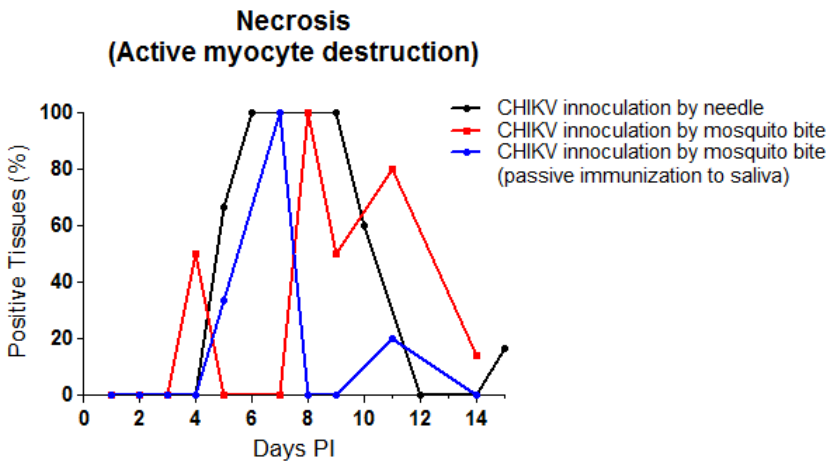


Figure 58: Necrosis presence in leg tissues of needle inoculated mice as compared to mosquito bite-inoculation

The presence of necrosis in leg tissue samples of young CD-1 mice after inoculation of CHIKV either by needle or *Ae. aegypti* bite. The third group received passive immunization of antibodies to *Ae. aegypti* 1 hour prior to inoculation of CHIKV by *Ae. aegypti*. Pathology samples were fixed, embedded and stained with H&E. Graph represents the percent of tissues that had necrotic myocytes upon examination.

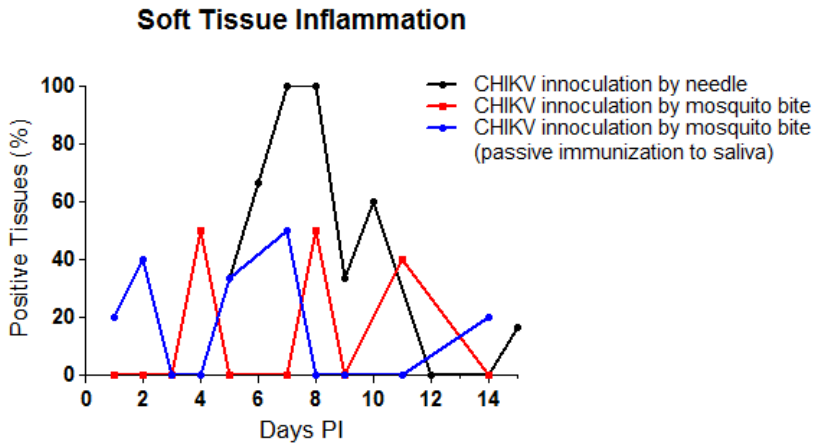


Figure 59: Inflammation of the soft tissue of leg samples of needle inoculated mice as compared to mosquito bite-inoculation

Inflammation of the soft tissue in leg tissue samples of young CD-1 mice after inoculation of CHIKV either by needle or *Ae. aegypti* bite. The third group received passive immunization of antibodies to *Ae. aegypti* 1 hour prior to inoculation of CHIKV by *Ae. aegypti*. Pathology samples were fixed, embedded and stained with H&E. Graph represents the percent of samples with inflammation in the soft tissue upon examination.

Overall, the pathology seen in the needle inoculated group was the most severe, while the pathology seen in the mosquito bite inoculated group with prior immunization to *Ae. aegypti* saliva was the least severe. This data is correlative to the viral loads found in the leg tissue, but was not correlative to those found in the liver or viremia levels.

Dose control studies with mosquito bites

When initiating these experiments, the amount of CHIKV that a single mosquito would deposit while probing and feeding was unknown. It was thought that by allowing 3-5 mosquitoes to feed, the viral inoculum would be similar to the $10^{4.5}$ PFU used as a needle inoculation as was seen with West Nile virus (Vanlandingham et al. 2004). A recent published report refuted this idea and stated that mosquitoes were more likely inoculating much less with a maximum of $10^{2.5}$ PFU/saliva for *Ae. aegypti* (Dubrulle et al. 2009). In order to test whether the differences we were seeing were due to the mosquito saliva itself or was just an artifact of a lower dose of virus, a second set of experiments was initiated with known doses of virus at both a high ($10^{4.5}$ PFU) and low ($10^{2.4}$ PFU) doses of CHIKV. In order to coordinate mosquito feeding and needle inoculations, CHIKV was injected into the footpad of the mice and this was done either without any mosquito feeding, or immediately after three to 5 uninfected *Ae. aegypti* were allowed to feed on the same footpad. When taking necropsy samples, the inoculated foot/leg was not used for either the histopathology analysis or viral titers. The front leg was used for histopathological sampling and the opposite rear leg was used for the viral titers of leg tissue.

Viral load in the leg tissues followed the same trend as before both in the high titer and low titer inoculated CHIKV. In the high dose groups, the needle inoculated without mosquito bite viral load was higher at every time point than that of the mosquito bitten group with the exception of one time point (Figure 60). In general, the viral titer decreased slightly each day until 6 dpi, when it was lower than the limit of detection. In the lower dose groups, the mosquito bite group had significantly less virus than the non-bitten group on 1, 2 and 4 dpi (Figure 61). The viral load remained almost constant in both groups for 5 days, until 6 dpi when the level significantly dropped off and on 7 dpi it was below the limit of detection. Between the high and low dose groups, the higher dose group exhibited viral loads approximately tenfold higher on 1 dpi; but by 4 dpi, the viral titers in the legs were at similar levels. The duration of viral load in the leg was similar in both the high and low dose groups.

Viremia levels were also assessed in all four groups of mice. In the high dose group, viremia levels were above 10^5 PFU/mL on 1 dpi in both the needle-inoculated group with and without mosquito bite (Figure 62). Viremia levels in both groups were very similar and were below the limit of detection by 4 dpi. In the low dose groups, very similar viremia levels were observed as those in the higher dose groups (Figure 63). On 1 to 3 dpi the viremia levels in both the needle inoculated with and without mosquito bite groups were above 10^5 PFU/mL and on 4 dpi they dropped dramatically. Again, on dpi 5 the levels of viremia were undetectable. When comparing the high and low dose groups, it is interesting to note that the level and duration of the viremia varied very little in comparison to each other. The overall trend when comparing the two doses was that the lower dose group had higher levels of viremia.

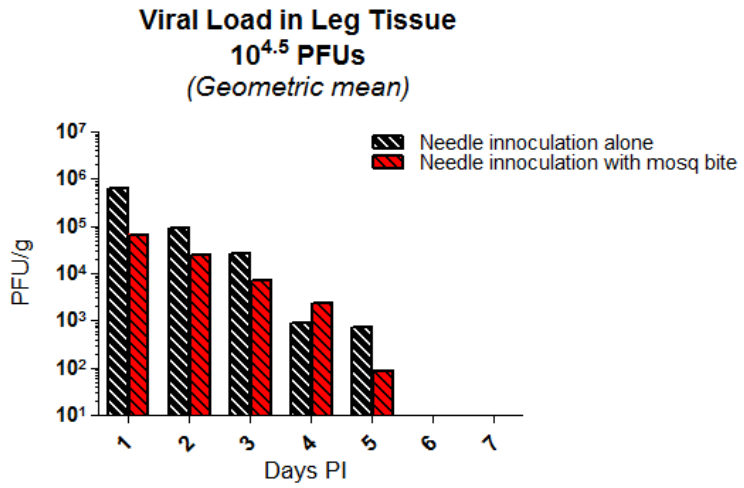


Figure 60: Viral load in leg tissues of mice needle inoculated with CHIKV at $10^{4.5}$ PFU with prior mosquito feeding

Viral load in leg tissue in young CD-1 mice needle inoculated with $10^{4.5}$ PFU of CHIKV in the footpad. The second group of mice were fed upon by *Ae. aegypti* mosquitoes in the same footpad immediately prior to needle inoculation. Bars represent the geometric mean of the individual mouse samples from two experiments.

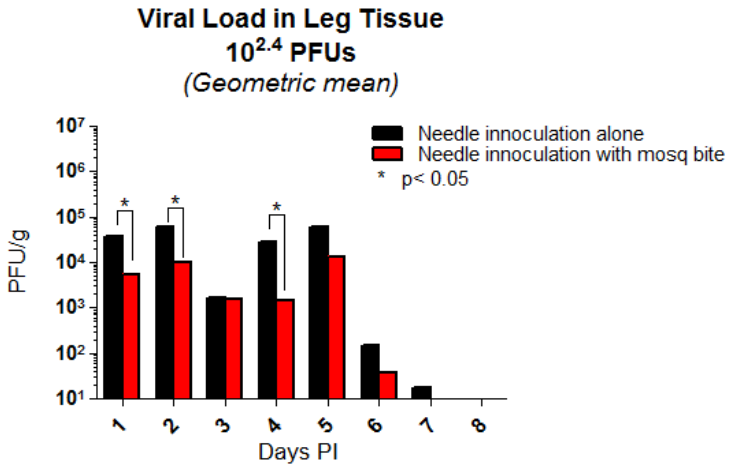


Figure 61: Viral load in leg tissues of mice needle inoculated with CHIKV at $10^{2.4}$ PFU with prior mosquito feeding

Viral load in leg tissue in young CD-1 mice needle inoculated with $10^{2.4}$ PFU of CHIKV in the footpad. The second group of mice were fed upon by *Ae. aegypti* mosquitoes in the same footpad immediately prior to needle inoculation. Bars represent the geometric mean of the individual mouse samples from two experiments.

Viral load in the spleen was also assessed in both dose groups. In both high dose groups, the viral load in the spleen was greater than 10^6 PFU/gm on dpi one and remained higher than 10^4 PFU/gm until dpi 5 (Figure 64). On 6 dpi, the viral titers were below the limit of detection. There was very little difference between the needle inoculated with and without mosquito bite groups as compared to viral load in the spleen, but on most days the titer has higher in the group without mosquito bites. In the low dose groups, viral load in the spleen peaked at 2 dpi at was approximately 10^6 PFU/gm (Figure 65). Viral loads in the low dose group remained higher than 10^4 PFU/gm for 4 dpi and were below the limit of detection on 6 dpi. In the low dose groups, the viral load was not significantly different between the mice receiving mosquito bites and those that did not.

Leg samples were taken from all groups of mice daily and the pathological changes were scored. Overall, the amount and severity of the pathology seen was very low in comparison to earlier studies in all groups analyzed. In the low dose group, the lesion distribution in the muscle tissue peaked at 21% on 7 dpi in the group not receiving mosquito bites and at 17% on 9 dpi in the mosquito bitten group (Figure 66). In the high dose group, the lesion distribution was also lower than expected (Figure 67). With the high dose, lesion distribution peaked at 7 dpi at 28% and 22% in the needle-inoculated group without and with mosquito bites respectively. In both the high and low dose groups there were no differences between the mosquito bitten and non-mosquito bitten groups. It is also surprising at how similar the lesion distribution was in both the high and low dose groups.

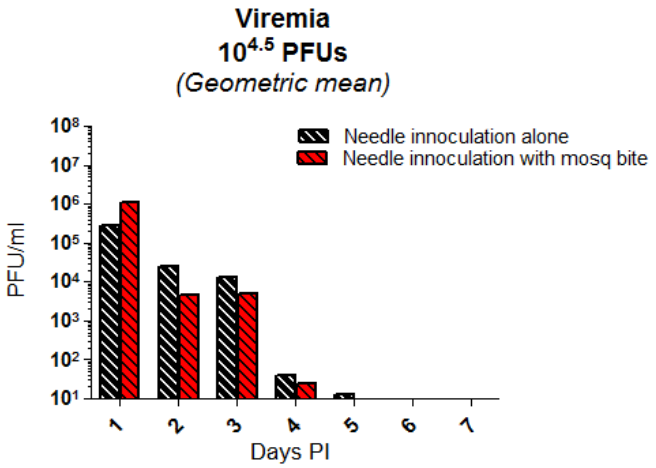


Figure 62: Viremia of mice needle inoculated with CHIKV $10^{4.5}$ PFU with prior mosquito feeding.

Viremia of young CD-1 mice needle inoculated with $10^{4.5}$ PFU of CHIKV in the footpad. The second group of mice were fed upon by *Ae. aegypti* mosquitoes in the same footpad immediately prior to needle inoculation. Bars represent the geometric mean of the individual mouse samples from two experiments.

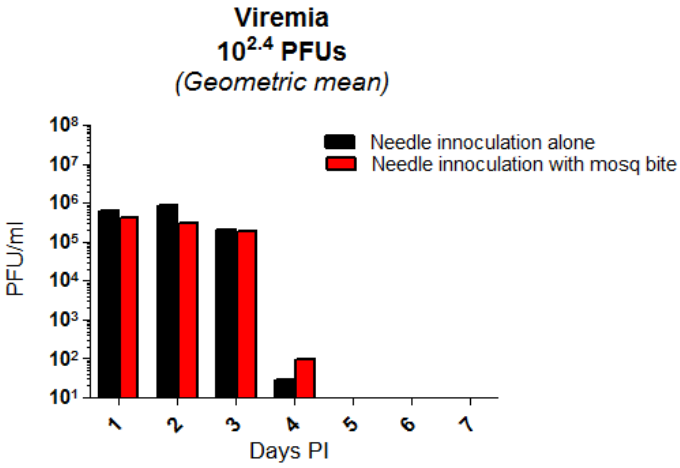


Figure 63: Viremia of mice needle inoculated with CHIKV $10^{2.4}$ PFU with prior mosquito feeding.

Viremia of young CD-1 mice needle inoculated with $10^{2.4}$ PFU of CHIKV in the footpad. The second group of mice were fed upon by *Ae. aegypti* mosquitoes in the same footpad immediately prior to needle inoculation. Bars represent the geometric mean of the individual mouse samples from two experiments.

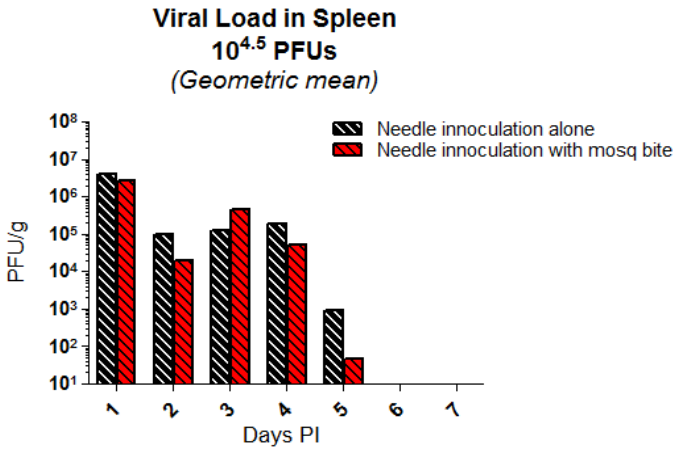


Figure 64: Viral load in spleens of mice needle inoculated with CHIKV at $10^{4.5}$ PFU with prior mosquito feeding

Viral load in spleens of young CD-1 mice needle inoculated with $10^{4.5}$ PFU of CHIKV in the footpad. The second group of mice were fed upon by *Ae. aegypti* mosquitoes in the same footpad immediately prior to needle inoculation. Bars represent the geometric mean of the individual mouse samples from two experiments.

