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Comparison of Chikungunya Virus Strains in Disease Severity and Susceptibility to T-705 (Favipiravir), *In vitro* and *In vivo*

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COMPARISON OF CHIKUNGUNYA VIRUS STRAINS IN DISEASE SEVERITY AND SUSCEPTIBILITY TO T-705 (FAVIPIRAVIR), *IN VITRO* AND *IN VIVO*

by

Makda S. Gebre

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ABSTRACT

Comparison of Chikungunya Virus Strains in Disease Severity and Susceptibility to T-705 (Favipiravir), *In vitro* and *In vivo*

By

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Utah State University, 2017

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Chikungunya virus (CHIKV) is an arbovirus that typically causes painful arthralgia, fever and rash in infected patients and is currently listed as a priority pathogen by the National Institute of Allergy and Infectious Diseases (NIAID), NIH. Various CHIKV strains have been classified into three phylogenetic groups or clades, namely West African, Asian and East/Central/South African (ECSA). Currently, there are no FDA approved therapeutics or vaccines used to treat or prevent Chikungunya. In order to understand inter-strain differences in disease phenotype and antiviral response, we compared two CHIKV strains from each clade in cell culture and in mouse models.

There was a difference in replication kinetics between different CHIKV strains. West African strains replicated more robustly and were slightly less sensitive to treatment with the test antiviral, T-705. This was further supported by studies in mice where West African strains, and similarly ECSA strains, caused a

more severe disease phenotype and were less readily protected by T-705 as compared with Asian lineage strains. These studies suggest there is a difference in disease severity and response to antiviral treatment between CHIKV lineages.

(114 Pages)

PUBLIC ABSTRACT

Comparison of Chikungunya Virus Strains in Disease Severity and Susceptibility to T-705 (Favipiravir), *In vitro* and *In vivo*

Makda S. Gebre

Chikungunya is a mosquito-transmitted disease caused by Chikungunya virus (CHIKV). Symptoms of Chikungunya include debilitating joint pain and swelling, fever and rash. CHIKV was first discovered in 1953 in Tanzania, and has since caused periodic outbreaks of disease. The virus reemerged recently in 2004 and has since spread around the world affecting more than 3 million people. The different strains of CHIKV have been grouped into three phylogenetic clades: West African, Asian and East/Central/South African (ECSA). There are no FDA approved medicines or vaccines used to treat or prevent CHIKV infection. The antiviral drug, T-705 (commercially known as Favipiravir), has recently been shown to have activity against CHIKV. T-705 has already been approved in Japan for the treatment of influenza and is currently going through clinical trials in the US.

Since there may be phenotypic differences between the clades of CHIKV, it is important to first characterize distinctions between the strains and determine the susceptibility of these strains to treatment. To do this, we obtained two different CHIKV strains from each of the three phylogenetic groups. These CHIKV strains displayed differences in their ability to replicate in cell culture and exhibited only slight differences in susceptibility to T-705 treatment. However,

more profound differences were observed in mouse models where differences in disease severity and response to T-705 treatment were observed.

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Makda S. Gebre

CONTENTS

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Chikungunya virus (CHIKV) is a mosquito-borne virus that causes Chikungunya (CHIK). The key symptoms of CHIK include fever, rash and joint pain that can last from weeks to several years. Other symptoms include joint swelling, headache and muscle pain. Although CHIK has a low mortality rate (one death occurring in 1,000 clinical cases), 50–97% of infected individuals show clinical symptoms (Gerardin et al., 2008b). Chronic symptoms that persist two years after initial infection have also been observed in 43–75% of CHIK patients (Gerardin et al., 2011).

CHIKV was first identified in 1953 in Tanzania (Powers and Logue, 2007). The word “Chikungunya” comes from the Makonde language spoken in south-east Tanzania and northern Mozambique. The word literally translates to mean “that which bends up” referring to the bent posture of CHIK patients (Mohan et al., 2010). CHIKV has caused several minor outbreaks in the decades following its discovery. However, within the last decade it has extensively spread throughout the world becoming a great public health concern.

In 2004, a virus strain emerged from the coast of Kenya to extensively spread to numerous regions in Africa, South-east Asia and Europe affecting millions of people (Graham et al., 2016). Between 2005-2007, CHIKV infected 300,000 people in the islands of the Indian ocean, 1.4 million people in India and 42,000 people in Thailand (Schwartz and Albert, 2010). A mutation that allowed the virus to adapt a new vector further expanded the geographic range of

infection of the virus to temperate areas (Tsetsarkin et al., 2007). In 2013, CHIKV reached the Americas causing 1.1 million cases within a year (Yactayo et al., 2016). The various strains of CHIKV currently present in different parts of the world have been grouped into three phylogenetic groups or clades, namely, West African, Asian and East/Central/South African (ECSA). The strains that emerged recently in the Americas are classified within the Asian lineage, likely introduced by an infected traveler.

Thus far, no approved antiviral therapies or vaccines exist for CHIKV. However, a broad-spectrum viral RNA polymerase inhibitor, T-705 (Favipiravir) has been identified as a prime candidate (Ahola et al., 2015).

1.1. Statement of the Problem

As CHIKV rapidly spreads around the world, it is critical to understand similarities and differences in disease manifestation that occur due to infection with different CHIKV lineages and to identify broad-spectrum vaccines and antiviral drugs that can be implemented globally. We have previously been working with two strains of CHIKV, S27 and LR2006_OPY1 (LR06), and have demonstrated different levels of disease severity in a mouse model. Briefly, mice infected with S27 or LR06 strains of CHIKV had significantly different levels of footpad swelling at various times after infection (Fig. 1). In fact, infection with 10,000-fold less virus inoculum of LR06, as compared with S27 challenge, resulted in a similar footpad swelling profile (Fig. 1).

This led us to question if differences in disease phenotype and antiviral response exist between strains from different virus clades. Thus far, no published studies have been conducted to address this question. Therefore, this project is primarily conducted to fill that knowledge gap.

In addition to S27 and LR06, which are grouped together in the ECSA clade, we obtained British Virgin Islands (BVI) and Indonesia (IND) strains of the Asian clade and Senegal (SEN) and Nigeria (NGR) strains that fall within the West African clade. The BVI strain is of particular interest as it is an Asian strain currently circulating in the Caribbean. The differences in disease phenotype

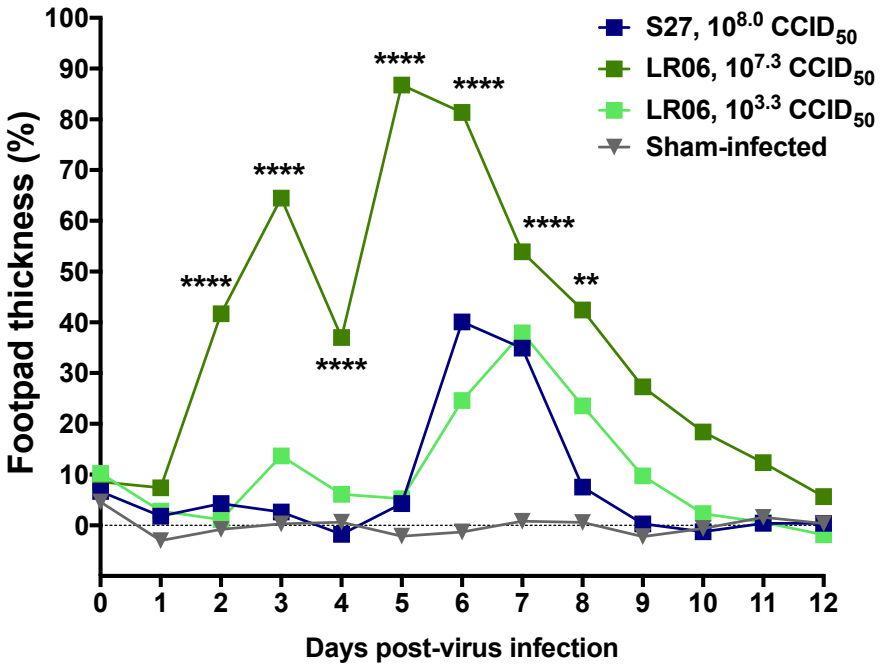


Fig. 1. Percent change in footpad thickness between inoculated foot and non-inoculated foot of DBA/1J mice infected with 10^8 $CCID_{50}$ of S27 or $10^{7.3}$ $CCID_{50}$ and $10^{3.3}$ $CCID_{50}$ of LR06 of CHIKV. (****P<0.0001, **P<0.01, $10^{7.3}$ $CCID_{50}$ of LR06 compared to $10^{8.0}$ $CCID_{50}$ of S27; $CCID_{50}$: 50% cell culture infectious dose)

observed in mice infected with S27 and LR06, which are within the same clade, suggest that strain differences may not strictly be based on phylogenetic grouping. As shown in the phylogenetic tree (Fig. 2), each clade is represented in our study by two strains. Geographic distribution of the strains is shown in Figure 3 and information on their origins and history is provided in Table 1.