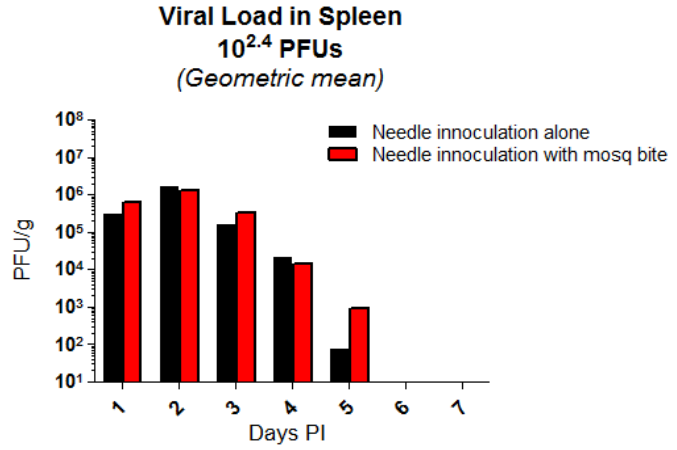


Figure 65: Viral load in spleens of mice needle inoculated with CHIKV at $10^{2.4}$ PFU with prior mosquito feeding

Viral load in spleens of young CD-1 mice needle inoculated with $10^{2.4}$ PFU of CHIKV in the footpad. The second group of mice were fed upon by *Ae. aegypti* mosquitoes in the same footpad immediately prior to needle inoculation. Bars represent the geometric mean of the individual mouse samples from two experiments.

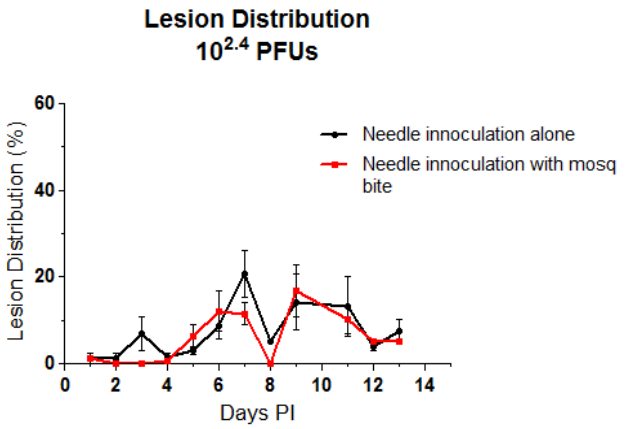


Figure 66: Distribution of lesions in leg tissues of mice needle inoculated with CHIKV at $10^{2.4}$ PFU with prior mosquito feeding

Lesion distribution in leg tissue samples of young CD-1 mice needle inoculated with $10^{2.4}$ PFU of CHIKV in the footpad. The second group of mice were fed upon by *Ae. aegypti* mosquitoes in the same footpad immediately prior to needle inoculation. Pathology samples were fixed, embedded, sectioned and stained with H&E. Samples were graded and the mean was determined from two experiments.

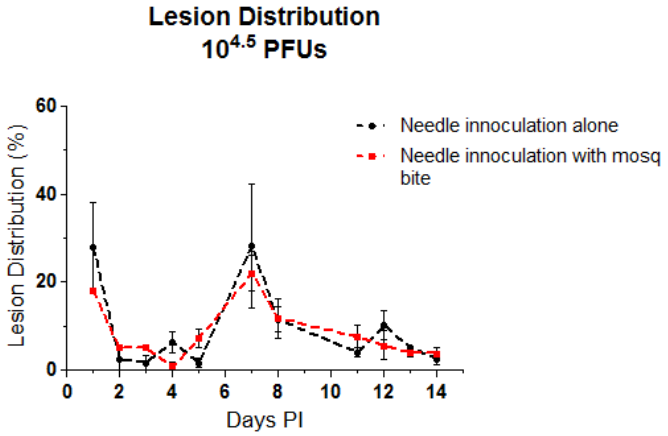


Figure 67: Distribution of lesions in leg tissues of mice needle inoculated with CHIKV at $10^{4.5}$ PFU with prior mosquito feeding

Lesion distribution in leg tissue samples of young CD-1 mice needle inoculated with $10^{4.5}$ PFU of CHIKV in the footpad. The second group of mice were fed upon by *Ae. aegypti* mosquitoes in the same footpad immediately prior to needle inoculation. Pathology samples were fixed, embedded, sectioned and stained with H&E. Samples were graded and the mean was determined from two experiments.

Inflammation severity was less severe in all groups, as compared with earlier findings. In the low dose group, severe pathology (score of 3 or greater) was only seen on dpi 7 in the needle inoculated without mosquitoes and on dpi 11 in the mosquito bitten group (Figure 68). In the experimental period, neither of the low dose groups had an average score greater than three. In the higher dose groups, similar non-severe pathology was seen (Figure 69). Inflammation severity peaked at a score of 3.2 on 8 dpi for the needle-inoculated group without mosquito bites with similar results seen in the mosquito bitten group. Overall, there was no difference in the severity of inflammation between the mosquito bitten and non-bitten groups or the high and low dose CHIKV groups.

The presence of neutrophils, necrosis and soft tissue inflammation was assessed to distinguish severe inflammation in the four experimental groups. In all of these analyses, no significant differences were observed. In the low dose group, the highest percent of tissues with neutrophils was 80% at 11 dpi in the mosquito bitten group (Figure 70). The needle-inoculated group, without mosquito bite peaked at 60% on 7 dpi. The two groups had very similar neutrophil amounts on all days sampled. In the high dose group, the mosquito bitten group peaked at 80% on 7 dpi and the group without mosquito bites peaked at 67% on 8 dpi (Figure 71). The mosquito bitten group had a slightly higher peak of neutrophil presence and was slightly earlier than the needle inoculated group without mosquito bites. But at later time points the two groups had almost the same amounts of neutrophils present.

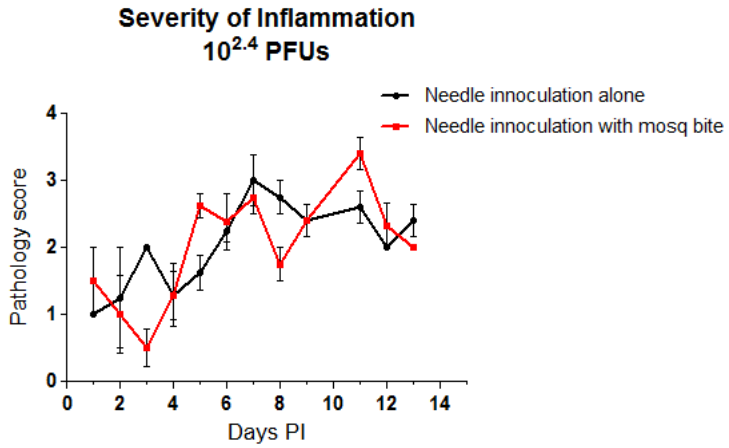


Figure 68: Inflammation severity in leg tissues of mice needle inoculated with CHIKV at $10^{2.4}$ PFU with prior mosquito feeding

Severity of inflammation in leg tissue samples of young CD-1 mice needle inoculated with $10^{2.4}$ PFU of CHIKV in the footpad. The second group of mice were fed upon by *Ae. aegypti* mosquitoes in the same footpad immediately prior to needle inoculation. Pathology samples were fixed, embedded, sectioned and stained with H&E before grading. The mean was determined from two experiments.

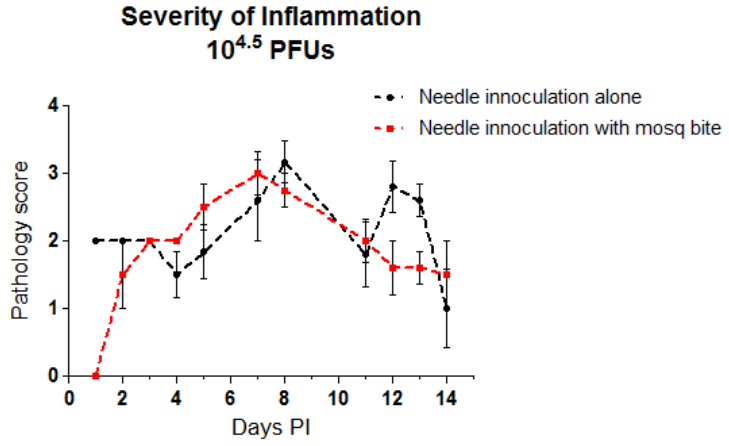


Figure 69: Inflammation severity in leg tissues of mice needle inoculated with CHIKV at $10^{4.5}$ PFU with prior mosquito feeding

Severity of inflammation in leg tissue samples of young CD-1 mice needle inoculated with $10^{4.5}$ PFU of CHIKV in the footpad. The second group of mice were fed upon by *Ae. aegypti* mosquitoes in the same footpad immediately prior to needle inoculation. Pathology samples were fixed, embedded, sectioned and stained with H&E before grading. The mean was determined from two experiments.

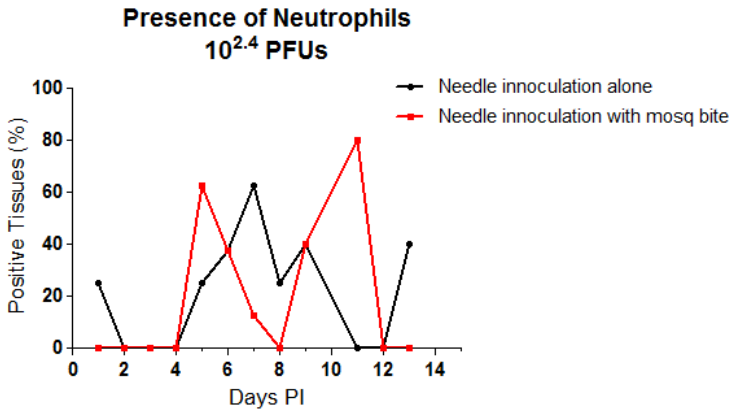


Figure 70: Neutrophil presence in leg tissues of mice needle inoculated with CHIKV at $10^{2.4}$ PFU with prior mosquito feeding

The presence of neutrophils in leg tissue samples of young CD-1 mice needle inoculated with $10^{2.4}$ PFU of CHIKV in the footpad. The second group of mice were fed upon by *Ae. aegypti* mosquitoes in the same footpad immediately prior to needle inoculation. Pathology samples were fixed, embedded, sectioned and stained with H&E. Graph represents the percent of tissues that neutrophils were present upon examination from two experiments.

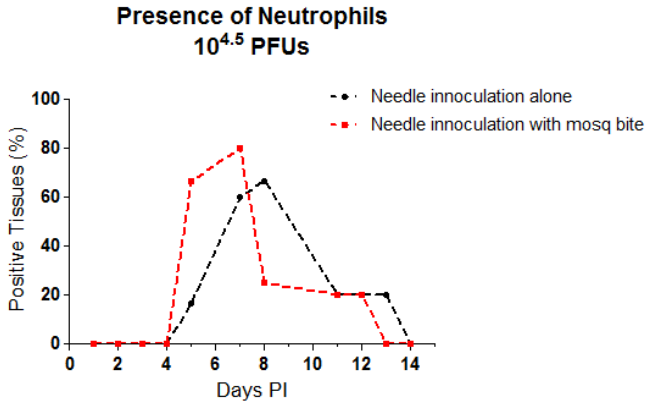


Figure 71: Neutrophil presence in leg tissues of mice needle inoculated with CHIKV at $10^{4.5}$ PFU with prior mosquito feeding

The presence of neutrophils in leg tissue samples of young CD-1 mice needle inoculated with $10^{4.5}$ PFU of CHIKV in the footpad. The second group of mice were fed upon by *Ae. aegypti* mosquitoes in the same footpad immediately prior to needle inoculation. Pathology samples were fixed, embedded, sectioned and stained with H&E. Graph represents the percent of tissues that neutrophils were present upon examination from two experiments.

In the low dose groups, the needle inoculation without mosquito bite had a slightly higher amount of necrosis seen on 7 and 8 dpi with 75% of the tissues having myositis (Figure 72). The needle-inoculated group with mosquito bites peaked at 63% on 7 dpi and 60% on 12 dpi. By 13 dpi neither group had any signs of necrosis. In the high dose groups, the needle inoculated without mosquito bites peaked at 60% on 12 dpi with 50% necrosis was observed on 8 dpi (Figure 73). The mosquito bitten group had very similar amounts of necrosis with a peak of 40% on 7 dpi. On 13 dpi, both groups had no observable myositis. The amount of soft tissue inflammation seen in the low dose groups was low, with the needle-inoculated group without mosquito bites having a peak of 75% on 8 dpi, but otherwise not having more than 20% of the tissues with soft tissue inflammation (Figure 74). In the mosquito bitten group the peak of soft tissue inflammation was at 38% on 7 dpi and 13% on 8 dpi, otherwise no soft tissue inflammation was observed on the days sampled in this group. Overall, in the lower dose group, the non-bitten mice had a higher amount of soft tissue inflammation. In the higher dose group, the mosquito bitten group peaked at 60% on 7 dpi and 50% on 1 dpi, but otherwise was less than 20% of the tissue had inflammation in the soft tissue on the experimental days (Figure 75). In the non-bitten needle inoculated group, the peak of soft tissue inflammation was at 40% on 7 dpi and 11 dpi. On 14 dpi, the mosquito bitten group had no soft tissue inflammation, but 25% of the tissue samples from the unbitten group still displayed inflammation.

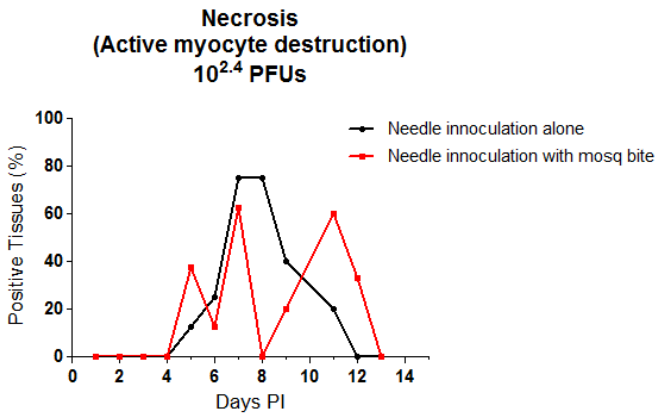


Figure 72: Necrosis presence in leg tissues of mice needle inoculated with CHIKV at $10^{2.4}$ PFU with prior mosquito feeding

The presence of necrosis in leg tissue samples of young CD-1 mice needle inoculated with $10^{2.4}$ PFU of CHIKV in the footpad. The second group of mice were fed upon by *Ae. aegypti* mosquitoes in the same footpad immediately prior to needle inoculation. Pathology samples were fixed, embedded, sectioned and stained with H&E. Graph represents the percent of tissues that had necrotic myocytes upon examination from two experiments.

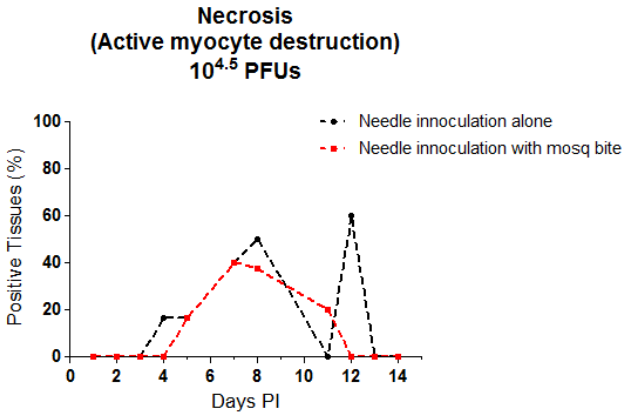


Figure 73: Necrosis presence in leg tissues of mice needle inoculated with CHIKV at 104.5 PFU with prior mosquito feeding

The presence of necrosis in leg tissue samples of young CD-1 mice needle inoculated with $10^{4.5}$ PFU of CHIKV in the footpad. The second group of mice were fed upon by *Ae. aegypti* mosquitoes in the same footpad immediately prior to needle inoculation. Pathology samples were fixed, embedded, sectioned and stained with H&E. Graph represents the percent of tissues that had necrotic myocytes upon examination from two experiments.

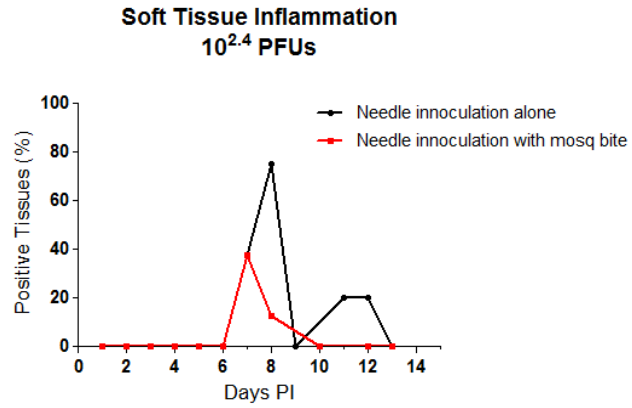


Figure 74: Inflammation of the soft tissue of leg samples of mice needle inoculated with CHIKV at $10^{2.4}$ PFU with prior mosquito feeding

Inflammation of the soft tissue in leg tissue samples of young CD-1 mice needle inoculated with $10^{2.4}$ PFU of CHIKV in the footpad. The second group of mice were fed upon by *Ae. aegypti* mosquitoes in the same footpad immediately prior to needle inoculation. Pathology samples were fixed, embedded, sectioned and stained with H&E. Graph represents the percent of tissues that had soft tissue inflammation upon examination from two experiments.

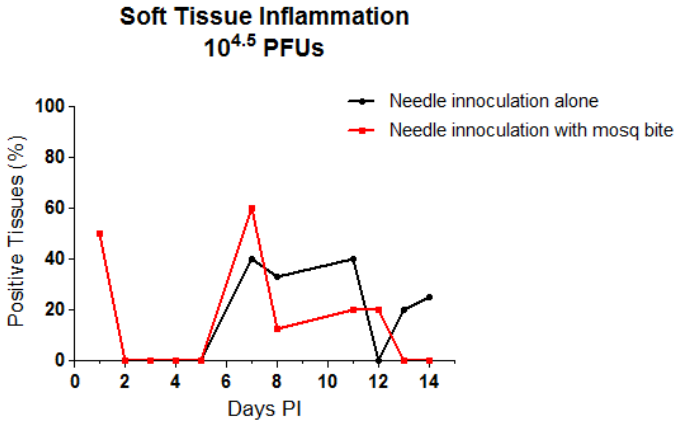


Figure 75: Inflammation of the soft tissue of leg samples of mice needle inoculated with CHIKV at $10^{4.5}$ PFU with prior mosquito feeding

Inflammation of the soft tissue in leg tissue samples of young CD-1 mice needle inoculated with $10^{4.5}$ PFU of CHIKV in the footpad. The second group of mice were fed upon by *Ae. aegypti* mosquitoes in the same footpad immediately prior to needle inoculation. Pathology samples were fixed, embedded, sectioned and stained with H&E. Graph represents the percent of tissues that had soft tissue inflammation upon examination, from two experiments.

Discussion

CHIKV and RRV pathogenesis that causes severe pathology has been shown to be in part due to immune-mediated mechanisms (Couderc et al. 2008, Gardner et al. 2010, Lidbury et al. 2008, Morrison et al. 2007). Since mosquito saliva also has been shown to alter the immune response, it is reasonable to hypothesize differences in the pathogenesis of CHIVK inoculated in the absence and presence of mosquito saliva. The most interesting aspect of the interplay of mosquito saliva and CHIKV is that it decreases the pathogenesis seen in mice. Since mosquito saliva has the ability to decrease the antiviral response in mice, one might expect the opposite effect, similar to what has been shown with West Nile virus (Schneider et al. 2008, Schneider et al. 2007, Schneider et al. 2006, Styer et al. 2006). Mosquito saliva causes a shift in the Th1/Th2 response in mice when infected with CHIKV (Thangamani et al. 2010). Decreased CHIKV pathogenesis is expected as previous studies with RRV have shown that decreased activation of macrophages and complement result in decreased amounts of myositis (Morrison et al. 2007, Morrison et al. 2008, Rulli et al. 2009).

The results shown in this chapter suggest that the differences seen are due in part to mosquito delivery and to viral dose. The initial experiments with natural transmission of CHIKV to mice with infected mosquitoes showed a dramatic decrease in viral load in the leg tissue that correlated well to a decrease in pathology. Lesion distribution, neutrophil presence and soft tissue inflammation were all dramatically decreased in response to mosquito bite-inoculation. This difference, in most cases, was even more significant when the mice which received HIAF generated against mosquito saliva. Viremia levels were significantly higher on 2 dpi in both the mosquito bite inoculated groups as

compared to the mosquito bitten group, suggesting that even if a lower dose was given the virus was still able to replicate to similar levels and for a similar duration as compared to the higher needle inoculum.

When repeating these experiments with a known dose of virus and footpad inoculation, the differences were not as notable between the mosquito bitten and non-bitten groups. There were still significant differences in the viral loads in the leg tissue in the lower dose group and the trend was similar in the higher dose groups. Viremia levels and viral load in the spleen was very similar for all groups looked at.

The pathological differences between the mosquito bite and non-bitten groups were far less remarkable when CHIKV was inoculated into the footpad. One reason for this could be the changing of the location of the needle inoculation. It was shown previously with the IVIS technology that CHIKV does not disseminate well to other limbs after footpad inoculation. This phenomenon has also been shown by other groups, namely that footpad inoculation of CHIKV results in localized swelling and pathology, but virus does not disseminate to the other limbs (Gardner et al. 2010, Morrison et al. 2011). This finding is also supported by the remarkably low amount of lesion distribution in the high dose group that did not receive mosquito bites (Figure 66). These results should be directly comparable to previous work with needle inoculated CHIKV (Figure 54). If footpad inoculation was the same as the SC inoculation, one would expect to see lesions in over 40% of the tissue for multiple days: but instead, it was never higher than 28% in the footpad inoculated group. Unfortunately, the footpad inoculation of virus was chosen because the mosquito bite area had to be contained so that it could be followed by needle inoculation in the same area. If

this experiment were to be repeated, it would be interesting to either examine the pathology of the leg that was inoculated with virus, or to localize the mosquito bites so that CHIKV could be injected in the skin of the back.

It is also important to note that histopathology scoring of samples is a qualitative measure more than a quantitative measure. Trying to make statistical significance out of these scores is extremely difficult. Lesion distribution seems to be the most robust of these measures, with inflammation severity being the least robust and more of a qualitative measure. Lesion distribution changes can be seen easily in Figure 54, where there are drastic differences in the amount of the muscle tissue affected by the inflammation. It was not uncommon to see small areas of severe pathology in the mosquito bitten groups, but extensive areas of inflammation and necrosis were absent.

While the second half of my experiments suggests that dose has more of a role to play in differences than the mosquito impact, the role of the saliva cannot be discounted. This is best demonstrated in the mice that were immunized against mosquito saliva but showed an even lower amount of pathology and lower viral loads in the leg tissue. Since all the mosquito bitten groups received approximately the same dose, the differences cannot be a dose affect. This difference between pathology may also be due to other aspects of mosquito transmission and not just the saliva. Glycosylation patterns of the virus may also play a key role in the pathogenesis seen. The second set of experiments that were done with needle inoculated virus in a mosquito bitten area would not have tested for this factor. Repeating these experiments with virus from a mosquito cell line could easily be done to look at key differences in glycosylation and cholesterol within CHIKV when derived from different cell

types. I would expect that different glycosolation patterns does not have an effect on pathogenesis of CHIKV in mice.

While the titer of virus in mosquito saliva has been reported, these reports utilize an artificial system of collecting saliva and it is truly unknown how much the mosquito is inoculating as it probes and feeds on a host. Mosquitoes are capable of infecting a host without feeding and becoming engorged, but by probing alone. During the act of probing the host, the mosquito deposits saliva into the dermis and the capillaries. Furthermore, in studies with collected saliva, it has been shown that the viral titers are widely variable between different mosquitoes, sometimes varying by a factor of 100. To control for this in these studies, when inoculating mice by route of mosquito, only 5 mosquitoes were placed in each carton and the mouse was not removed until at least three of the mosquitoes were engorged. By doing this, it was hoped that a more constant dose would be inoculated into the mouse. Overall, this data has shown that both dose and mosquitoes play a role in the severe myositis seen in CHIKV-infected mice.

These studies are useful to look at differential responses in mice, but may be different than responses in humans. In mice, systemic immune changes occur due to mosquito feedings, but this may not be applicable to humans. The body mass to mosquito saliva ratio is very different when comparing mice and humans. To have a comparable saliva/mass ratio, a human would have to be bitten by more than 300 mosquitoes in a period of 45 minutes. This number of mosquito bites is possible in tropical areas during the rainy season, but is certainly more than average for an urban setting (Billingsley et al. 2006). While people living in tropical areas with large numbers of mosquitoes would inevitably

possess antibodies in response to mosquito saliva, it is unknown if these antibodies modulate the immune response the same way passive immunization does in naïve mice. While systemic responses are seen in mice, it is probable that responses in humans would be localized to mosquito bite areas. For this reason, these studies have more importance on understanding CHIKV pathogenesis in the context of Th2 immune responses than mosquito bites.

These studies looking at the effects of mosquito saliva are, in my opinion, a good tool to demonstrate how modulation of the mouse immune system has an effect on CHIKV pathogenesis. I believe that the mosquito saliva and the inoculation site on the mouse are the key factors that modulate the differences in myositis severity. While dose and glycosolation may also have an impact, I think that these factors are minor as compared to the mosquito saliva and the location of inoculation. If these studies were repeated with inoculation of the virus in the skin of the back, it could be hypothesized that disseminated myositis could be observed and that mosquito bite would greatly decrease the severity. Due to the similarities in the viremia levels in the mice when the dose of the virus was controlled leads to a conclusion that viremia levels are not dependent on the dose of virus, which may be hypothesized that mysotis may also be indepenten of dose. Overall these studies have shown the importance of Th1/Th2 immune response and the role of the innate immune system in CHIKV pathogenesis in mice.

Chapter 6: The affects of immune modulation and CHIKV pathogenesis

Abstract

Immunocompromised individuals are at risk for increased disease severity from many viral infections. While this is sometimes the case for arthralgic alphaviruses, a growing body of work has also shown that some types of immune suppression actually decrease muscle pathology in mouse models (Couderc et al. 2008, Gardner et al. 2010, Morrison et al. 2008, Rulli et al. 2009, Zaid et al. 2011). In the studies presented in this chapter, different types of immune suppression were used to study their effects on CHIKV-infected mice. Both knockout mouse models and chemotherapeutic agents were used to decrease the immune response. Interferon deficient mice had 100% mortality when infected with CHIKV by needle inoculation. Chloroquine treatment, while it decrease viral replication in cell culture, did not change the outcome of CHIKV infection in mice. Cyclophosphamide treatment of mice decreased the white blood cell counts, but did not cause an increased disease state or mortality in CHIKV-infected mice. Dexamethasone treatment, when given at 2 or 4 dpi, decreased the lesion distribution seen in the muscles of CHIKV-infected mice. Collectively these results suggest that while interferon is important early on in CHIKV infection, lymphocytes, macrophages and neutrophils are not imperative to clear CHIKV infection and may be involved in CHIKV immune-mediated disease.

Introduction

The interplay of virus and the immune system is an important dynamic that with any virus needs to be studied. Since disease development and severity can be determined by the interaction of the virus and the host immune response in people, it is important to understand how different immune competencies react to viral infections in animal models. It becomes even more important with CHIKV and related viruses, when the immune system seems to be both fighting off the virus and enhancing disease. Intuitively it would seem that by decreasing the immune response or biasing it to a Th2 response there would be a increased disease severity with CHIKV infection, which in some cases is true (Couderc et al. 2008), but we wanted to explore if there was a way to decrease part of the immune system which would lead to a decrease in myositis in mice. It has been shown that in RRV that by decreasing either macrophages or complement results in a decrease in severe myositis in mice (Morrison et al. 2007, Morrison et al. 2008, Rulli et al. 2009). We applied this concept to a known pharmaceutical that could be used in humans and hypothesized that dexamethasone, an immune suppressant, could be used as a CHIKV treatment.

In the human population, there exists a subset of people that are in an immune compromised state, whether it be medically induced with pharmaceuticals or disease related. This population is at greater risk to many viruses and it is important to know what impact this condition may have on disease pathogenesis with CHIKV. For these reasons, the effects of a potent immunosuppressant were also investigated, cyclophosphamide, that may mimic an immune compromised state in people. Also, further studies were performed with interferon deficient mice and mosquito bite-inoculation. Chloroquine has

been shown to have some success in decreasing the disease severity of CHIKF and decreasing CHIKV replication in cell culture (Brighton 1984, Sourisseau et al. 2007). Chloroquine is known for its inhibition of endosomal maturation and alkylating qualities in cell culture, but *in vivo* it can stimulate nitric oxide(NO) release from activated macrophages and down regulates TNF-α, IL-1β and IL-6 expression (Ghigo et al. 1998, Legssyer et al. 2003, Weber et al. 2001). Further studies were performed in mice and cell culture with chloroquine to confirm these previous findings.

The objective of this aim was to characterize how modulating the immune system in mice would influence CHIKV pathogenesis. The hypothesis of this aim was that by down regulating the innate immune response you would decrease CHIKV-induced pathology.

Results

Chloroquine therapies and chikungunya virus pathogenesis

Chloroquine in cell culture has been shown to inhibit virus replication presumably due to its inhibition of endosomal trafficking (Ozden et al. 2008, Sourisseau et al. 2007). This work was repeated and confirmed on Vero cell culture (Figure 76). Vero cells were inoculated with CHIKV with chloroquine added to media and showed a significant decrease in viral replication. At 36 hpi Vero cell cultures had reached a viral titer of $10^{7.2}$ PFU/mL in the supernatant, while cells in the presence of chloroquine had a viral titer of 10^5 PFU/mL. The cells in the presence of chloroquine were never able to reach the same level of virus titer that untreated cells did.

Chloroquine was also preliminarily screened in mice for its therapeutic effects against CHIKV. Young CD-1 mice were infected SC as previously described at 24 hpi, one group received an IP inoculation of 80mg/kg of chloroquine phosphate in PBS. Viremia levels in both the untreated and treated mice were very similar (Figure 77). At 1 dpi the viremia levels were at 10^5 PFU/mL, by 4 dpi they were below the level of detection. The viral load in the leg tissue was higher and a longer duration of the viremia levels and the chloroquine treated group had much higher levels than that of the untreated group with a titer of $10^{6.9}$ PFU/mL on 5 dpi. By 8 dpi the viral load in the leg was below the limit of detection. Histopathological samples were also analyzed and it was clear that the chloroquine was not inhibiting the myositis seen in CHIKV (data not shown). If any conclusion could be drawn from the histopathology analysis, it was that the most severe myositis was seen in the chloroquine treated mice. With the preliminary results in mice showing that chloroquine did not lessen the disease in mice and new reports showing chloroquine was not beneficial in CHIKF patients, these studies were not repeated (De Lamballerie et al. 2008).

Chikungunya virus pathogenesis in interferon deficient mice

Interferon and STAT knockout mice have been used to increase the disease pathogenesis of CHIKV and other viruses. AG129 mice are lacking in IFN-α , IFN-β and IFN-γ , while A129 mice are only lacking IFN-α and IFN-β. Two AG129 mice were inoculated with 10^5 PFU SC in the back and were both dead by 3 dpi (Figure 78). On 1 dpi viremia levels were at an average of $10^{6.8}$ PFU/ml and on 2 dpi the viremia titers were greater than 10^8 PFU/mL. Five A129 mice were infected by exposure to 5 infected *Ae. albopictus* and 2 of these mice

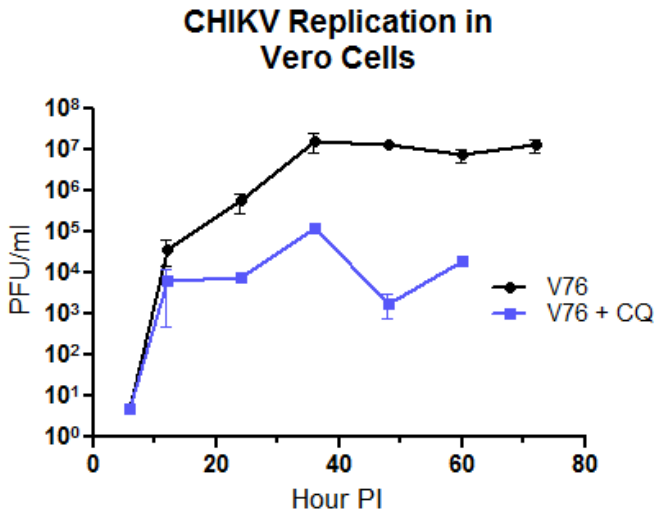


Figure 76: CHIKV replication is inhibited by chloroquine

CHIKV replication in Vero cells determined by plaque assay. Chloroquine phosphate was added to Vero cell cultures at the same time CHIKV was added. Samples were taken every 12 hpi.