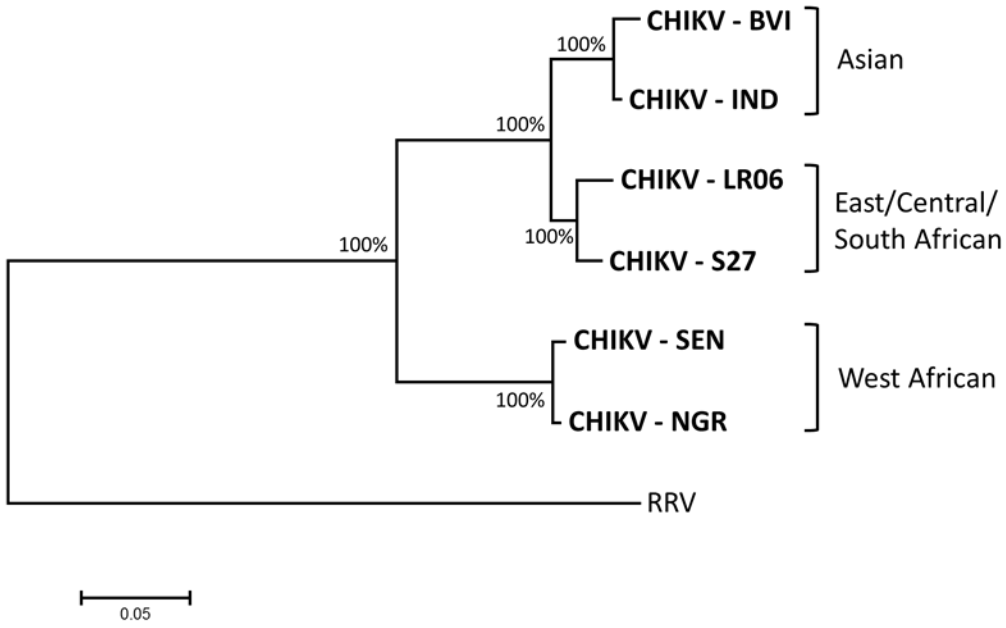


Fig. 2. Phylogenetic tree of strains of CHIKV used for *in vitro* and *in vivo* strain comparison experiments. Evolutionary history was constructed with MEGA7 software using complete genome sequences obtained from the GenBank database. Maximum Likelihood method was used for analysis based on Tamura-Nei model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of bootstrap replicates (1,000 bootstrap replications, confidence probability 100%) are indicated at the nodes. The analysis included CHIKV strains from British Virgin Islands (BVI, **KJ451624.1**, 2013), Indonesia (IND, **HM045797.1**, 1985), La Reunion (LR06, **DQ443544.2**, 2006), Tanzania (S27, **AF369024.2**, 1953), Senegal (SEN, **AY726732.1**, 1983) and Nigeria (NGR, **HM045786.1**, 1964). Ross River Virus (RRV, **NC_001544.1**, 2012), a closely related alphavirus that also belongs in the Semliki Forest virus complex, was introduced to add correct rooting of tree.

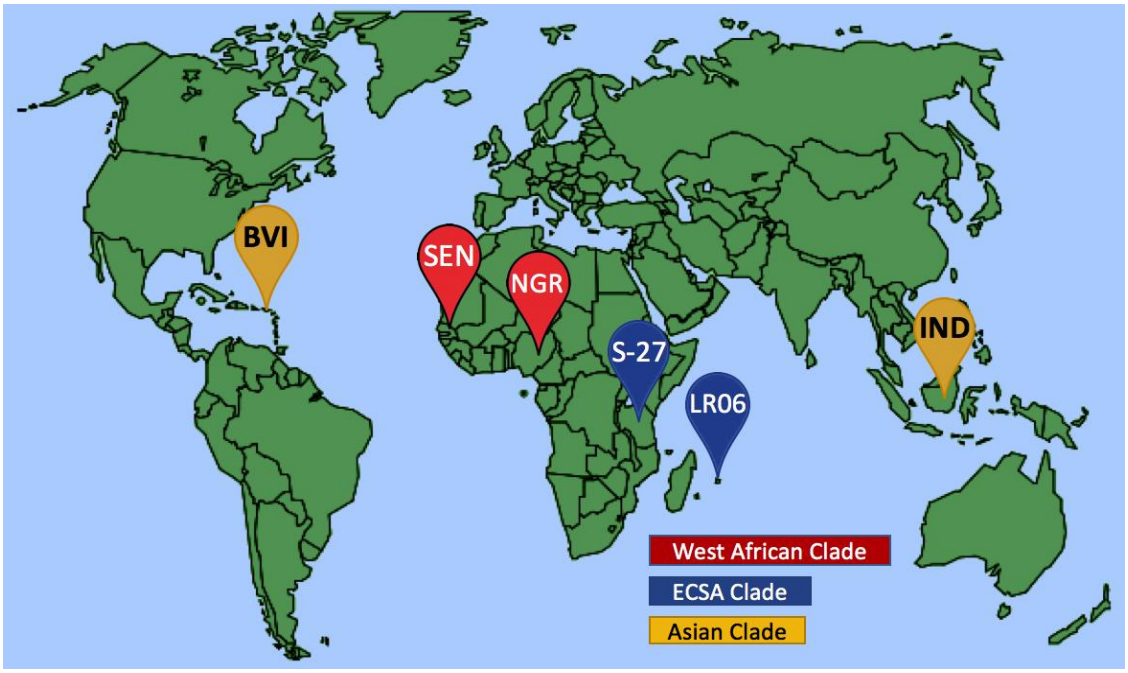


Fig. 3. Geographic distribution of strains from three phylogenetic groups of CHIKV.

Table 1
History and origins of BVI, IND, SEN, NGR, S27 and LR06 CHIKV strains used for *in vitro* and *in vivo* comparison.

Clade	**Asian**		**ECSA**		**West African**	
Abbreviation	**BVI**	**IND**	**S27**	**LR06**	**SEN**	**NGR**
Strain ID	**R 99659**	**RSU 1**	**S27**	**LR2006_OPY1**	**37997**	**IB H 35**
Country of origin	British Virgin Island	Indonesia	Tanzania	La Reunion	Senegal	Nigeria
Collection Date	2013	1985	1953	2006	1983	1964
Source	Human (Serum)	Human (Serum)	Human (Serum)	Human (Serum)	Mosquito (Aedes)	Human (Serum)
Serogroup	A	A	A	A	A	A
Passage history	Vero 2	Vero 2	C6/36	Vero E6	Vero 3	Vero 1

Hypothesis**:** Based on our preliminary data we hypothesized that CHIKV strains differed in disease phenotype and response to antiviral treatment.

Specific aims:

1. Compare different CHIKV strains in replication kinetics and susceptibility to T-705 in cell culture.
    1.1. Conduct growth curve experiment to determine infectivity and replication kinetics of CHIKV strains in various cell lines.
    1.2. Compare conversion of T-705-RTP in these cell lines to identify suitable cell lines to test antiviral activity.
    1.3. Test antiviral activity of T-705 against different strains of CHIKV belonging to the 3 phylogenetic clades.
2. Determine differences between CHIKV strains in disease severity and susceptibility to T-705 *in vivo* in arthralgia and lethal mouse models.
    2.1. Characterize CHIKV strains in DBA/1J (arthralgia model) and AG129 (lethal model) mice to determine disease phenotype and severity.
    2.2. Examine antiviral activity of T-705 against CHIKV strains in both models.

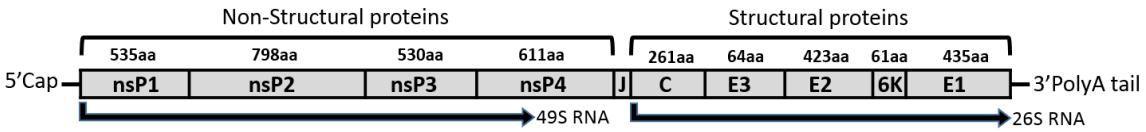
CHAPTER 2

LITERATURE REVIEW

2.1. Molecular Biology of CHIKV

As a member of the *Alphavirus* genus in the *Togaviridae* family, CHIKV contains a linear positive-sense, single-stranded RNA genome with a size of about 11.6 kb (Powers and Logue, 2007). In neutral pH, the diameter of CHIKV measures around 70nm (Lum and Ng, 2015). The virus is unstable above 58°C.

The genome of CHIKV contains a 5' methylated terminal cap and a 3' terminal poly-A tail. It encodes 4 non-structural proteins (nsP1-4) and 5 structural proteins (6K, C and E1-3). Together, the four non-structural proteins form the replication complex. nsP1 is involved in the initiation of the negative strand RNA synthesis. It also participates in the methylation and capping of positive RNA strands (Schwartz and Albert, 2010).



nsP1	Guanylyl- & Methyl- transferase	**C**	Capsid
nsP2	Helicase, Triphosphatase	**6K**	Virus assembly
nsP3	Replicase	**E3+E2+E1**	Envelope proteins
nsP4	Polymerase (RdRP)	**nsP1+nsP2+nsP3+nsP4**	Replication Complex

Fig. 4. Structural organization of the single-strand RNA genome of CHIKV. The junction region (J) is a non-coding joining gene segment. (nsP = non-structural protein; E = Envelope; aa= amino acids)

nsP2 functions as an RNA helicase, but also has triphosphatase and proteinase activities. It shuts off host cell transcription. nsP3 is a replicase unit, while nsP4 functions as an RNA-dependent RNA polymerase enzyme (Lum and Ng, 2015). The 6K protein is involved in virus assembly (Leung et al., 2011). The capsid protein forms the icosahedral nucleocapsid of CHIKV along with 80 trimeric spikes. A trimeric spike is composed of three heterodimers, each containing both E1 and E2 proteins. The E3 protein interacts with the E2 glycoprotein, and directs other structural proteins towards the ER of the cell for assembly (Snyder and Mukhopadhyay, 2012). It is also involved in the budding out of CHIKV (Uchime et al., 2013).