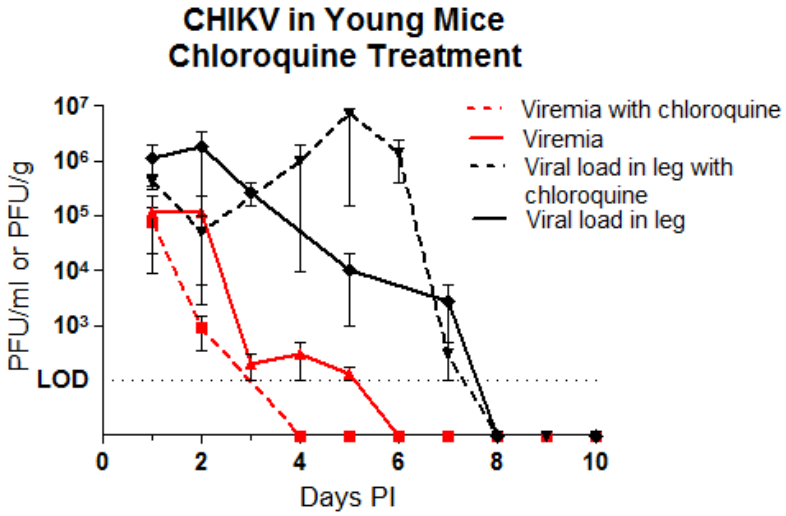


Figure 77: Viral titers in CHIKV-infected mice with chloroquine treatment

Young CD-1 mice were infected with CHIKV SC in the skin of the back and one group received chloroquine at 24hpi at 80mg/kg IP. Mice were sampled every days for 10 days and viral titers were determined by plaque assay.

survived. These two mice did not have detectable levels of HI antibody 2 weeks post infection and when re-challenged with $10^{4.5}$ PFU of CHIKV by needle inoculation they succumbed 4 dpi. It is unclear why these mice behaved as if they were uninfected and why the mosquitoes apparently did not transmit the virus since the mosquitoes were infected by IT inoculation and 5 mosquitoes fed on each mouse. To understand the difference between the mosquito bite infected mice and the needle-inoculated virus, studies were done with needle inoculation of a low dose of virus immediately following uninfected mosquito feeding. Two groups of 6 mice received a low dose of CHIKV ($10^{2.4}$ PFU/mL) needle inoculated into the footpad either directly following mosquito bites or no mosquito bites. In these two groups, all the mice died by day 4 and 5 post

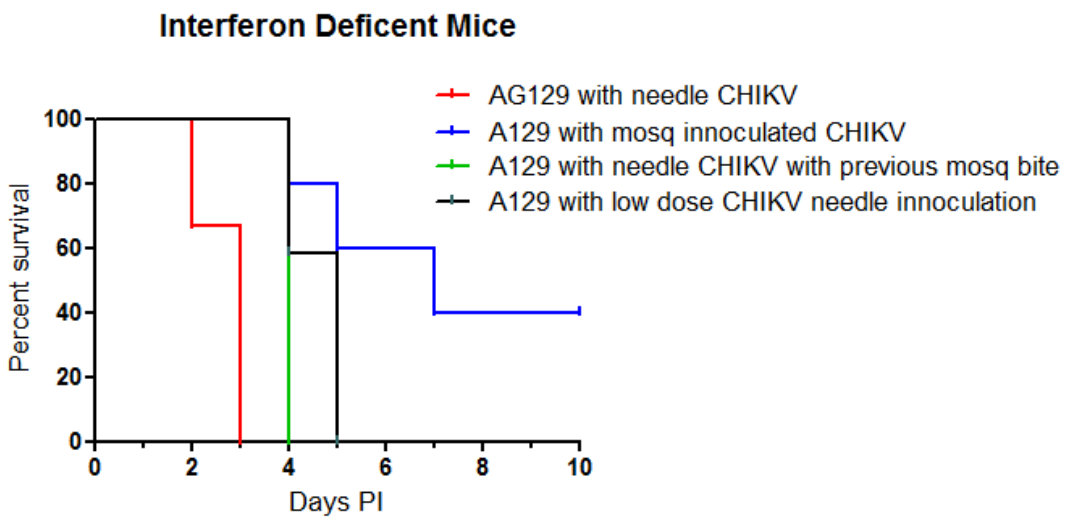


Figure 78: Survival of interferon deficient mice

Both AG129 and A129 mice were infected with CHIKV. Needle inoculation was at $10^{4.5}$ PFU or $10^{2.4}$ PFUS (low dose) SC in the back. *Ae. albopictus* mosquitoes IT inoculated with CHIKV were used to inoculate mice. Uninfected mosquitoes were allowed to bite the footpad immediately prior to needle inoculation with $10^{2.4}$ PFU CHIKV.

infection. While using a lower dose of virus did delay mortality by 2 days, it did not decrease the mortality rate.

Cyclophosphamide immune suppression in CHIKV-infected mice

Cyclophosphamide is an immune suppressant that causes a decrease in white blood cells including B lymphocytes and antibodies and induces a Th1/Th2 switch (Matar et al. 2002, Sistigu et al. 2011). Cyclophosphamide was given every four days to have continuous suppression of the immune system. This dosing strategy was confirmed by assessing the white blood cell counts in the treated mice and naïve mice. By 5 days after the first dose of cyclophosphamide there was a significant decrease in the white blood cell count. For these studies, mice received cyclophosphamide 5 days prior and 1 day prior to CHIKV inoculation and this continued every four days until the end of the experiment. The negative control group consisted of mice receiving the same dose of cyclophosphamide. These mice were monitored for mortality for 2 weeks post infection and showed no signs of disease or mortality, similar to CHIKV-infected mice that were not immune suppressed. Histopathology analysis of mice 11 dpi showed large amounts of neutrophils, necrosis and severe inflammation in the skeletal muscle of the mice that was comparable to that of CHIKV mice that were not immune suppressed. Overall, cyclophosphamide immunosuppression did not increase the mortality or myositis seen in CHIKV-infected mice.

Dexamethasone as a treatment strategy for CHIKV pathogenesis

Dexamethasone is a corticoid steroid that is readily prescribed for people to treat a number of conditions including arthritis, inflammation, allergies and asthma (Shih et al. 2007). It comes in multiple forms including oral tablets,

topical creams and an injectable solution. Serious side effects are rare for short term usage, but can occur if used for an extended period of time. Corticoid steroids decrease TNF-α, IFN-γ, lymphocytes, monocytes and eosinophils while increasing neutrophils in the peripheral blood (Machado et al. 2011). These mechanisms work to stop the infiltration of inflammatory cells into damaged tissues.

The purpose of these experiments was to use dexamethasone as a treatment to lessen the myositis seen in mice and to determine the safety of giving an immunosuppressive to CHIKV-infected mice. Since the goal of using dexamethasone would be as a treatment, pre-treatment to suppress the immune system prior to CHIKV infection was not analyzed. These experiments also focused on using a single high dose of steroid at different time points after CHIKV infection, specifically immediately following CHIKV inoculation, 2 dpi and 4 dpi. CHIKV was inoculated SC in the back at a dose of $10^{4.5}$ PFU as described previously.

Importantly, in all treatment groups no adverse side effects or clinical signs of disease were seen in any of the mice. There was no mortality, hind limb paralysis or hair loss in any of the mice up to 3 weeks post infection. Weight gain was assessed as a marker of severe CHIKV-induced disease. CHIKV-infected young mice rarely show any difference in weight gain as compared to naïve mice. This was confirmed in the CHIKV-infected mice with and without dexamethasone treatment (Figure 79). Dexamethasone treatment did not affect the weight gain in any of the treatment groups looked at and all mice continued to gain weight as expected.

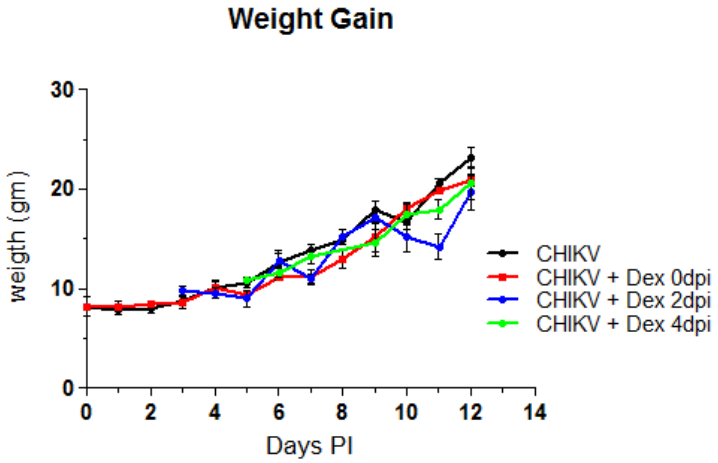


Figure 79: Weight gain in CHIKV-infected mice with dexamethasone treatment

Young CD-1 mice were infected with CHIKV SC in the back with $10^{4.5}$ PFU. Treatment groups received a single dose of 200µg of dexamethasone IP at 0, 48 or 96 hpi. Treatment groups were not sampled until 24 hours after dexamethasone injection.

Viremia and viral loads were analyzed in various tissues of infected mice. In dexamethasone treatment groups, mice were not sampled until 24 hour after dexamethasone treatment. Viremia levels in CHIKV-infected mice were $10^{3.9}$ PFU/ mL 1 dpi and were below the limit of detection at 3 dpi (Figure 80). Dexamethasone treatment concurrent with CHIKV infection resulted in slightly higher viremia levels with a peak at $10^{4.8}$ PFU/mL on 1 dpi and decreased by a factor of ten every 24 hours and were below the limit of detection by 4 dpi. The dexamethasone treatment on 2 and 4 dpi never had viremia levels above the level of detection.

Viral load in the leg was seen for 5 dpi in the CHIKV-infected mice (Figure 81). The titer was consistently above 10^4 PFU/gm on 1, 2, 4 and 5 dpi and was below the limit of detection on 6 dpi. The peak viral titer in the leg tissue was $10^{5.3}$ PFU/gm on 4 dpi. In the dexamethasone treatment group at 0 dpi, the viral

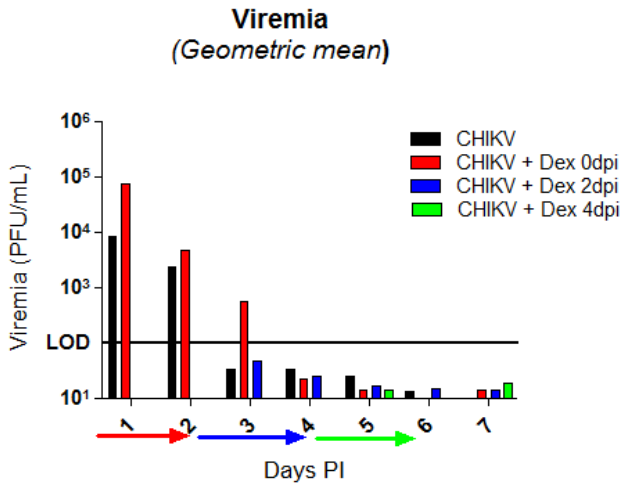


Figure 80: Viremia levels in CHIKV-infected mice treated with dexamethasone

Young CD-1 mice were infected with CHIKV SC in the back with $10^{4.5}$ PFU. Treatment groups received a single dose of 200µg of dexamethasone IP at 0, 48 or 96 hpi. Treatment groups were not sampled until 24 hours after dexamethasone injection. Viremia levels were assessed by plaque assay and presented as geometric means.

titer was $10^{4.4}$ PFU/gm on 1 dpi and then dropped close to, and below, the limit of detection for the rest of the time points. In both the 2 and 4 dpi treatment groups, viral load in the leg was below the limit of detection throughout all the sampling period. Viral load in the brain were also measured to confirm that giving the dexamethasone treatment was not causing a more neurovirulent disease in mice (Figure 82). Dexamethasone did not cause any significant increase in viral titers in the brain with individual samples never reaching more than 100 PFU/gm. This is typical of CHIKV infection in young mice where virus is rarely found in the brain after SC infection (Ziegler et al. 2008).

Dexamethasone is not believed to affect antibody production during treatment, but it does have some ability to decrease T cell function (Machado et

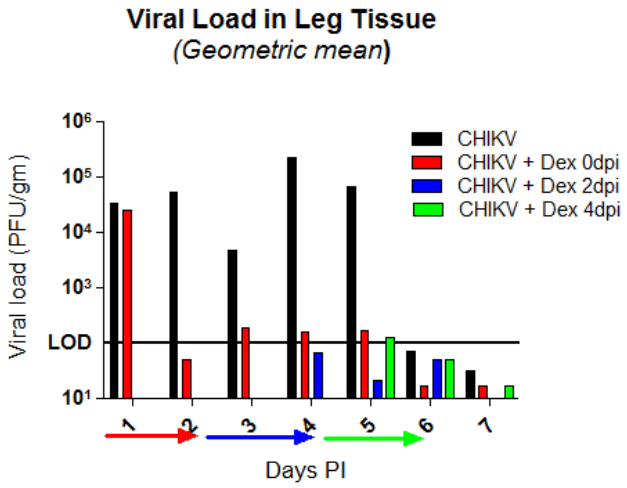


Figure 81: Viral load in leg tissue from CHIKV-infected mice with dexamethasone treatment

Young CD-1 mice were infected with CHIKV SC in the back with $10^{4.5}$ PFU. Treatment groups received a single dose of 200µg of dexamethasone IP at 0, 48 or 96 hpi. Treatment groups were not sampled until 24 hours after dexamethasone injection. Viral loads were assessed by plaque assay and presented as geometric means.

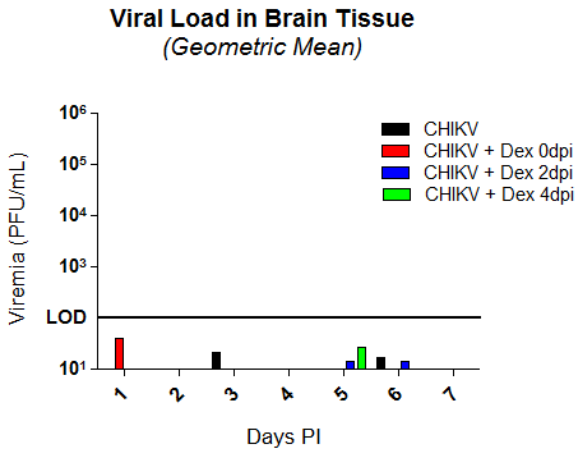


Figure 82: Viral load in brains from CHIKV-infected mice with dexamethasone treatment

Young CD-1 mice were infected with CHIKV SC in the back with $10^{4.5}$ PFU. Treatment groups received a single dose of 200µg of dexamethasone IP at 0, 48 or 96 hpi. Treatment groups were not sampled until 24 hours after dexamethasone injection. Viral loads were assessed by plaque assay and presented as geometric means.

al. 2011). It was important to confirm that CHIKV mice treated with dexamethasone were able to produce a robust antibody response and have immunity to further CHIKV infections. HI antibodies were assessed in CHIKV-infected mice (Figure 83). Most mice had detectable antibody on 5 dpi and all mice from all groups had measurable antibody responses by 6 dpi. The titers in all groups was comparable, with the highest tiers being seen in the 2 and 4 dpi treatment groups with a greater than 1:640 dilution being able to abolish the heamglutination reaction. The highest antibody titer reached in the untreated CHIKV mice and the zero dpi treatment was 1:320. This would suggest that dexamethasone in not inhibiting the production of antibodies and may in part be stimulating a more robust antibody response in mice.