According to Bernard et al (2010), CHIKV enters the cell through an Eps15-dependent, clathrin-independent endocytosis. Successful infection requires a Rab5-positive endosomal compartment (Bernard et al., 2010). Although neither mosquito nor human cell receptors for CHIKV entry have been identified, C-type lectins and heparin sulfate proteoglycans are believed to be involved (Leung et al., 2011).

Once in the cellular endosome, low pH environment activates conformational changes in the E1 glycoprotein which allows fusion of the CHIKV membrane with the endosomal membrane (Lum and Ng, 2015). This results in the release of the nucleocapsid into the cytoplasm of the cell. Free viral genome is then translated by the cellular machinery into a replication complex that eventually generates a full-length negative sense RNA intermediate. The negative sense RNA intermediate is transcribed by the replication complex into

subgenomic (26S) and genomic (49S) positive sense viral RNA. The 26S subgenomic mRNA is translated into the structural proteins: C(Capsid), 6K, E1 and pE2. pE2 is further cleaved into E2 and E3 glycoproteins in the cellular Golgi-apparatus. The capsid protein auto-assembles into an icosahedral nucleocapsid incorporating the 49S viral genome in the cytoplasm. Near the plasma membrane of the cell, glycoproteins E1 and E2 hetrodimerize and are incorporated onto the nucleocapsid as trimeric spikes to form a complete virion. As the newly mature virion buds out, it adds on a double membrane from the host cell membrane (Lum and Ng, 2015). Although the exact budding mechanism is not completely understood, it is known to require the binding of the nucleocapsid with E2 proteins present on the cell membrane (Solignat et al., 2009).

2.2. Pathogenesis of CHIKV

When CHIKV first infects a human host through a mosquito bite, it invades dermal fibroblasts and macrophages where initial rounds of replication occur (Kam et al., 2009). Peripheral blood mononuclear cells (PBMCs) such as Natural killer cells, B cells and T cells are also susceptible (Sourisseau et al., 2007). From the site of infection, the virus enters the lymphatic system and consequently, the blood where it disseminates to peripheral tissues/organs such as the spleen, liver, brain, muscles and joints (Lum and Ng, 2015).

CHIKV replicates in various types of cells, including epithelial, endothelial and fibroblast cells (Sourisseau et al., 2007). The virus uses Macrophages as

reservoirs to continue to manifest symptoms chronically (Wintachai et al., 2012). Ross River virus, a closely related alphavirus within the Semliki Forest virus complex (Powers et al., 2001), was found to persist in synovial Macrophages 10 months after disease onset in chronically ill patients (Hoarau et al., 2010). It is believed that Chikungunya has a similar persistence in Macrophages localized in joints (Lum and Ng, 2015).

After 2-10 days post-infection, viremia is cleared in human patients. This is primarily due to the type I interferon (IFN) pathway of the innate immune system (Gardner et al., 2010). The pathway is initiated when pattern recognition receptors (PRRs) such as Toll-like receptors, RIG-I-like receptors (MDA5) and RIG-I, recognize pathogen-associated molecular patterns (PAMPS) including double-stranded RNA formed during replication of CHIKV in the cytoplasm of the cell (Stetson and Medzhitov, 2006). After recognition, downstream signaling cascades involving Caspase Recruitment Domain (CARD) adaptor, IFNβ adapter protein (CARDIF) and crucial interferon-regulatory factors (IRF-3 and IRF-7) collectively result in type I IFN production (Stetson and Medzhitov, 2006). Through JAK-STAT signaling, IFNs induce the expression of a multitude of genes called interferon-simulated genes (ISGs), including inflammatory cytokines and chemokines resulting in an anti-viral state within the infected host (van Boxel-Dezaire et al., 2006).

ISGs including Viperin, Mx proteins, protein kinase R (PKR) and 2', -5'-oligoadenylate synthase 3 (OAS3) have anti-viral properties (Brehin et al., 2009; Lum and Ng, 2015; Schoggins and Rice, 2011; Werneke et al., 2011). For

example, OAS3 activates RNase L, which is a latent nuclease that degrades viral RNA (Lohofener et al., 2015).

In addition to IFNs α/β/γ, other cytokines elevated in patients experiencing acute CHIKV infection include IL-2, IL-2R, IL-6, IL-7, IL-12, IL-5 IL17 and IL-17. Chemokines such as granulocyte colony-stimulating factor (GM-CSF), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP) and fibroblast growth factor (FGF) have also been shown to have been elevated during CHIKV infection (Teng et al., 2015).

The adaptive immune system plays an important role in the control and elimination of CHIKV. CD4+ T cells were found to be significantly elevated in the spleen and feet of mice infected with CHIKV (Teo et al., 2013). CHIKV specific CD4+ T cells have been found to be critical in mediating joint swelling and inflammation in mice in the absence of IFN-γ (Teo et al., 2013). The function of CD8+ T cells in the immune response to CHIKV infection still remains to be examined. In mice, regulatory T cells (Tregs) have been shown to ameliorate CHIKV induced pathology by driving activated T cells into anergy (Lee et al., 2015).

B cells participate in limiting CHIKV virus replication by producing anti-CHIKV immunoglobulins (Igs). In mice lacking B cells, viremia has been shown to persist over a year, underlining the importance of B cells in CHIKV clearance (Lum et al., 2013). The primary target of anti-CHIKV antibodies is the E2 glycoprotein. In a study conducted by Kam et al (2015), 72-100% of CHIK patients have anti-CHIKV antibodies that recognized the E2EP3 epitope on the

E2 glycoprotein (Kam et al., 2015). The E2EP3 peptide is conserved across various CHIKV strains, and has successfully been used in a pre-clinical vaccination study where it significantly reduced joint pathology in mice (Kam et al., 2012).

2.3. Clinical Symptoms of CHIK

CHIKV is one of 30 viruses that belong to the genus *Alphavirus*. Alphaviruses are divided into New World and Old World viruses. New World alphaviruses cause encephalitis while infection with Old World alphaviruses primarily results in polyarthritis and rash (Schwartz and Albert 2010). Although CHIKV is classified as an Old World virus and primarily causes the symptoms typical of this group, it has also been documented to cause meningoencephalitis in rare instances (Gerardin et al., 2008b). In these cases, CHIKV infects stromal cells lining the choroid plexus rather than directly infecting neurons of the central nervous system.

When first isolated in Tanzania, symptoms of CHIKV were considered indistinguishable from Dengue (Robinson, 1955). Currently, the disease is primarily diagnosed through viral RNA or antibody in patient samples. Symptoms of CHIK set in 2-4 days post-infection and include fever (92%), arthralgia (87%), backache (67%), headache (62%) and maculopapular rash (50%) (WHO, 2008). Infrequent symptoms include stomatitis (25%) and oral ulcers (10%) (WHO, 2008). Symptoms are observed 4-7 days-post infection (Lum and Ng, 2015). Viremia persists 5 days after symptoms begin. Although arthralgia could persist

for years, most of the symptoms disappear within a week (Kam et al., 2009). Other symptoms such as myeloradiculopathy and meningoencephalitis have also been associated with CHIKV (Chandak et al., 2009). Rarely, meningeal syndrome, vomiting and diarrhea are observed in CHIK patients.

Although CHIK has a very low mortality rate (one death occurring in 1,000 clinical cases), infected individuals have a morbidity rate between 45-97% (Rezza et al., 2007). Specifically, polyarthralgia is observed in 87-98% of patients (Thiberville et al., 2013). Chronic symptoms that persist two years after initial infection are observed in 43 – 75% of CHIK patients (Gerardin et al., 2011).

2.4. Transmission of CHIKV

Chikungunya virus is an arbovirus transmitted by female mosquitoes. The virus has two transmission cycles, sylvatic and urban. In Africa, the virus circulates primarily in the sylvatic cycle infecting wild primates, birds, rodents and squirrels. It is transmitted through Aedes mosquitoes including *A. africanus*, *A. furcifer-taylori*, and *A. dalzieli*. In the urban cycle, which is primarily prevalent in Asia, the virus is transmitted between humans through *A. aegypti and A. albopictus* (Thiboutot et al., 2010).

All age groups are susceptible to CHIKV. There is no evidence of transmission of CHIKV through breastfeeding or sexual contact. Transplacental transmission of CHIKV does not likely occur (Chen et al., 2010). In extremely rare conditions, the virus is transmitted from mother to newborn during the perinatal period through exposure during parturition. In a multidisciplinary study

conducted by Gerardin et al., vertical CHIKV transmission was investigated in 7,504 pregnant women and 7,629 neonates. Only 19 out of 749 (2.5%) neonates born to mothers with CHIKV infection became infected with CHIKV (Gerardin et al., 2008a). CHIKV infected neonates have a high rate of mortality. Symptoms in neonates include fever, pain, rash and edema in the periphery (Ramful et al., 2007).

2.5. Epidemiology and Emergence

CHIKV was first isolated from blood of febrile patients and mosquito species in October 1952 during an epidemic affecting villages in the Makonde Plateau in the Southern province of Tanzania (Robinson, 1955). For the next 50 years, CHIKV re-emerged in different parts of Africa and South-east Asia causing minor outbreaks affecting people in the hundreds and thousands. It is estimated that CHIKV may have been introduced from Africa to Asia 80 – 100 years ago (Volk et al., 2010). Most outbreaks occurred between 1960s and 1980s and a significant decrease in number of outbreaks was observed in the 1990s (Zeller et al., 2016).

However, in 2004, CHIKV re-emerged from the coast of Kenya and extensively spread to multiple islands of the Indian ocean, India and Southeast-Asia affecting millions of people. Initially, 13,500 cases were reported in Lamu island of Kenya, which comprised 70% of the island population. The epidemic spread to the most notable La Reunion island, where 260,000 people were affected (35% of the island's population).

During the La Reunion outbreak CHIKV was associated with an atypical vector, *A. albopictus*. Previously, *A. aegypti* was known to be the classical vector for CHIKV. After genomic analysis of strains obtained from outbreaks along the Indian Ocean, a single amino acid mutation in the E1 glycoprotein, Ala226Val, was identified. This mutation improved the ability of the virus to infect insect cells by removing the need for cholesterol in the target membrane for entry (Gibbons et al., 2003). As a result, it enabled the La Reunion CHIKV strain to utilize *A. albopictus* as a new host. The transmission of CHIKV by *A. albopictus* results in a more efficient transmission of CHIKV, as this mosquito species has a flight radius of 400-600 m (*A. aegypti* females generally fly 100-500 m) and can survive both rural and urban environments. It also feeds on various animal species as well as humans and its eggs are viable through the dry season (Alto and Juliano, 2001). Furthermore, the mosquito is more prevalent in the US and European countries where the typical vector (*A. aegypti*) isn't present.

From 2005 – 2006, CHIKV reemerged in India, after being inactive for 32 years, with more than 1.3 million cases reported (Zeller et al., 2016). In 2007, the virus further spread to Europe during a small outbreak in Northeast Italy, where 217 cases occurred. In 2010, a limited outbreak was also documented in southern France (Gould et al., 2010).

CHIKV has also emerged in the Americas. It was first identified in October 2013 on the island of St. Martin in the Caribbean (Cassadou et al., 2014). The virus quickly spread to other Caribbean islands, as well as central, north and south American countries owing to intense travel and presence of competent

Table 2
The yearly number of cases of CHIKV reported in the Americas (2013 – 2016).

2013	2014	2015	2016	Total (2013-2016)
111	1,147,515	726,478	114,199	1,988,303

vectors in the regions. As a result, the Pan American Health Organization reported close to 1.2 million autochthonous cases in the Americas between 2013 and 2014. The CHIKV strain that emerged in the Americas was later found to be of Asian origin and was unrelated to the better adapted virus that emerged in India and Europe in preceding years (Gay et al., 2016). According to Rodriguez et al., between 2015 – 2016, 840,677 cases of CHIKV were reported in the Americas. The yearly number of reported cases of CHIKV in the Americas between 2013 and 2016 is summarized in Table 2 (Rodriguez-Morales et al., 2016). The yearly number of reported cases of CHIKV in the Americas between 2013 and 2016, as reported by Rodiguez-Morales et al, is summarized in Table 2. Although the number of CHIKV cases may have gone down, there is still the risk that the virus might become endemic to new regions and cause future outbreaks. Currently, CHIKV is present in 80 countries (Pietromonaco and Powers, 2015).

2.6. Clades/Phylogenetic Groups

The various strains of CHIKV are categorized into three distinct phylogenetic groups or clades namely, West African, East-Central-South African

(ECSA), and Asian (Mohan et al., 2010). Phylogenetic analysis for classification was primarily based on complete ORF and partial NS4 and E1 sequences of the virus strains.

The three phylogenetic groups are estimated to have evolved from a common ancestor in Africa 500 years ago. Despite their geographical proximity, genetic lineages of ECSA and West African strains don't closely overlap (Volk et al., 2010). Therefore, it is difficult to distinguish where in Africa CHIKV first emerged. The cause of divergence between the two African clades has yet to be investigated. Evolution of CHIKV strains is mainly driven by nucleotide substitutions that occur predominantly during the urban transmission cycle of the virus in contrast to the sylvatic cycle (Volk et al., 2010).

Diversity among the clades includes differences in genome length. ECSA (11,557 – 11,789nt) strains are found to be shorter than West African (11,843 to 11,881) and Asian clade viruses (11,777 to 11,999) (Volk et al., 2010). ORFs are highly conserved among the clades, however; 5' and 3' untranslated regions (UTRs) and 26S junctions were highly variable. There is a poly A tail insertion in the 3' UTRs of ECSA strains. Although, Volk et al (2010) have found the same insertion in the genome of the Senegal, West African, strain. The clades also differ in nucleotide substitution rates. The Asian lineage has shown the highest mean rate of 4.16×10^{-4} nucleotide substitutions per site per year (subs/nt/yr), while ECSA and West African strains exhibit rates of 2.3×10^{-4} and 2.39×10^{-4} subs/nt/yr, respectively (Volk et al., 2010).

2.7. Animal Models of Infection

Animal models utilized to investigate CHIKV infection include zebrafish, mouse strains (including AG129, C57BL/6, DBA/1J, RAG -/-), Golden Syrian hamsters and primates. Each of these models replicates some aspect of disease, but none fully reproduce human disease completely.

Zebrafish have been used to study host-pathogen interactions during CHIKV infection. Although they are the furthest removed model from humans, they allow for ease of use of imaging and genetic techniques which are instrumental in monitoring viral immune response. In zebrafish larvae infected with CHIKV, upregulation of IFN-stimulated genes (ISGs) was observed. This is consistent with antiviral signaling that occurs in humans and indicated that INFs were orthologous genes that were highly conserved across vertebrates (Briolat et al., 2014).

Adult wild type C57BL/6 mice have been infected with serially passaged CHIKV to demonstrate rheumatic symptoms and virus titers in spleen, lymph nodes, liver and muscles (Gardner et al., 2010). Footpad swelling peaked between days 6 to 8 post infection. Persistent infection was detected 14 – 21 days post infection where virus was found in mononuclear cell infiltrates in subcutaneous and connective tissues of infected feet (Gardner et al., 2010). Less than 12 day old neonatal C57BL/6 mice exhibit severe disease and age dependent lethality (Couderc et al., 2008).

Infection of AG129 mice, lacking type I and II interferon receptors, results in a lethal disease phenotype. These mice are highly susceptible to infection and

die rapidly. However, they provide a highly sensitive challenge model for evaluating the efficacy of antiviral agents and vaccines (Haese et al., 2016). This model also demonstrates the importance of the host interferon response to viral control.

RAG1-/- mice lack the recombination activating gene 1 and are deficient in T and B lymphocytes (Seymour et al., 2015). Use of the RAG-/- mouse model has provided a better understanding of the role of the adaptive immune system in CHIKV infection (Hawman et al., 2013). This model can be used to study chronic CHIKV infection to understand the mechanisms that lead to prolonged arthralgia and to evaluate vaccine safety.

In the DBA/1J mouse model, clinical signs observed in humans including joint swelling, arthralgia, high virus titers and increased cytokine levels are observed (Dagley et al., 2014). This model is useful in studying pathogenesis of arthritis and to evaluate the efficacy of antiviral compounds and vaccines. Compared to the other CHIKV arthritis models, the DBA/1J model is more sensitive to CHIKV infection and consistently exhibits robust foot pad swelling across different strains (Dagley et al., 2014).

Golden Syrian hamsters are also susceptible to CHIKV infection and can serve as a useful outbred model with an intact immune system. Inflammation and lesions in skeletal muscle, tendon and fascia are observed in CHIKV-infected hamsters (Bosco-Lauth et al., 2015). Clinical signs are typically not observed in this model and viremia is cleared in 4 days. Hamsters make suitable models for

CHIKV transmission studies as they develop high viremia titers above the experimental mosquito infectivity threshold (Bosco-Lauth et al., 2015).

For pre-clinical assessment of antiviral therapies and vaccines, nonhuman-primates (NHPs) serve as excellent models as they are genetically and physiologically closest to humans. They are also natural reservoirs of CHIKV. Symptoms including joint pain, fever and rash were observed in primates infected with CHIKV (Higgs and Ziegler, 2010). Viremia peaks 2-3 days post infection and CHIKV is detected in spleen, lymph nodes, liver, muscles, and joints (Chen et al., 2010). Persistent CHIKV infection is best investigated in NHPs as the virus has been collected in from spleen, liver, and muscle tissues 44 days post infection in Cynomolgus macaques (Broeckel et al., 2015).

2.8. Antiviral Therapy and Vaccine Development

Currently there are no approved antiviral therapies or vaccines available for CHIK. Patients suffering from CHIK have predominantly been treated to partially alleviate their symptoms by the use of non-steroidal anti-inflammatory drugs (NSAIDs) and nonsalicylate analgesics.

CHIK patients of the 2006 La Reunion outbreak experiencing chronic arthritis were treated with Methotrexate (MTX), a NSAID mainly developed for rheumatoid arthritis (RA) (Hoarau et al., 2010). There appeared to be some benefit after treatment. However, the mechanism of MTX in treating viral induced arthritis is not understood. Taylor et al (2013) also provide evidence against using MTX to treat arthritis induced by alphaviruses as the drug caused early

onset of disease when used to treat Ross River virus in mice (Taylor et al., 2013). Furthermore, serious gastrointestinal complications have been observed in patients treated with MTX. Therefore, the use of MTX is currently controversial.