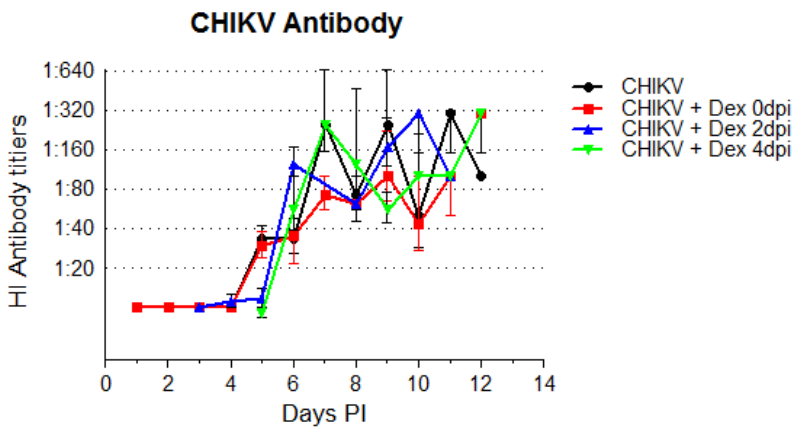


Figure 83: HI antibody levels in CHIKV-infected mice treated with dexamethasone.

Young CD-1 mice were infected with CHIKV SC in the back with $10^{4.5}$ PFU. Treatment groups received a single dose of 200µg of dexamethasone IP at 0, 48 or 96 hpi. Treatment groups were not sampled until 24 hours after dexamethasone injection. Antibody levels were assessed in mouse serum by HI assay and are presented as means with error. The limit of detection was 1:20.

Complete blood chemistries of CHIKV-infected mice were performed to see if dexamethasone created any adverse affects and decreased any of CHIKV-induced pathologies (Figure 84). The white blood cell counts were not significantly different in any of the treatment groups (Figure 84: A). The untreated CHIKV mice had decreased white blood cells across the experimental period as compared to naïve mice. The treated groups tended to have a lower level of white blood cells, but no significant differences can be seen. Circulating neutrophil amounts tended to be the same or lower in the dexamethasone treated groups as compared to the untreated group 24 hours after treatment, but at 48 and 72 hours after treatment, they were increased, specifically in the zero and 2 dpi treatment groups (Figure 84: B). This correlates with the action of the dexamethasone which can increase circulating neutrophils, but decrease tissue neutrophils (Machado et al. 2011).

Lymphocytes were slightly decreased in the treatment groups as compared to naïve and untreated CHIKV mice (Figure 84: C). In the zero dpi treatment, both 24 and 48 hours after treatment, the lymphocyte counts were below the level of the untreated mice. In the 2 dpi treatment the 24 and 72 hour post treatment samples had fewer lymphocytes as compared to the untreated mice. In the 4 dpi treatment group, every time point had fewer lymphocytes present as compared to the untreated group. Circulating monocytes also tended to be higher in the untreated group as compared to the treatment groups (Figure 84: D). The untreated mice had fewer monocytes than naïve mice for 2 dpi and then the level increased and was similar to naïve mice in the number of monocytes. On every day, except 4 dpi, the untreated group had a higher number of circulating monocytes as compared to any of the treatment groups.

Both eosinophils and basophils were in very low concentrations in all groups, with no definitive trends to be seen (Figure 84: E and F). All groups were at very similar levels with the treatment groups slightly higher on a few of the experimental days.

Erythrocyte counts were fairly consistent across all groups (Figure 85: A). Throughout the experimental period the trend was for there to be a slight increase in the number of red blood cells with an unusual dip in the number on 5 dpi. Hemoglobin levels also tended to increase over time with all treated and untreated mice having a fairly similar level (Figure 85: B). Again, there is an unusual dip in the level on 5 dpi. Hematocrit levels were also very stable during the experiment with the dexamethasone not having an effect on the concentration (Figure 85: C). Red cell distribution width was fairly constant during the experiment, with no differences seen after dexamethasone treatment (Figure 85: D). Platelet counts were slightly lower 1 dpi in the untreated group, but by 2 dpi they were increased (Figure 85: E). Both the untreated and treated mice after 2 dpi were at a comparable platelet count. In untreated CHIKV-infected mice for 3 dpi, there was a decreased platelet volume (Figure 85: F). This did not occur in the dexamethasone treated mice and by 4 dpi the untreated mice were back at a level comparable to treated and naïve mice.

Liver enzymes and electrolyte levels were also analyzed in the CHIKV-infected mice (Figure 86). Some liver enzymes including aspartate transaminase (AST) and alanine transaminase (ALT) have been shown to be increased in CHIKV-infected people and non-human primates. It was not possible to study the AST levels in mice, but a similar liver enzyme, alkaline phosphatase (ALP), was measured as a marker of liver damage (Figure 86: A).

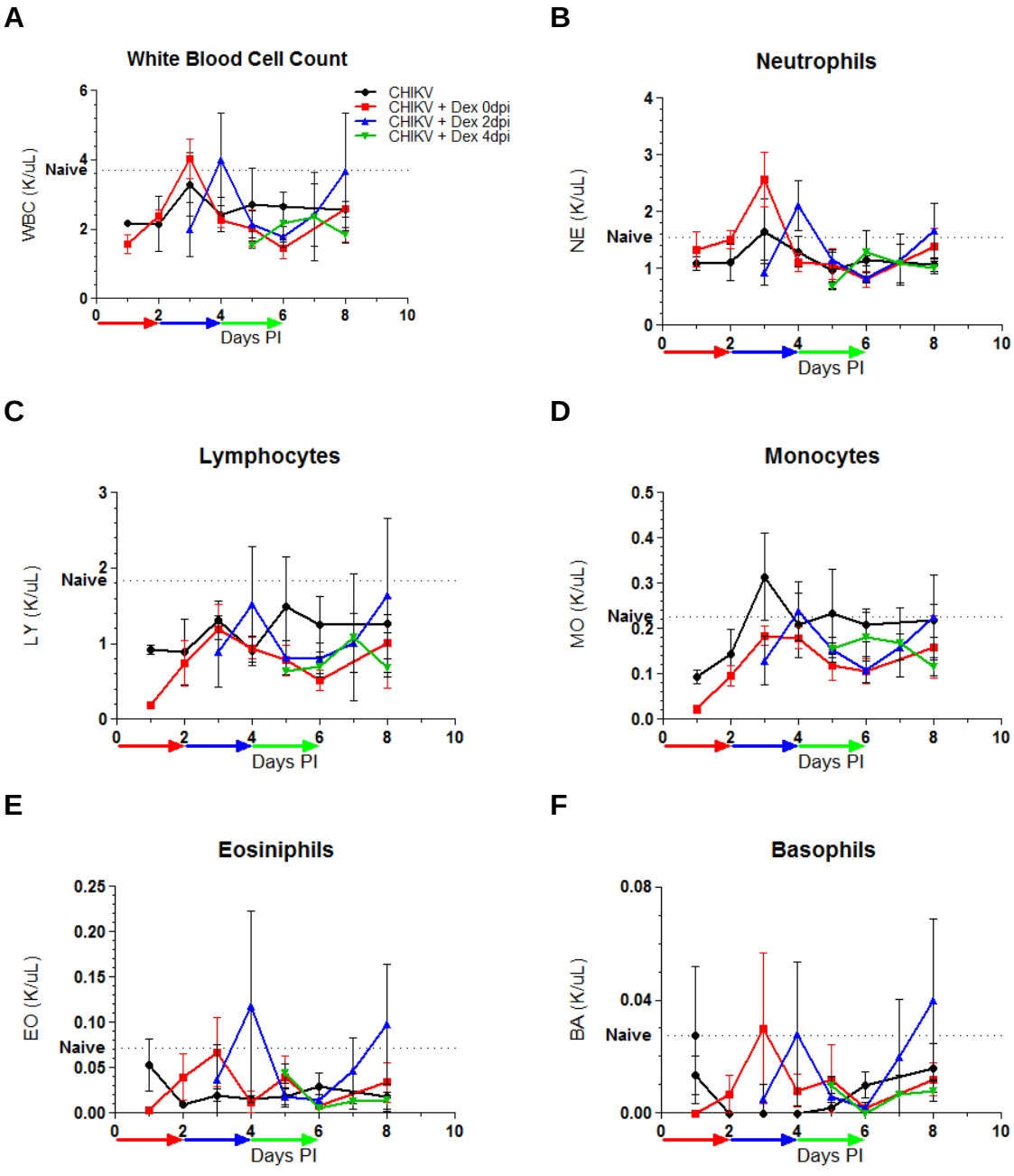


Figure 84: Analysis of the white blood cells in CHIKV-infected mice treated with dexamethasone

Analysis of the complete blood chemistry profile of young CD-1 mice infected with CHIKV SC in the back with $10^{4.5}$ PFU. Treatment groups received a single dose of 200µg of dexamethasone IP at 0, 48 or 96 hpi.

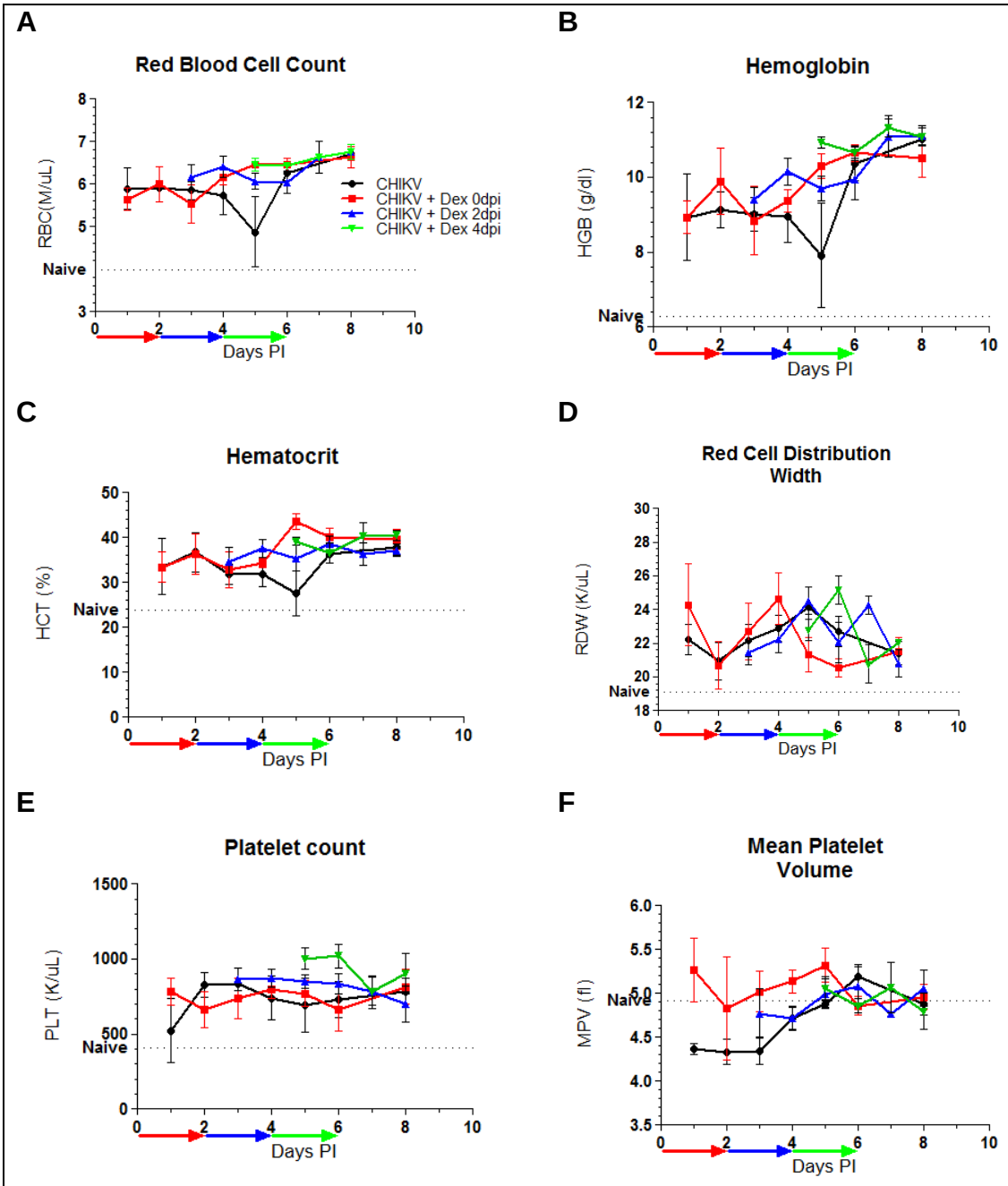


Figure 85: Analysis of the red blood cells in CHIKV-infected mice treated with dexamethasone

Analysis of the complete blood chemistry profile of young CD-1 mice infected with CHIKV SC in the back with $10^{4.5}$ PFU. Treatment groups received a single dose of 200µg of dexamethasone IP at 0, 48 or 96 hpi

In untreated CHIKV-infected mice, the levels of ALP were elevated for 3 dpi. In CHIKV-infected mice receiving dexamethasone treatment on 0 dpi, the levels of ALP were not increased and on 3 dpi there was a statistical differences between the untreated and treated mice ($p< 0.05$). The mice which received treatment on 2 dpi were slightly lower in ALP values on 3 dpi as compared to untreated mice. By 4 dpi, all mice were at comparable levels of ALP. Albumin levels were also analyzed in the mice (Figure 86: B). It is not surprising that 48-72 hours after treatment the mice exhibited increased levels of albumin, since steroids are known to increase levels of albumin. In untreated infected mice there was very little change in albumin levels and 3-4 days after dexamethasone treatment albumin levels were back to the level of infected mice. ALT was also assessed in CHIKV-infected mice (Figure 86: C). Surprisingly these levels were unchanged in the infected mice with and without treatment. These were expected to be increased with CHIKV infection as was seen with ALP. Amylase levels were also analyzed and like albumin, were increased due to the dexamethasone treatment (Figure 86: D). Corticosteroids increase amylase levels and in the mice this was transient and the levels were comparable to the naïve mice within 72 hours of treatment. CHIKV infection alone did not have any effect on the amylase levels. Total bilirubin levels were also increased with dexamethasone treatment (Figure 86: E). Bilirubin levels were increased for 24 hours after treatment and also for at least 24 hours after CHIKV infection. It is possible that the bilirubin level remained higher in the zero dpi dexamethasone treatment group for 48 hours as a synergistic affect between the virus and the steroid, but if this is the case it again was transient. Increased bilirubin levels can also be a sign of liver disease. Blood urea nitrogen levels were increased with 2

and 4 dpi treatment, but were not elevated with the zero dpi treatment group (Figure 86: F). CHIKV infection caused a slight decrease in this level while all treatment groups had an increased level of urea nitrogen. Low urea nitrogen levels are an indicator of liver failure.

Electrolytes are monitored to asses underlying nutritional problems or disease in people. For the most part in the CHIKV-infected mice, there was very little difference in the concentrations of these over the experimental period (Figure 87). Calcium values were increased with dexamethasone treatment, but this was a transient affect (Figure 87: A). Phosphorous, sodium and potassium showed little change over time with either CHIKV infection of dexamethasone treatment (Figure 87: B, C, D). Total protein levels were greatly increased with dexamethasone treatment and returned back to a normal level within 72 hours after treatment (Figure 87: E). CHIKV-infected mice had a slight decrease in total protein levels that was not resolved 7 dpi. Decreased protein levels can indicate bleeding or liver disease. The albumin:globulin ratio is another level used to indicate signs of disease (Figure 87: F). This level was transiently increased in CHIKV-infected mice for less than 48 hpi. The level in the dexamethasone treated mice at 0 dpi was not elevated at 1 dpi, but was elevated at 2 dpi as compared to untreated mice.