A nonsalicylate analgesic that has been used most for CHIKV treatment is paracetamol. It has also been used by La Reunion patients as an analgesic. Extensive use of the drug (>3g/day) resulted in severe liver disease in patients. (Parashar and Cherian, 2014).

Aside from symptomatic treatments, there are also several direct acting antiviral therapies that have shown activity against CHIKV in cell culture or in animal models. These drugs act by inhibiting viral entry, protein synthesis and/or genome replication.

Antivirals that interfere with viral entry, such as Chloroquine, Arbidol, Epigallocatechin gallate, Phenothiazines, and Flavaglines, are active against CHIKV *in vitro* (Abdelnabi et al., 2015). However, most of them have not been evaluated in animal studies. Of the few examined *in vivo*, Chloroquine has been found to be ineffective in macaques infected with CHIKV (Chopra et al., 2014).

Antiviral drugs that inhibit CHIKV protein synthesis include Small interfering RNA (siRNA) sequences and cephalotaxine alkaloids such as Harringtonine and Homoharringtonine (Abdelnabi et al., 2015). siRNAs that target nsP1 and E2 genes of CHIKV result in more than 90% inhibition of CHIKV reduction *in vitro* when administered up to 24 hours post infection. Use of multiple siRNAs have lowered viral titers by 99.6%. However, because of the instability and degradation of siRNAs, virus titer rebounds 72 hours post

treatment (Dash et al., 2008). Harringtonine and its analog homoharringtonine, interfere with host cell translation machinery to inhibit CHIKV replication (Kaur et al., 2013). The 90% cytotoxic concentration of Harringtonine is 1 µM, and it has an EC_{50} of 0.24 µM (Kaur et al., 2013). Both harringtonine and homoharringtonine are specifically more efficacious against La Reunion CHIKV strain, with the A226V mutation in the E1 glycoprotein, than CHIKV strains with wildtype E1 sequences (Abdelnabi et al., 2015). The cause for the elevated efficacy has yet to be examined.

Compounds that target CHIKV genome replication include ribavirin, mycophenolic acid, suramin, 6-azauridine and favipiravir (T-705). Ribavirin, a guanosine analogue with a broad-spectrum antiviral activity against flaviruses acts by inhibiting inosine monophosphate dehydrogenase (IMPDH), an enzyme that regulates the intracellular guanine nucleotide pool. (Leyssen et al., 2005). It is efficacious against CHIKV (EC_{50} = 341µM) *in vitro*. It has also been evaluated in mice in combination with doxycycline to significantly reduce viral titer (Rothan et al., 2015). An even more potent inhibitor of IMPDH, mycophenolic acid, is also efficacious against CHIKV *in vitro* (Abdelnabi et al., 2015). Suramin, an anti-parasitic drug that has shown efficacy against CHIKV *in vitro*, inhibits viral replication of CHIKV and other alphaviruses by interfering with RNA binding enzymes such as viral polymerases and helicases (Albulescu et al., 2015). The uridine analog, 6-azauridine, inhibits CHIKV replication by interfering with orotidine monophosphate decarboxylase enzyme and depleting intracellular

UTP-pools (Rada and Dragun, 1977). *In vitro*, 6-azauridine proved efficacious against CHIKV at very low (0.8-1.6μM) concentrations (Briolant et al., 2004).

Favipiravir (T-705) is a pyrazinecarboxamide derivative with broad spectrum activity against RNA viruses including influenza virus, Rift Valley fever virus, West Nile virus, yellow fever virus, foot-and-mouth disease virus and other flaviviruses, arenaviruses, bunyaviruses and alphaviruses (Julander et al. 2009; Abdelnabi et al. 2015; Caroline et al. 2014; Scharton et al. 2014; Gowen et al. 2013; Furuta et al. 2013). T-705 inhibits viral RNA-dependent RNA polymerase (RdRp) without interfering with host DNA and RNA synthesis (Furuta et al., 2013). Although the mechanism by which it interferes with CHIKV replication is not yet determined, suggested mechanisms include chain termination and induction of lethal mutagenesis (Furuta et al., 2013).

Different types of CHIKV vaccines currently under evaluation include live attenuated, inactivated, chimeric, recombinant subunit, DNA, adenoviral vector and virus-like particle (VLP) vaccines. In 1986, Levitt et al, developed an attenuated vaccine after serially passaging the CHIKV strain 15561 in MRC-5 cells. The vaccine successfully produced neutralizing antibody in mice and rhesus monkeys providing protection against the parent virus (Levitt et al., 1986). However, although the vaccine proved to be highly immunogenic, in a Phase II clinical study, it caused mild temporary arthralgia (Bettadapura et al., 2013). Other live attenuated vaccines have been constructed via large deletions in nsP3, 6K and E2 CHIKV genes (Hallengard et al. 2014; Piper et al. 2013; Gardner et al. 2014). The most advanced live attenuated vaccine, currently in

Phase II clinical trials, is the TSI-GSD-218 (Thailand/1556) 2ΔE2 vaccine (Smalley et al., 2016).

The development of chimeric virus vaccines involves combining genomes of multiple viruses. Chimeric CHIKV vaccines created by combining genomes of CHIKV and Venezuelan Equine Encephalitis (VEE) or Eastern Equine Encephalitis (EEE) virus have been tested in mice and found to be immunogenic (Wang et al., 2011). Alternatively, recombinant virus vaccines are made by introducing a piece of the genome of one virus into another by using genome recombinant technology. Recombinant, vesicular stomatitis and measles viruses carrying structural (C, E1 and E2) CHIKV protein genes have generated immunity that protected mice from lethal challenge (Chattopadhyay et al. 2013; Brandler et al. 2013). The most recent recombinant vaccine utilizes a modified vaccinia virus Ankara (MVA) strain. MVA is widely known to be safe as it has been clinically used as a recombinant vaccine for other diseases (Volz and Sutter, 2013). The vaccine has been shown to be immunogenic and protective in mice (Garcia-Arriaza et al., 2014).

DNA vaccine development for CHIKV involves genetically engineering cells to produce CHIKV antigens. A prominent CHIKV DNA vaccine, pMCE321, is a single plasmid envelope-based DNA vaccine that expresses C, nsP2, E2 and E1 CHIKV proteins. pMCE321 has successfully protected mice and rhesus macaques from CHIKV challenge (Mallilankaraman et al., 2011). Adenovectors modified to carry structural protein genes of CHIKV have also proven

immunogenic and have protected mice from developing viremia and arthralgia (Wang et al., 2011).

Lastly, virus-like particles (VLPs), have also been used in CHIKV vaccine development. The leading VLP vaccine currently under investigation is produced by transfecting human embryonic kidney (HEK) 293 cells with plasmid DNA coding for C-E3-E2-6K-E1 CHIKV proteins. It has been efficacious in primates against wild-type CHIKV (Akahata and Nabel, 2012) and has recently entered ~~a~~ phase I clinical trials (Kramer et al., 2013).

CHAPTER 3

IN VITRO COMPARISON OF CHIKUNGUNYA STRAINS IN REPLICATION KINETICS AND RESPONSE TO T-705 TREATMENT

3.1. Introduction

Chikungunya (CHIK) is a disease mainly characterized by acute febrile arthralgia. It is caused by Chikungunya virus (CHIKV), which is an alphavirus belonging to the Togaviridae family. CHIKV was first discovered in 1952 in Tanzania. The virus reemerged in 2004, causing outbreaks in Africa, Asia and territories on the Indian Ocean, resulting in millions of disease cases in these regions. Virus outbreaks also occurred in Europe in 2007. In 2013, the virus was introduced to the Caribbean, further spreading to countries in North and South America. According to the Pan American Health Organization (PAHO), close to 1.2 million people were affected with CHIKV in the Americas in its first year of expansion alone.

Currently there is no FDA-approved antiviral treatment or vaccine available for the treatment or prevention of CHIKV. However, T-705 ($C_5H_4FN_3O_2$ – 6-fluoro-3-hydroxy-2-pyrazinecarboxamide), a purine analog, has been identified as a potential candidate for the treatment of CHIKV (Delang et al., 2014).

T-705, also known by the trade name Favipiravir, is an antiviral drug that is effective against a diverse group of RNA viruses *in vitro* and *in vivo*. It is active against influenza viruses, as well as arenaviruses, bunyaviruses, flaviviruses, alphaviruses, picornaviruses and noroviruses (Furuta et al., 2013). It is approved

for the treatment of influenza in Japan, and is currently going through phase III clinical trials in the US. T-705 has also been shown to be efficacious against Chikungunya virus (Ahola et al., 2015).

The mechanisms of action of T-705 include inhibition of viral replication by interfering with viral RNA dependent RNA polymerase and introduction of lethal mutagenesis (Baranovich et al., 2014; Baranovich et al., 2013; Furuta et al., 2005).