In summary, there were many factors that were increased for less than 72 hours due to the dexamethasone treatment and most were left unchanged because of CHIKV infection. The ALP was the only enzyme that was significantly changed due to CHIKV and dexamethasone treatment nullified this affect. All other changes that occurred with CHIKV in mice are indicators of liver disease or dysfunction.

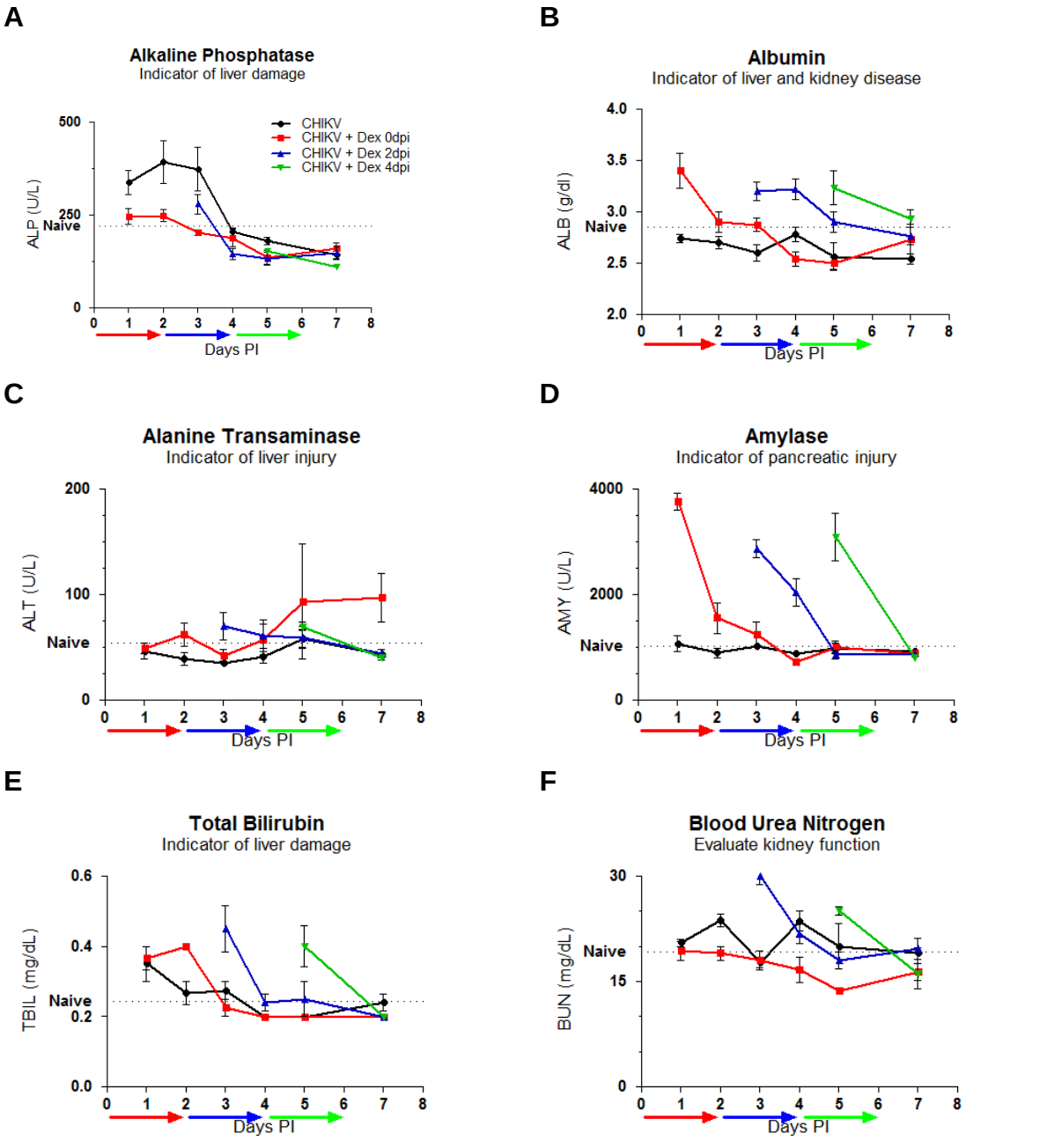


Figure 86: Analysis of liver enzymes in CHIKV-infected mice treated with dexamethasone

Analysis of liver enzymes of young CD-1 mice infected with CHIKV SC in the back with $10^{4.5}$ PFU. Treatment groups received a single dose of 200µg of dexamethasone IP at 0, 48 or 96 hpi

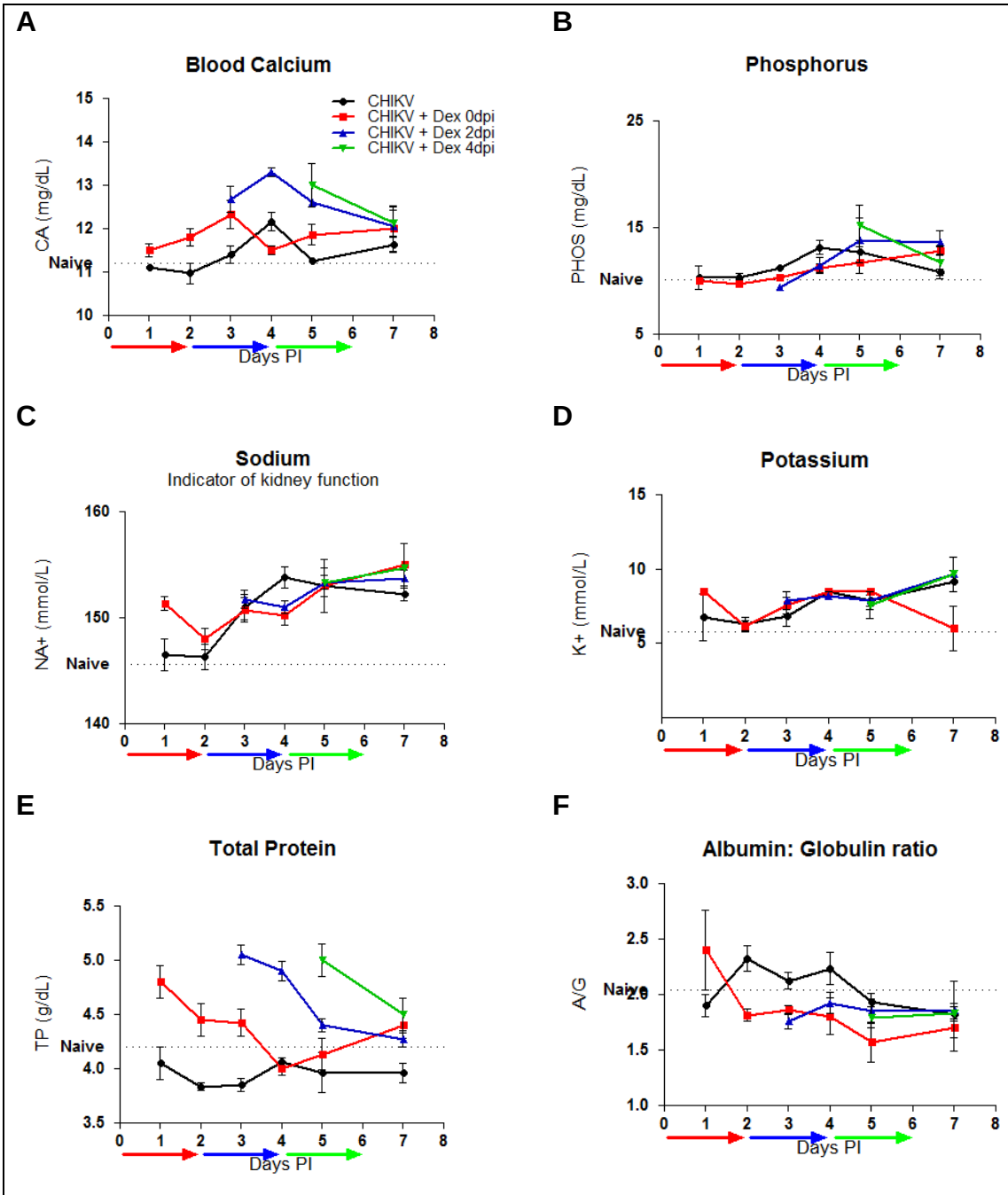


Figure 87: Analysis of serum chemistries in CHIKV-infected mice treated with dexamethasone

Analysis of serum chemistries of young CD-1 mice infected with CHIKV SC in the back with $10^{4.5}$ PFU. Treatment groups received a single dose of 200µg of dexamethasone IP at 0, 48 or 96 hpi

Histopathology analysis of CHIKV-infected mice with and without dexamethasone treatment was done. Lesion distribution was less than expected in the CHIKV-infected mice without treatment (Figure 88). Previously this value was greater than 60%, but in this experimental group the lesion distribution peaked at 38% on 7 dpi. The zero dpi treatment did not have any noticeable effect on the lesion distribution, which peaked on 7 dpi at 53%. The other two treatment groups had markedly less lesion distribution with the 2 dpi treatment having a peak distribution at 21% on 5 dpi. The 4 dpi treatment group had a peak distribution of 32% on 8 dpi. Severity of inflammation was virtually unchanged between the treatment groups and the untreated group (Figure 89). All groups had severe inflammation from 5 dpi to 9 dpi or longer. These results in conjunction with the distribution results signify that even though there is markedly less lesion formation in the treated groups, those lesions still have severe inflammation.

Interestingly, even though the dexamethasone should have reduced the amount of neutrophils in the sights of inflammation, there were still many neutrophils found in the treated groups, if not more in some groups (Figure 90). The presence of neutrophils peaked at 88% in the untreated CHIKV-infected mice on 6 dpi. In the zero and 4 dpi treatment groups the neutrophil presence peaked at 100% on 7 dpi. In the 2 dpi treatment group the neutrophil peak was at 60% on 5 dpi. Necrosis was also widely seen in the treatment groups (Figure 91). Necrosis began to be observed starting on 3 dpi in the untreated groups and 4 dpi in the treatment groups. All groups reached 100% necrosis over the experimental period, with many samples still having necrosis 12 dpi. Soft tissue inflammation was less predominant in the leg samples (Figure 92). Inflammation

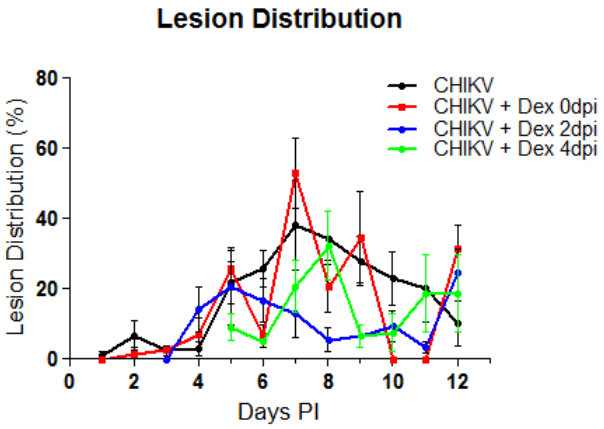


Figure 88: Lesion distribution in leg samples of CHIKV-infected mice treated with dexamethasone

Lesion distribution in leg samples of young CD-1 mice infected with CHIKV SC in the back with $10^{4.5}$ PFU. Treatment groups received a single dose of 200µg of dexamethasone IP at 0, 48 or 96 hpi. Treatment groups were not sampled until 24 hours after dexamethasone injection. Samples were fixed, embedded and H&E stained and graded as previously described.

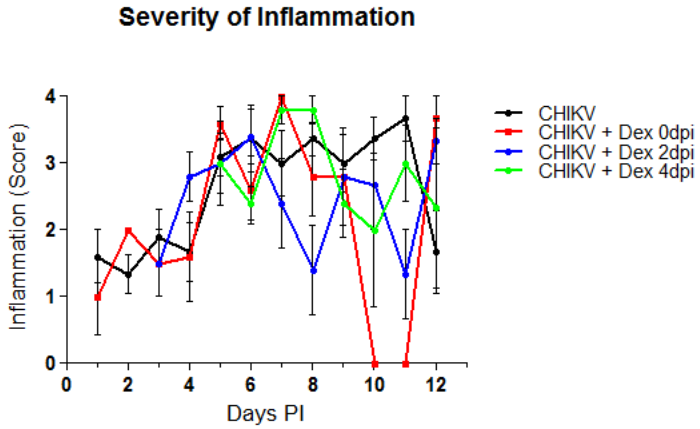


Figure 89: Inflammation severity in leg samples of CHIKV-infected mice treated with dexamethasone

Inflammation severity in leg samples of young CD-1 mice infected with CHIKV SC in the back with 104.5 PFU. Treatment groups received a single dose of 200µg of dexamethasone IP at 0, 48 or 96 hpi. Treatment groups were not sampled until 24 hours after dexamethasone injection. Samples were fixed, embedded and H&E stained and graded as previously described.

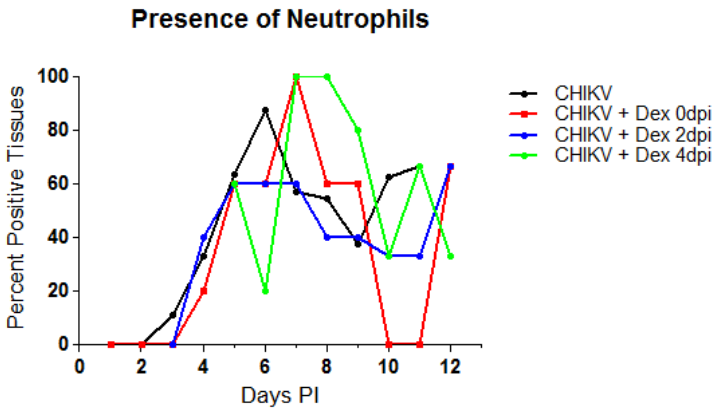


Figure 90: Neutrophil presence in leg samples of CHIKV-infected mice treated with dexamethasone

Neutrophil presence in leg samples of young CD-1 mice infected with CHIKV SC in the back with $10^{4.5}$ PFU. Treatment groups received a single dose of 200µg of dexamethasone IP at 0, 48 or 96 hpi. Treatment groups were not sampled until 24 hours after dexamethasone injection. Samples were fixed, embedded and H&E stained and graded as previously described.

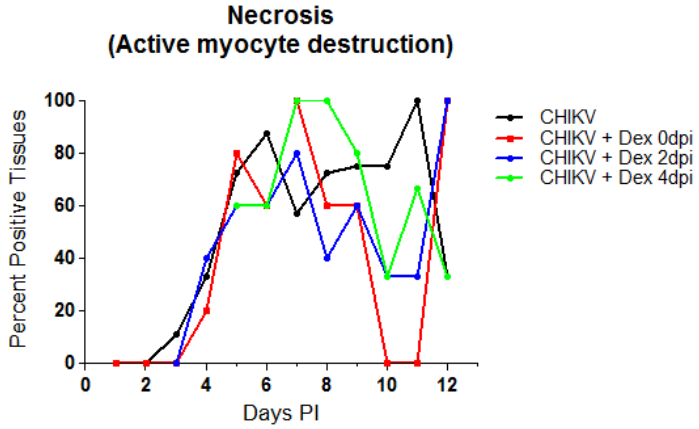


Figure 91: Necrosis presence in leg samples of CHIKV-infected mice treated with dexamethasone

Necrosis presence in leg samples of young CD-1 mice infected with CHIKV SC in the back with $10^{4.5}$ PFU. Treatment groups received a single dose of 200µg of dexamethasone IP at 0, 48 or 96 hpi. Treatment groups were not sampled until 24 hours after dexamethasone injection. Samples were fixed, embedded and H&E stained and graded as previously described.

in the soft tissue began to be observed on 4 dpi in the untreated and 0 dpi treatment group and reached a peak of 100% in the untreated group on 11 dpi. The treatment groups had less soft tissue inflammation with peaks in the zero and 4 dpi treatment groups at 80% on 7 dpi and in the 2 dpi treatment group the peak was 66% on 12 dpi. Overall, the differences in the treated groups and untreated group were subtle in the terms of pathological changes. The most effective treatment group in terms of pathological changes was the dexamethasone treatment given on 2 dpi.