T-705 acts as a pseudo-purine. The structure of T-705 mimics that of guanine and adenine nucleotides (Fig. 4). Furuta et al. and Mendenhall et al. have shown that addition of excess purine nucleotides significantly reduces the efficacy of T-705 against influenza virus and some arenaviruses (Arias et al., 2014; Furuta et al., 2005; Mendenhall et al., 2011)

The antiviral activity of T-705 is dependent on its enzymatic intracellular conversion to the ribofuranosyl-triphosphate species (T-705-RTP). According to a pathway proposed by Furuta et al (2005), T-705 is converted to T-705 ribofuranosyl monophosphate (T-705-RMP) by the host enzyme Phosphoribosyl-transferase. Nucleotide kinases further phosphorylate T-705-RMP into ribofuranosyl-diphosphate (T-705-RDP) and ribofuranosyl-triphosphate (T-705-RTP) derivatives (Fig. 5) (Furuta et al., 2005). T-705-RTP interferes with RdRp by being incorporated in nascent viral RNA and partially preventing the extension of the RNA strand (Furuta et al., 2013). Enzymatic kinetic analysis done by Sandawe et al. has shown that T-705-RTP inhibited the incorporation of ATP and GTP competitively (Sangawa et al., 2013).

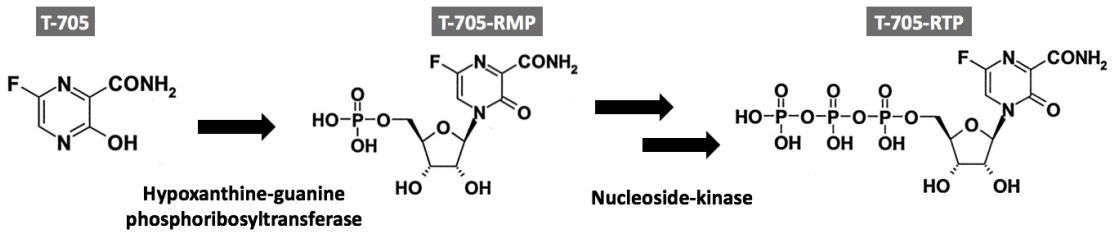


Fig. 5. The intracellular metabolism of T-705 into its active ribosylated, triphosphorylated form (T-705-RTP)

It is unknown how T-705-RTP terminates chain elongation since it contains a natural 3' OH group. It is possible that the unnatural base may contribute to chain termination by affecting the hydrogen bond of viral RNA bases (Sangawa et al., 2013).

Another proposed antiviral mechanism of T-705 is lethal mutagenesis. T-705 induces a high level of mutation that renders viral RNA non-viable. The compound has been shown to elevate mutation frequency, increase the number of G → A and C →T transversion mutations and result in shift of nucleotide profiles in influenza A H1N1 viruses (Arias et al., 2014; Baranovich et al., 2013).

Time of addition studies have shown that T-705 has no antiviral effect when added to virus adsorption and release stages. Early to intermediate stages of viral replication are affected by the compound (Furuta et al., 2005).

Since RdRps are exclusive to RNA viruses and absent in human cells, T-705 presents very minimal toxicity to the host. The effect of T-705-RTP against human RNA and DNA polymerases as well as viral RdRp has been investigated. T-705-RTP inhibited the RdRp of influenza virus at a minimal 50% effective

concentration (EC_{50}) of 0.341µM. However, the EC_{50} of T-705 against human RNA polymerase II was 905µM and 50% inhibition against human DNA polymerases (α, β or γ) was not observed as high as 1000µM (Kiso et al., 2010). The specificity of T-705 in targeting viral polymerase without affecting host polymerases makes it a very ideal antiviral compound.

In our lab, T-705 has shown variable efficacy against S27 and LR06 CHIKV strains *in vivo* (unpublished data). This presented the question whether T-705 displayed differential efficacy against different strains of CHIKV *in vitro*. To address this, four additional CHIKV strains, BVI, IND, SEN and NGR were obtained, and tested for response to T-705 alongside S27 and LR06 strains.

To conduct T-705 antiviral testing, a study was first conducted to identify suitable cell lines for *in vitro* assays. Previously in our laboratory, T-705 was found to have limited efficacy against influenza in MA104 cells (unpublished data). Variable EC_{50} values of T-705 against different arenaviruses and bunyaviruses have also been observed (Gowen et al., 2007). The efficacy of T-705 is largely dependent on cellular enzymes to metabolize it into its active triphosphate form (Fig. 4), therefore the level of T-705 phosphorylation in various cell lines was investigated.

Next, the replication kinetics of the S27 strain of CHIKV in different cell lines was examined. After suitable cell lines for CHIKV replication were identified, the replication kinetics of different CHIKV strains (BVI, IND, SEN, NGR, S27 and LR06) were evaluated. Finally, comparative experiments were conducted to test the response of the six CHIKV strains to T-705 treatment.

3.2. Materials and Methods

3.2.1. Virus

The parental East African CHIKV isolate, S27 (VR-64), was obtained from the American Type Culture Collection (ATCC) in Manassas, VA. La Reunion Island (LR06 – LR2006-OPYI), Nigeria (NGR – IB H 35), Senegal (SEN – 37997), British Virgin Island (BVI, R 99659), and Indonesia (IND – RSU 1) strains were obtained from Robert Tesh at the University of Texas Medical Branch (UTMB). The S27, LR06, NGR, SEN, BVI and IND stock virus vials had virus titers of $10^{8.5}$ $\log_{10}$, 10^{9} $\log_{10}$, $10^{9.67}$ $\log_{10}$, 10^{9} $\log_{10}$, 10^{8} $\log_{10}$, and 10^{8} $\log_{10}$ 50% cell culture infectious dose ($CCID_{50}$)/1mL respectively. Virus stocks were prepared by harvesting the supernatant from C6/36 cells that were grown in RPMI at 28°C in 5% CO_2 with 5% fetal bovine serum (FBS). Virus was diluted in Minimal Essential Media (MEM) containing 2% fetal bovine serum (FBS) and 50mg/L gentamicin for *in vitro* testing.

3.2.2. Compound

T-705 was obtained from Toyama Chemical Company, Ltd. (Toyama, Japan). The powder was dissolved in MEM containing 50µg/mL gentamicin immediately prior to assays.

3.2.3. Cells

The cells utilized in this study included RD (Human – muscle – rhabdomyosarcoma), Vero-76 (Primate – kidney epithelial), HEK 293 (Human –

kidney epithelial), HEL 299 (Human – embryonic lung) and MA104 (Primate – kidney epithelial) cells. They were obtained from ATCC and cultured in Eagle's medium with 10% FBS at 37°C and 5% CO_2.

3.2.4. T-705 Phosphorylation and HPLC Analysis

Cell lines including Vero-76, HEK 293, HEL 299, RD and MA104 were seeded in 25cm^2 T-25 flasks in MEM containing 5% FBS. After 24 hours, confluent flasks were treated with 100µM, 320µM and 1,000µM dilutions of T-705 in MEM containing 2% FBS and 50mg/L gentamicin. One flask was used for each concentration of T-705 for each cell line. Control flasks were treated with the same medium without T-705. The cell concentrations of the flasks ranged between 4 x 10^6 – 6 x 10^6 of cells/mL at confluency. After a 24 hour incubation, medium was aspirated and 540µL of 3.5% perchloric acid was added. After a 5 min refrigeration at 4°C, 270µL of 1N KOH/1 M imidazole solution was added to neutralize lysate solution. After another 5 minute incubation at room temperature, lysate was collected and stored in -80°C until HPLC analysis.

For HPLC analysis, a Waters 510 HPLC (Waters Corp., Milford, MA, USA) fitted with a Kinetex 2.6µm (50X4.6mm) reverse phase C18 column (Phenomenex Inc., Torrance, CA, USA) was used for the chromatographic separation of T-705-RTP. A linear gradient of 0 to 5mM magnesium sulfate in 30mM TEA phosphate buffer (6.5 pH) was run for 30 minutes at 0.6mL/min. A volume of 200µL of each sample was injected for analysis. Between sample runs, the column was re-equilibrated with a 10 min wash with 60% acetonitrile. T-

705-RTP eluted at ~18 min and was detected at a wave length of 360nm using a Waters LC Spectrophotometer. The UV detector was set for 0.02 absorbance units. A 10 µM T-705 standard was used to back calculate pmols of T-705-RTP observed in samples. The number of cells in the untreated flasks containing each cell line was determined using Countess II FL Automated Cell Counter (ThermoFisher Scientific, Logan, UT) and used to calculate the concentration of T-705-RTP/0^6 cells. Conversion of T-705 standard concentration to T-705-RTP concentration was done by using the T-705:T-705-RTP ratio of 1:3.8 previously specified by Smee et al. (2009).

3.2.5. Virus Replication Kinetics

Three replication kinetics experiments were conducted. The first was conducted to measure the ability of CHIKV, specifically S27, to replicate in V-76, HEK 293, HEL 299, RD and MA104 cell lines. The second was conducted to quantify reduction of virus replication by T-705 in Vero-76 and MA104 cells. The third was carried out to determine the abilities of the six CHIKV virus strains to replicate in RD cells.

Both replication kinetics experiments were conducted using cells seeded at a concentration of 4 x 10^4 cells/well on 96-well plates. Plates were infected with the same 0.01 multiplicity of infection (MOI) or virion/cell. For the second experiment, cells were treated with half log dilutions of T-705 starting at 1000µM. Three separate experiments were conducted. In both experiments, 200µL of supernatant from three wells were pooled for virus titration at 0, 6, 12, 18, 24, 30,

26, 42, 48, 54, 60, 66, 72 hours post infection. Virus titers were assayed in 96-well microplates infected with approximately 50 $CCID_{50}$ per 0.1 ml and quantified by the end-point dilution method (Reed and Muench, 1938).