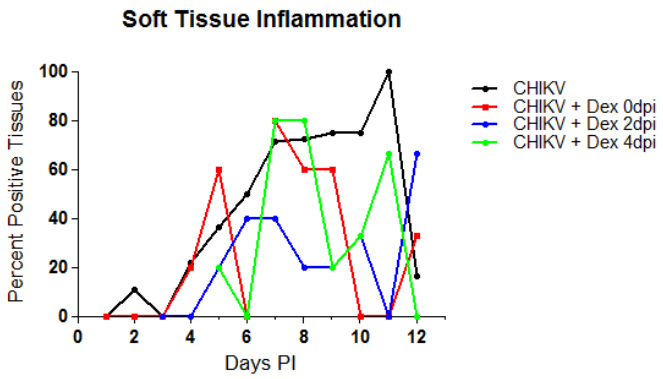


Figure 92: Soft tissue inflammation in leg samples of CHIKV-infected mice treated with dexamethasone

Soft tissue inflammation in leg samples of young CD-1 mice infected with CHIKV SC in the back with $10^{4.5}$ PFU. Treatment groups received a single dose of 200µg of dexamethasone IP at 0, 48 or 96 hpi. Treatment groups were not sampled until 24 hours after dexamethasone injection. Samples were fixed, embedded and H&E stained and graded as previously described.

Discussion

Immunosuppression of mice had interesting results in the context of CHIKV infection of mice (Table 11). Interferon deficient mice died quickly with high titer viremias. This has also been seen with other groups (Couderc et al. 2008). Mosquito bite-inoculation of CHIKV in these mice did resulted in significantly less mortality, but that may have been a result of the inability of the mosquitoes to transmit the virus at a level that caused an infection in the mice. This is indicative by the lack of viremia, HI antibody and re-infection of CHIKV in these mice resulting in mortality. In people, CHIKV infection in the immune compromised or severely ill is usually an indicator of worse disease outcome. In these knockout mice, the mortality seen is not due to specific myositis in the

Table 11: Immune modulators and their effects on pathogenesis

Immune Modulator	**Physiological function**	**Effects on Pathogenesis**
Chlorodonated liposomes	Decreased macrophages	Decreased myositis
Dexamethasone	Decreased lymphocytes, neutrophils, monocytes	Decreased myositis
Mosquito saliva	Decreased Th1 response	Decreased myositis
Cyclophosphamide	Decreased B cells and antibody	No difference in pathology
Chloroquine	Inhibited TNF-α, increased NO production, decreased IL-6	Same or increased myositis
Enteracept	Decreased TNF-α	Increased myositis in RRV
AG129 and A129 mice	Interferon deficient	Mortality

(Gardner et al. 2010, Ghigo et al. 1998, Machado et al. 2011, Matar et al. 2002, Sistigu et al. 2011, Zaid et al. 2011)

skeletal muscle, but due to the inability of the body to control the viral replication.

Chloroquine treatment, while it was thought to have therapeutic effects against CHIKV infection, more recently has been shown in people to not be effective (Brighton 1984, De Lamballerie et al. 2008, Ozden et al. 2008, Savarino et al. 2007, Sourisseau et al. 2007). Chloroquine has been shown to inhibit IL-6, IL-1β and TNF-α while increasing nitric oxide production from macrophages where it accumulates in the phagolysosome (Ghigo et al. 1998, Khan et al. 2010, Legssyer et al. 2003, Weber et al. 2001). The results in both cell culture and mice agree with published work with CHIKV in cell culture and CHIKF patients. The effects seen *in vitro* are not correlative to the mouse model. This could be due to *in vitro* affect that inhibits virus replication, but not *in vivo*. The results seen actually suggest that chloroquine may have negative effects on disease outcome in mice, but would need to be repeated with more mice to make that conclusion. This is supported by a recent publication that reports that people who received chloroquine treatment with CHIKV infection were more affected by recurrent arthralgia (De Lamballerie et al. 2008). The interesting part of the work with chloroquine is that in vivo it has both a stimulatory and inhibitory affect on activated macrophages. Chloroquine is able to down regulate the Th2 cytokine, IL-6 and Th1 cytokine TNF-α, but increases the ability of macrophages to release NO. If repeated experiments truly showed an increased amount of myositis in chloroquine treated mice, some definite conclusions could be formed about the role of TNF-α and NO in CHIKV-induced pathology. Overall, this work with chloroquine supports the fact that macrophages play a critical role in causing CHIKV pathogenesis and that increased production of NO may cause increased myositis in mice.

Cyclophosphamide is an immunosuppressant that targets the humoral immune system. In recent reports, cyclophosphamide immunosuppressed hamsters showed increased disease severity and mortality when infected with West Nile virus and Yellow fever virus (Mateo et al. 2006, Mateo et al. 2007). This was not the case in these studies with CHIKV. Immunosuppressed mice did not have any signs of increased disease or mortality. Rigorous studies of daily viral titers and histopathology were not done when the preliminary results were unremarkable. This study was repeated with an increased dosing of cyclophosphamide and white blood cell counts were assessed to confirm immunosuppression, but the results were the same, with no mortality seen.

Dexamethasone as a treatment for CHIKV infection looks promising with the current studies. Importantly there was no indication of a worsening of the disease in the mice with the dexamethasone treatment. The laboratory values that became abnormal with the dexamethasone treatment were all transient and know to be side effects of corticosteroid use. The viral load in the legs was decreased and the amount of inflammation and the severity of the pathology in the leg muscle were decreased with the dexamethasone treatment. While the results are not as striking as one would like, the dexamethasone treatment given was a single dose. Not only was the dexamethasone treatment beneficial to the mice, it was beneficial when given after infection. This would suggest that in people, getting treatment after symptoms have occurred may lessen the severity of the muscle pain and arthralgia. If these experiments were to be repeated, giving multiple dexamethasone injections should be used. One of the benefits of using dexamethasone is that it is readily used in humans and does not need further regulatory approval. While the above work does not show a complete

loss of CHIKV-induced symptoms with dexamethasone treatment, it does show improvement on various aspects of disease.

Chapter 7: Discussion

The goal of this research was to better understand CHIKV pathogenesis and CHIKV-induced immune-mediated pathology. Over the course of these studies, I developed a small animal model of CHIKV infection and utilized this model to investigate locations of CHIKV replication and the effects of mosquito bite-inoculation and immune modulators on the course of disease. It was shown that young mice are a good model of CHIKV pathogenesis and that they developed an acute febrile illness that resulted in severe myositis. CHIKV replicated in muscle cells and lymph nodes of infected mice as well as endothelial cells in the brain and spleen. Mosquito bite-inoculation of CHIKV was shown to cause less severe disease in mice than needle inoculation. The degree of pathology was dependent both on viral dose and presence of mosquito saliva. Work was presented indicating that immunosuppression, while sometimes detrimental to CHIKV-infected mice, decreased muscle pathology. It was also shown that the dexamethasone dose used did not have a negative effect on CHIKV disease outcome in mice and actually lessened the severity of CHIKV-induced myositis.

The young mouse model of CHIKV infection presented in this work has many parallels to human disease, including limited mortality, severe myositis, viremia and liver enzyme changes; but for some laboratory analyses, it still has faults. Infection of young mice with CHIKV is a good model to study disease pathogenesis; but because of the young age of the mice and the lack of mortality, it is not a good testing platform for vaccines. Also, due to the limited signs of

CHIKV-induced disease in mice of this age, mainly pathology, testing therapeutics are problematic.

The most striking change in CHIKV-infected mice was the severe myositis, involving widespread lessons and inflammation in skeletal muscle. This could only be quantitated through histopathological analyses. Unfortunately, while grading pathology slides can show striking differences, it is still a qualitative measure. In using such a scoring system, subtle differences were lost, but it was still possible to see dramatic changes. Possibly, with more refined or quantitative systems, statistical differences could be obtained. A second drawback with the young mouse model is that while SC inoculation in the back led to a disseminated infection in all four limbs, footpad inoculation did not lead to a disseminated infection. This lack of dissemination caused problems when doing experiments with mosquito bites and IVIS technology. In future experiments, footpad inoculation should be avoided and comparisons to SC inoculations with footpad inoculations should be done carefully.

One reason for the difference in footpad and SC inoculation might be due to the draining lymph nodes that are infected. Inoculation of CHIKV SC in the back allowed the virus to infect multiple draining lymph nodes and to have multiple points of origin; conversely, inoculation of the footpad caused virus to be sequestered to a small subset of draining lymph nodes in the foot and leg that was infected. The question remains as to how this correlates to human infections by mosquito bite. One could hypothesize that the site of initial infection in a person has a dramatic effect on the ultimate course of the disease. Infected mosquito bites are more likely to occur on areas of the skin that are exposed, including the arms and legs. It could be hypothesized that an increased rate of

mosquito bites on the limbs explains why the wrist, fingers, knees and ankles are more likely to be affected by CHIKF-induced arthralgia. However, this hypothesis remains difficult to test at this time.

The animal model presented here is comparable to the other animal models of CHIKV and RRV infection. The use of young CD-1 mice was first utilized with RRV (Morrison et al. 2007, Morrison et al. 2006, Rulli et al. 2007). RRV infected mice develop a more severe disease, as compared to CHIKV-infected mice. RRV infection was manifested by loss of weight, ruffled fur and hind limb dragging (Morrison et al. 2006). The histopathological studies of the skeletal muscle in the RRV infected mice showed severe myositis on 7 and 10 dpi, but very little myositis by 30 dpi, which is similar CHIKV infection. CHIKV infection in mice deficient in interferon has also been tested with both complete and partial knockout mice (Couderc et al. 2008). In this model system, mice with complete deletion of the IFN-α and IFN-β gene exhibited 100% mortality, similar to studies that were presented here. Mice that had incomplete deletion of the genes (+/-) were susceptible to CHIKV infection but exhibited no mortality. These mice also had very low viral titers in tissues, while no pathology was seen in the muscle tissue. Adult wild type mice have also been shown to have a limited amount of pathology when infected with CHIKV in the footpad (Gardner et al. 2010, Morrison et al. 2011). One advantage of this model is the older age of the mice; but the pathology seen in these mice is limited to the foot that is inoculated and it is not a disseminated infection. A similar mouse model used young C57BL/6J mice with footpad inoculation of CHIKV. In the C57BL/6 model, the foot that was inoculated had severe edema and swelling, while the opposite

non-injected foot had some pathological changes, but no obvious swelling or edema (Morrison et al. 2011).

Many aspects of CHIKV pathogenesis remain a mystery even with rigorous examination in animal models and clinical studies. One thing that has become clear is that CHIKV does not behave as one might expect, causing disease through cell targeted cytopathic effects. The role of the immune system is still undefined with CHIKV pathogenesis. It has become apparent that the immune system is double-edged during CHIKV infection. The interferon and antibody responses are essential for controlling CHIKV replication and inhibiting disseminated infection. What still remains unknown is the role of the innate immune system including macrophages and complement as well as neutrophils and NK cells in disease pathogenesis. It could be hypothesized that neutrophils and NK cells are equally important for the myositis observed in CHIKV infection. Neutrophils were almost always present in a large number in observed myositis. If neutrophils were decreased in CHIKV-infected mice, a decrease in severe myositis was observed.

The role of complement in arthralgic alphavirus infections has only been studied in RRV-infected mice. Due to the time limitations of this work, complement specifically was not studied. This is an important aspect of the innate immune system that has been shown with RRV infected mice to be a key to myositis severity (Morrison et al. 2007). It can be hypothesized that complement also plays an important role in CHIKV-induced myositis. By decreasing complement receptor 3 in mice infected with CHIKV, it can be assumed that there would be a decrease in myositis, based on RRV studies. This may not be due to the modulation of the immune system. I have speculated

that the complement receptor may also be a secondary receptor for CHIKV, as it is found on a wide range of cells that CHIKV replicates within, including macrophages, muscle cells and endothelial cells. While this remains untested, further work may help to understand the complex role of complement and alphavirus pathogenesis.

I propose that there are three phases of CHIKV pathogenesis in people and in animals (Figure 93). The acute phase of CHIKV infection begins with replication at the site of infection and the draining lymph nodes, and results in a high viremia. At this time people have non-specific symptoms common with many viral infections (fever, headache, malaise, etc.). During this initial stage of viral infection, the interferon response is important in controlling viral replication. High mortality in interferon deficient animals is during this initial phase of disease and is due to complications that may exist in many different viral infections. CHIKV is capable of cytopathic effects within cells during viral replication, and high viremia levels can lead to non-specific cellular death. The second phase of CHIKV infection is when the immune system begins to cause damage. At this stage, CHIKV-induced myositis occurs and the DTH response starts to cause myositis. Macrophages and complement systems are activated and are two of the keys to causing this damage. At this point, suppression of the innate immune system might be helpful to the infected individual. In mice, dexamethasone given 2 and 4 dpi decreased the innate immune response during this subacute phase, decreasing the immune-mediated pathology. The last stage of CHIKV infection is the persistent and recurrent stage. Unfortunately, this was not modeled in our animals, and it is still unknown how the virus is able to evade the immune system. There is some evidence that CHIKV replicates within macrophages

within the synovial fluid of people (Hoarau et al. 2010). If this is the case, there are many physiological factors that could cause a localized recurrent event in these individuals including stress, immunosuppression or injury. Antibody levels would stop a systemic affect, but in localized areas, there could still be inflammation and edema.

A proposed mechanism for CHIKV pathogenesis includes the activation of macrophages and complement that result in a DTH response (Figure 94). This model represents both the work presented here and published work with RRV and CHIKV. The work with mosquito saliva has shown that a Th2 response in mice has a decreased affect on the CHIKV-induced myositis. This corresponds well with the proposed model. If macrophage and complement activation are keys to CHIKV-induce pathology, a Th2 response down regulates these. It also explains why there may be an increased viremia in mosquito bitten mice. This is due to the decreased interferon response in a Th2 versus a Th1 type immune event. While a complete lack of interferon induces mortality in CHIKV-infected mice, a decrease in the level does not (Couderc et al. 2008). This proposed mechanism may give more opportunities for testing of therapeutic options

One important aspect of CHIKV pathogenesis that was lacking from the mouse model was arthralgia. While myositis was abundant in CHIKV infected mice, no appearance of joint pathology was seen. The synovial membrane and space in young mice infected with CHIKV was rarely seen to be inflamed. Arthralgia has been shown to be one of the most debilitating aspects of CHIKF in humans. It is unknown the mechanism that causes of arthralgia in people. One might hypothesize that the lack of arthralgia in the CHIKV infected mice was due the decreased severity of the virus in mice as compared to humans. It may also

be hypothesized that there is a threshold of viral replication in order for arthralgia to occur and in the mice infected in these studies that threshold may not have been met. This may also be one reason why there was a lack of persistence in the mouse model of CHIKV infection.

Future studies with CHIKV pathogenesis are needed to further our understanding of how CHIKV causes immune system dysfunction. The viral proteins that lead to the DTH response may help to further our understanding of the function of the immune system in many types of viral infections. There are very few viral proteins within the CHIKV genome, so while it may be difficult to tease out the exact mechanism, there is a limited number of proteins to work with. Also the alphavirus genome is easy to manipulate which has been shown by the production of infectious clones. These clones could be engineered to over express viral proteins for more detailed work with cells of the immune system.

Another important aspect that needs to be further researched is the fact that virus does not disseminate from footpad inoculations, but does with SC injections. If this phenomenon could be further related to people, it may be possible to pinpoint therapeutic approaches. If virus is not disseminating readily, a therapeutic might be applied to a single area to alleviate symptoms and not systemically. This may become important if we were to further study immunosuppressants as treatment strategies.

Vaccine development also remains a goal of work with CHIKV. Understanding the role of the immune system during pathogenesis may be a key to developing a vaccine that does not cause myalgia in its recipients. While CHIKV protection is from conventional antibody production, and lifelong immunity

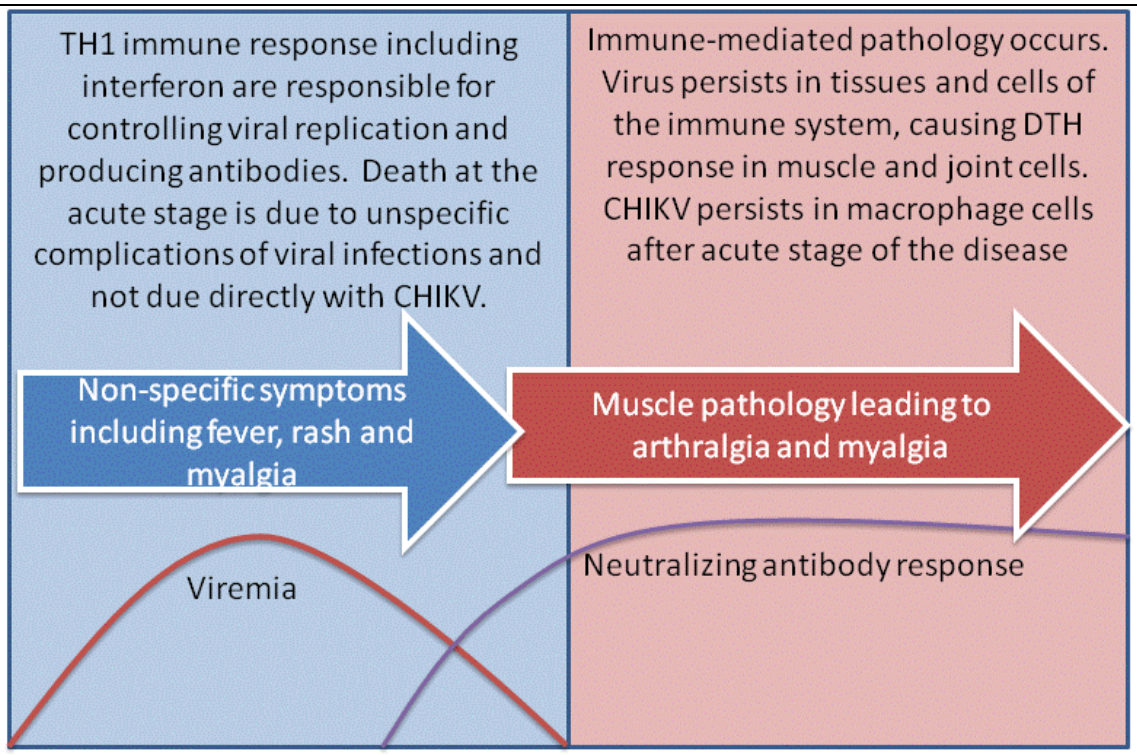


Figure 93: Proposed timeline of events for CHIKV pathogenesis in humans and animal models

is assumed to occur, the problem with vaccine development is having an appropriate model to test in and developing an attenuated virus that does not cause myalgia. If the proteins important to macrophage dysfunction could be pinpointed, myalgia might be able to be stopped and a vaccine candidate might emerge. An alternative, would be to give vaccine candidates an immune suppressant with the vaccine to help decrease the likelihood of arthralgic side effects.

Another question that still exists is the receptor that the virus uses *in vivo*. Heparin-sulfate had been shown to be a receptor for CHIKV *in vitro*, but it is ubiquitously produced in most cells. While CHIKV infects most adherent cells,

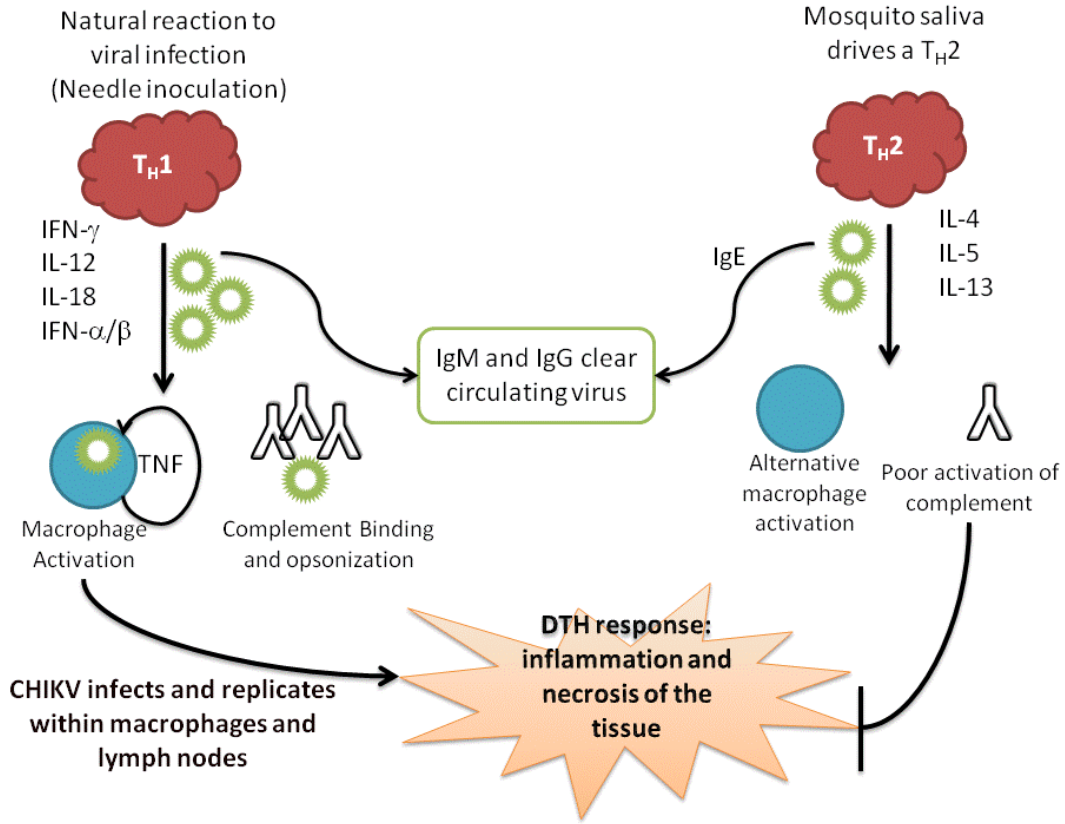


Figure 94: Proposed mechanism for CHIKV pathogenesis

in vitro, within the mouse model, CHIKV is not found in all tissues in the same amounts. This suggests that a second receptor to CHIKV may exist, or a helper of sorts greatly increases the likelihood of CHIKV infection. One would assume that this receptor would be found on muscle cells as well as on macrophages or other cells of the immune system.

This work was given proof of concept that immunosuppressants have the potential to be used as therapeutic options for CHIKF. While complete amelioration of myositis did not occur, there was a marked decrease in disease in CHIKV-infected mice. Similar studies have been done in RRV-infected mice

with an inhibitor of TNF-α with negative results (Zaid et al. 2011). In these RRV infected mice, application of etanercept caused a more severe disease in mice with 100% mortality. These results indicate that caution must be used when immunosuppressants are used in CHIKV or RRV infected individuals. One difference between dexamethasone and etanercept is that etanercept causes a complete reduction in TNF-α, while dexamethasone causes a decrease in multiple inflammatory cells. It was proposed that etanercept caused a decrease in the antiviral immunity which is why mortality occurred starting at 12 dpi. Dexamethasone has not been associated with a loss of antiviral immunity and these studies did not result in increased disease.

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Vita

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June 20, 2011

PRESENT POSITION AND ADDRESS: Graduate Student
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August 2006 - Present

BIOGRAPHICAL: Born in Las Vegas, Nevada, U.S. on March 2, 1980
Parents: William and Connie Roberts
Husband: Jason Ziegler
Children: Gabrielle, Daniel and Nathan

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EDUCATION:

Ph.D.	Biomedical Sciences, University of Texas, Medical Branch – **in progress**
M.S.	Biochemistry, University of Nevada, Las Vegas, August 2006
B.S.	Biochemistry, University of Nevada, Las Vegas, December 2003

PROFESSIONAL AND TEACHING EXPERIENCE:

Jan 2005	Graduate Teaching Assistant for the Biochemistry Laboratory; UNLV
Sept 2004	Graduate Teaching Assistant for Organic Chemistry Laboratory; UNLV
June 2004	Graduate Teaching Assistant for the Chemistry for Life Sciences Laboratory; UNLV
Jan 2004	Graduate Teaching Assistant for Organic Chemistry Laboratory; UNLV

WORK EXPERIENCE:

09/06 – Present Univ. of Texas Medical Branch Galveston, TX
Graduate Assistant
- Conducting research in pursuit of a Ph. D.

07/06 – 03/07 Gaido's of Galveston Galveston, TX
Waitress/Bartender

01/04 – 06/06 Univ. of Nevada, Las Vegas Las Vegas, NV
Graduate Assistant
• Taught laboratory courses in chemistry and biochemistry, along with grading and proctoring exams.
• Conducted research in pursuit of a Master's degree.

09/05 – 01/06 Upward Bound Las Vegas, NV
Science Instructor
• Taught Science Courses to high school students in a tutorial setting included developing curriculum.

08/03 – 12/04 Las Vegas Natural History Museum Las Vegas, NV
Education Department Intern
• Intern in the Education Department, helped to setup programs for school age children.
• Gave tours and educated children on the museum and live animals.
• Traveled to schools and presented science related material to elementary students

RESEARCH ACTIVITIES:

Ph. D Research: Studying biomedical research in the department of pathology at the Univ. of Texas Medical Branch. Investigating the pathogenesis of chikungunya virus with classic virological methods and biochemical assays. Working in biocontainment laboratories including animal biocontainment facilities.

M.S. Research: Utilized cell culturing, clonogenic survival, fluorescent microscopy, flow cytometry, spectrophotometery, western blot, DNA gel electrophoresis and HPLC.

COMMITTEE RESPONSIBILITIES:

2008 – pres. Member of the Education Committee, ASTMH

2010 – pres. Presidential Scholar's Committee, Student Mentor; UTMB
2008 – 2010. Co-President Experimental Pathology GSO; UTMB
2007 – 2009 Senior Co-Director, National Student Research Forum; UTMB
2007 – 2008 Program Representative, Graduate Student Organization; UTMB

2010 Scientific Symposium Organizer; ASTMH Annual Meeting
2009 Co-Chair of the Virus I Session; ASTMH Annual Meeting

MEMBERSHIP IN SCIENTIFIC SOCIETIES:

2009 – pres. American Society of Tropical Medicine and Hygiene Student Member
2005 – 2006 Associate Member, American Association for Cancer Research

HONORS:

2010 Robert Shope, M.D. Scholarship; UTMB
2010 Named to "Who's Who in American Colleges and Universities
2009 Sealy Center for Vaccine Development Fellowship: UTMB
2009 Ann and John Hamilton Endowed Scholarship Award; UTMB
2008 Stephen C. Silverthorne Memorial Award; UTMB

Ad hoc Journal Reviewer for Vector-Borne and Zoonotic Diseases

PUBLISHED:

A. Articles in Peer-Reviewed Journals:

Madsen, S. J., Angell-Petersen E., Spetalen S., Carper S. W., Ziegler S. A., Hirschberg H. Photodynamic therapy of newly implanted glioma cells in the rat brain. Lasers Surg Med. 2006 Jun;38(5):540-548.

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Long, K.C., Ziegler, S.A., Thangamani, S., Hausser, N.L., Kochel, T.J., Higgs, S., Tesh, R.B. Experimental transmission by Mayaro virus in *Aedes aegypti* mosquitoes. Am J Trop Med Hyg. 2011 Oct;85(4):750-757.

B. Other Publications:

Meacham, Susan L., Kyler E. Elwell, Sarah A. Ziegler, and Stephen W. Carper. "Boric Acid Inhibits Cell Growth in Breast and Prostate Cancer Cell Lines " In *Advances in Plant and Animal Boron Nutrition*, edited by F. Xu, H.E. Goldbach, P.H. Brown, R.W. Bell, T. Fujiwara, C.D. Hunt and S.; Shi Goldberg, L.: Springer, 2007.

Ziegler, Sarah A**.** "Photodynamic Therapy Induces Oxidation in Breast and Brain Cancer Cell Lines" Chemistry Department. University of Nevada, Las Vegas. Masters of Science in Biochemistry, 2006.

C. Abstracts:

Ziegler, Sarah A., Steen J. Madsen, and Stephen W. Carper. "Hsp27 Protects against ALA Mediated PDT Induced Cytotoxicity in Human Breast Cancer Cells." Poster presented at the American Society for Biochemistry and Molecular Biology Annual Meeting, San Diego, CA, April 2005.

Meacham, Susan L., Sarah A. Ziegler, Kyler E. Elwell, and Stephen W. Carper. "Boric Acid Inhibits Cell Growth in Breast and Prostate Cancer Cell Lines."

Paper presented at the The third international symposium on all aspects of plant and animal boron nutrition, Wuhan, China, September 2005.

Madsen, Steen J., Even Angell-Petersen, Signe Spetalen, Stephen W. Carper, Sarah A. Ziegler, and Henry Hirschberg. "ALA-PDT of Glioma Cell Micro-Clusters in Bd-Ix Rat Brain." Paper presented at the Photonic Therapeutics and Diagnostics II, San Jose, CA. January 2006.

Ziegler, Sarah A., Becky M. Cox, Cherisse R. Loucks, Steen J. Madsen, and Stephen W. Carper. "ALA and Photofrin Mediated PDT Induces Apoptosis and Necrosis in Human Breast Cancer Cells." Poster presented at the American Association for Cancer Research annual meeting, Washington, D.C., April 2006.

Madsen, Steen J., Even Angell-Petersen, Qian Peng, Stephen W. Carper, Sarah A. Ziegler, Olav Engebraaten, and Henry Hirschberg. "ALA-PDT in Experimental Glioma Models " Paper presented at the 5th International Workshop on Photodynamic Therapy and Photodetection with Porphyrin Precursors, Buenos Aires, Argentina, June 2006.

Ziegler, Sarah A., Casey Hall, Cherisse Loucks, Steen J. Madsen and Stephen W. Carper. "Comparison of ALA-PDT in two rat glioma models." Paper presented at the Photonic Therapeutics and Diagnostics III, San Jose, CA., January 2007.

Ziegler, Sarah A., Liang Lu, Shu-Juan Xiao and Robert B. Tesh. "Pathogenesis of Chikungunya Virus in Mice." Oral presentation at American Society of Tropical Medicine and Hygiene annual meeting, Philadelphia, PA, November 2007.

Ziegler, Sarah A., Amelia P.A. Travassos da Rosa, Shu-Yuan Xiao, Dana Vanlandingham, Stephen Higgs and Robert B. Tesh. "A Comparison of the Pathogenesis of Chikungunya Virus in Mice after Infection by Mosquito Bite or Needle Inoculation." Oral presentation at American Society of Tropical Medicine and Hygiene annual meeting, Washington, D.C., November 2009.

Ziegler, Sarah A., Amelia P.A. Travassos da Rosa, Dana Vanlandingham, Stephen Higgs and Robert B. Tesh. "Understanding the mechanism of immune-mediated pathology in chikungunya virus infected mice." Poster presentation at 2010 IHII/Mclaughlin Colloquium, Galveston, TX, April 2010.

Ziegler, Sarah A. Hilda Guzman, Amelia P.A. Travassos da Rosa, Robert B. Tesh "The effects of immune suppression on chikungunya virus pathogenesis in mice." Accepted as an oral presentation at American Society of Tropical Medicine and Hygiene annual meeting, Atlanta, GA., November 2010.