3.2.6. T-705 *In Vitro* Antiviral Testing

Antiviral activity of T-705 was evaluated in the strains by cytopathic effect (CPE) inhibition assay. 96-well plates, seeded at 4×10^4 cells/well, were infected with each CHIKV strain. T-705 was diluted in half logs starting at 1,000µM in MEM containing 2% FBS and gentamycin. Uninfected cells were also treated to test cytotoxicity. Plates were incubated at 37°C and 5% CO_2 until maximal viral CPE was observed on day 3 and scored visually for CPE and toxicity. Supernatants of wells containing virus were collected for virus yield reduction assay.

Inhibition of virus replication was determined by microscopic evaluation of infected cytopathic cells and quantified by neutral red (NR) uptake and virus yield reduction assay. The 50% effective concentration (EC_{50}) and the concentration where 50% reduction in cell viability was observed (CC_{50}) were calculated using regression analysis. The selectivity index (SI) was obtained by dividing CC_{50} by EC_{50}. Virus yield reduction results were determined by the concentration of drug that reduced the virus yield by 1 $\log_{10}$ unit (EC_{90}) based on regression analysis.

First the efficacy of T-705 against the S27 strain was evaluated in multiple cell lines. Next using a suitable cell line in which T-705 is active, antiviral efficacy was evaluated against 6 (BVI, IND, S27, LR06, SEN and NGR) CHIKV strains.

3.2.7. Statistical Analysis

Statistical analyses were done by two-way ANOVA using a Bonferroni group comparison (Prism 7, GraphPad Software, Inc.).

3.3. Results

3.3.1. S27 CHIKV Strain Does Not Replicate Uniformly in Different Cell Lines

As can be seen in Figure 6, S27 did not replicate well in HEL 299 cells although it displayed robust replication curves in V-76, HEK 293, RD and MA104 cells (Fig. 6). Virus titers obtained in Vero-76 cells between 18 – 72 hpi were significantly (****P>0.0001 and ***P>0.001) higher than titers obtained in HEL 293 cells. The virus reached peak titers more rapidly in V-76 cells at 12 hours post infection as compared with the other cell lines tested. Sufficient increase in virus titers was observed in V-76, HEK 293, RD and MA104 cell lines, indicating that these cell lines support suitable replication of CHIKV.

3.3.2. Replication of BVI, IND, S27, LR06, SEN and NGR CHIKV Strains in Cell Culture

The NGR strain, closely followed by LR06 and SEN, produced high titers throughout the incubation period compared to other strains (Fig. 7). The BVI and IND strains replicated slower and produced significantly less virus between 12 – 42 HPI. The most significant (****P>0.0001) difference was observed between the NGR and BVI CHIKV strains. Thus, these viruses were prime candidates for comparison in *in vivo* experiments.

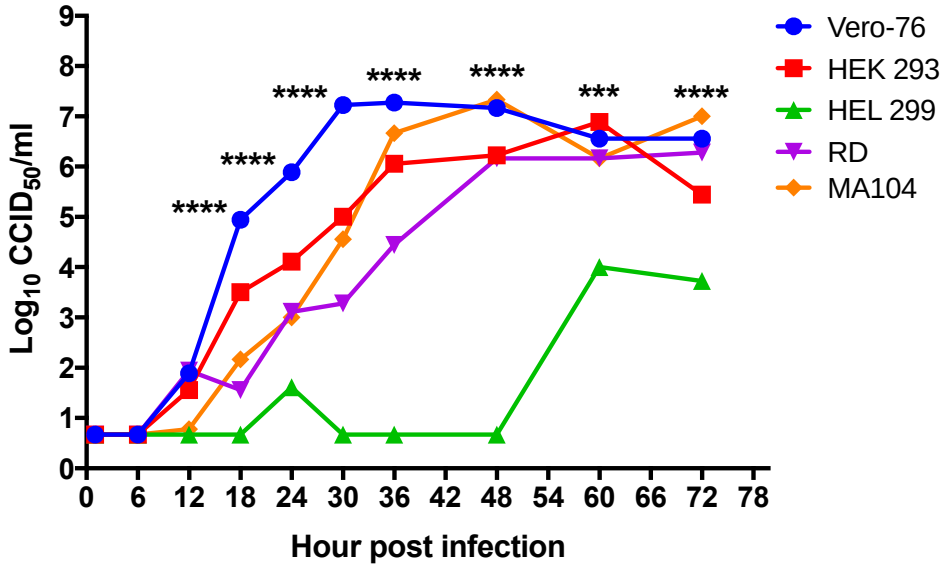


Fig. 6. *In vitro* replication curves of S27 CHIKV strain in Vero-76, HEK 293, HEL 299, RD and MA104 cell lines. (****P<0.0001, ***P<0.001; Virus titers of Vero-76 cells compared to HEL 299 cells)

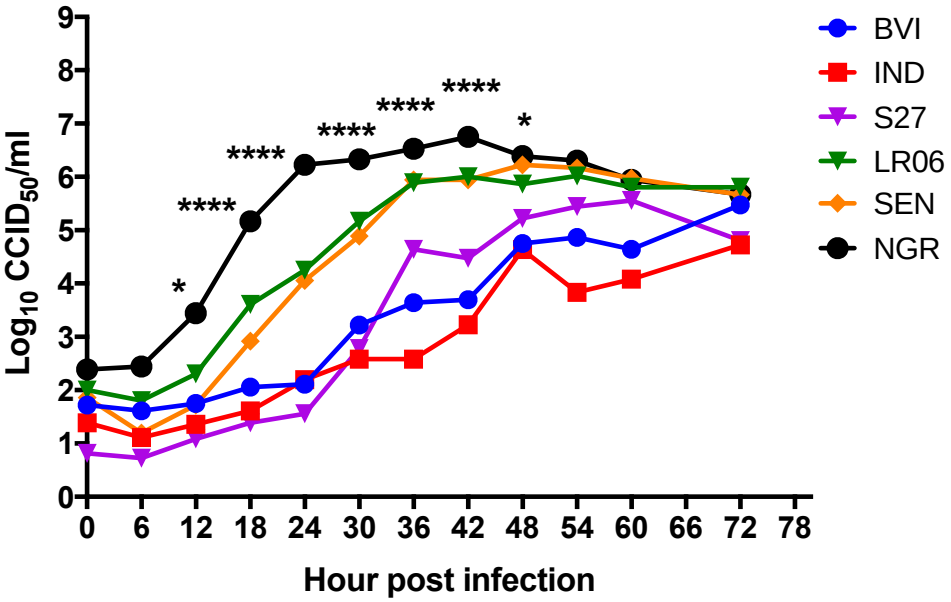


Fig. 7. Comparison of growth curves of BVI, IND, S27, LR06, SEN and NGR CHIKV strains in RD cells. (****P<0.0001, *P<0.05, Virus titers of NGR CHIKV strain compared to the BVI CHIKV strain)

3.3.3. Efficacy of T-705 Against CHIKV in Various Cell Lines

T-705 was effective (SI value >10) against CHIKV (S27) in Vero-76, HEK 293, HEL 299 and RD cells but was not efficacious in MA104 cells (Table 4). Furthermore, virus reduction was measured in Vero-76 and MA104 cells treated with half-log dilutions of T-705 up to 1,000µM. Concentrations between 32µg/ml and 1,000µg/ml reduced virus titers in Vero-76 but not in Ma104 cells. At 1,000µM of T-705, cell viability is partially compromised, thus, the reduction observed at that concentration in MA104 cells is not completely due to compound efficacy. This raised the question whether the compound was effectively metabolized into its active form in MA104 cells.

Table 3
Antiviral activity of T-705 against the S27 CHIKV strain in Vero-76, HEK 293, HEL 299, RD and MA104 cells.

Cell Line	CC_{50}[a]	EC_{50}[a]	SI_{50}	EC_{90}[a]	SI_{90}
Vero-76	260	7.5	34.9	6.1	43.1
HEK 293	150	6.8	22.1	4.16	36.1
HEL 299	280	6.7	41.8	16.0	17.5
RD	136	6.3	21.6	3.6	37.7
MA104	600	>600	0	>600	0

[a]CC_{90}, EC_{50}, EC_{90} values are in µg/ml

3.3.4. Phosphorylation of T-705 in Various Cell Lines

Using HPLC analysis, we demonstrated a dose dependent conversion of T-705 to its active ribosylated-triphosphorylated form in HEK 293, HEL 299 and Vero-76 cells (Fig. 8). However, T-705-RTP could not be detected in MA104 cells treated with 1000µM, 320µM and 100µM of T-705. These data, together with the antiviral inefficacy of T-705 in MA104 cells (Table 3), suggest that the lack of activity in these cells is due to their inability to convert T-705 in to its active phosphorylated form (T-705-RTP).

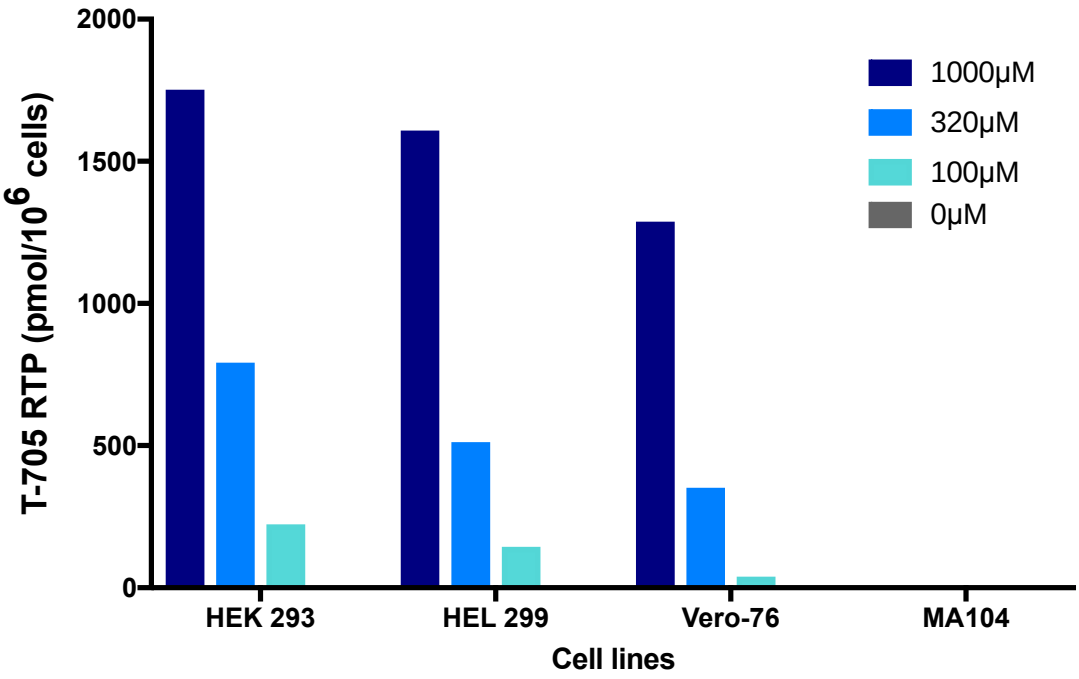


Fig. 8. Amount of T-705-RTP pmol/10^6 cells of HEK, HEL, V-76 and MA104 after being incubated for 24 Hrs with 1000µM, 320µM, 100µM and 0µM of T-705.

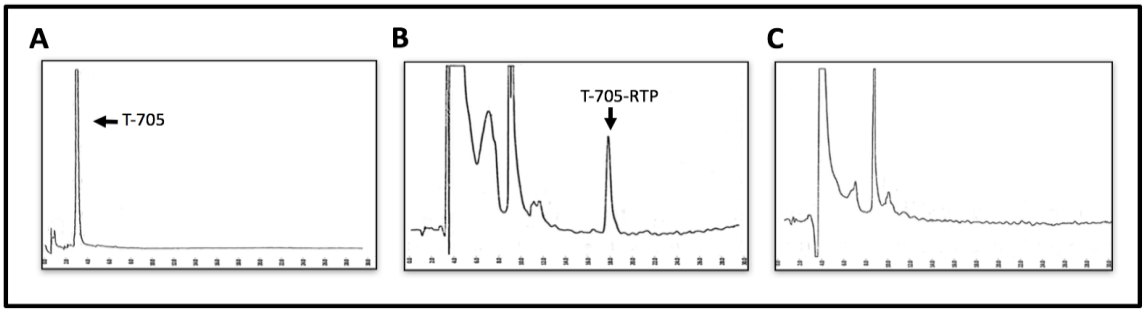


Fig. 9. HPLC Chromatogram of (A) 10μM of T-705 standard and lysates of (B) HEK 293 and (C) MA104 cells treated with 320 μM of T-705. The active ribosylated-triphosphorylated form of T-705, T-705-RTP was not detected in Ma104 cells treated with T-705.

Table 4
Antiviral activity of T-705 against BVI, IND, S27, LR06, SEN and NGR strains of CHIKV.

Clade	Virus Strain	T-705 (μM)	
		EC_{90} ± SD[a]	SI
Asian	British Virgin Islands (BVI)	14.0 ± 7.8	85.7
	Indonesia (IND)	16.0 ± 15.9	75.0
East/Central/ South African	Tanzania (S27)	24.5 ± 6.8	48.9
	La Reunion (LR06)	27.0 ± 11.4	44.4
West African	Senegal (SEN)	19.0 ± 14.8	63.1
	Nigeria (NGR)	33.0 ± 10.9	36.3

[a] Data are means and standard deviations from three separate experiments in RD cells

3.3.5. T-705 is Efficacious Against BVI, IND, SEN, NGR, S27 and LR06 CHIKV Strains *In Vitro*

T-705 was active against all strains (SI value >10) and significant difference in response to T-705 was not observed (Table 4). Difference in EC_{90} among the strains was not statistically significant. However, we do observe a slightly higher EC_{90} average for the NGR strain compared to the Asian strains.

3.4. Discussion

To identify a suitable cell line for use in measuring the efficacy of T-705 against CHIKV strains, replication kinetics of various cell lines were determined. From these experiments, we identified that replication of CHIKV in HEL 299 cells is less robust than Vero-76, HEK 293, RD and Ma104 cells. Efficient replication of CHIKV is observed in various types of cells, including epithelial, endothelial and fibroblast cells (Sourisseau et al., 2007). HEL299 cells are lung fibroblast cells, therefore, it was unexpected to observe less robust CHIKV replication in these cells.

Although CHIKV successfully replicated in MA104 cells, T-705 was found to be non-efficacious in the cell line. We hypothesized that this was due to the inability of MA104 cells to phosphorylate T-705, thus converting the compound into its active form. We obtained preliminary data to support this hypothesis. HPLC analysis revealed that MA104 cells treated with various concentrations of T-705 did not produce T-705-RTP. Based on preliminary phosphorylation results, as well as on CHIKV replication studies, Vero-76 and RD cells were selected for use in antiviral studies in cell culture. Additionally, RD cells are a human muscle cell line, thus representing a relevant cell type for investigating CHIKV.

There was some indication of a differential antiviral response among the various clades of CHIKV in cell culture studies, although these differences were not significant. T-705 appeared to be only slightly more efficacious against Asian strains (BVI and IND) compared to ECSA (S27 and LR06) and West African (SEN and NGR) clades. Thus, *in vivo* studies in mouse models were designed to

further evaluate differences in antiviral response between different strains or clades of CHIKV.

CHAPTER 4

ANTIVIRAL EFFECT OF T-705 AGAINST DIFFERENT STRAINS OF CHIKV IN AN ARTHRALGIA MOUSE MODEL

4.1. Introduction

Chikungunya virus (CHIKV), an alphavirus transmitted primarily by *Aedes* mosquito species. Acute CHIKV infection with in 3 - 7 days post-infection is characterized by fever, rash, joint swelling and debilitating polyarthritis. Within the last decade the virus has affected millions of people around the globe (Handler et al., 2017). Strains of CHIKV have currently been classified into three phylogenetic groups/clades: West African, Asian and East/Central/South African (ECSA).

Currently, there are no approved antiviral drugs or vaccines available for CHIKV. Meanwhile, CHIKV continues to spread to different parts of the world causing high morbidity in patients, and is expanding its geographical reach. As with other RNA viruses, various adaptive mutations have been reported, which have been important in increasing the range of the virus. Therefore, identifying compounds with broad-spectrum activity against different CHIKV strains is important.

T-705, an RNA-base analog with a broad activity against RNA viruses, has been identified as a promising antiviral candidate for CHIKV *in vitro* and *in vivo* (Ahola et al., 2015). However, the drug has been associated with different levels of activity against two ECSA clade strains, S27 and LR06, *in vivo*. T-705 was active against the S27 strain, but not the LR06 strain, in a lethal mouse

model (unpublished data). To further characterize the broad-spectrum activity of T-705 against various strains of CHIKV, the following strains were obtained and characterized in a mouse model of arthralgia: BVI and IND strains from the Asian clade, and SEN and NGR strains of the West African clade.

To understand the phenotypic consequences of CHIKV strains, such as rheumatic disease, and to evaluate differential response to antiviral treatment *in vivo*, the arthralgia DBA/1J mouse model was utilized.

The DBA/1J mouse models various aspects of natural CHIKV infection, including arthralgia and has been used for evaluating the efficacy of antiviral candidates against CHIKV (Dagley and Julander, 2013). Infection of this mouse strain with S27 (Dagley and Julander, 2013) or LR06 CHIKV results in significant footpad swelling, similar to other models that have been described (Gerardin et al., 2008b). Clinical symptoms, such as foot pad swelling, viremia and cytokine levels can be reduced after treatment with an immune modulator (Dagley et al., 2014). Herein, we describe the use of this mouse strain to identify phenotypic differences between various clades of CHIKV and determine the effect of T-705 against selected strains at high and low treatment doses.

4.2. Materials and Methods

4.2.1. Animals

Seven to eight-week old female DBA/1J mice from Jackson Laboratories were used after a quarantine period of 48 h. Animals were randomly assigned to cages and individually marked with ear tags.

4.2.2. Virus

Chikungunya virus isolates from British Virgin Islands (BVI, R99659 TVP 20811), Indonesia (IND, RSUI TVP-1336), Senegal (SEN, 37997 TVP-21089) and Nigeria (NGR, IbH 35 TVP-1337) were obtained from Bob Tesh (WRCEVA). The virus was passaged twice in C6/36 cells and virus stocks used in this study had titers of 10^8, 10^9, 10^8 and $10^{9.67}$ 50% cell culture infectious doses ($CCID_{50}$)/ml, respectively.

4.2.3. Test Compound

T-705 was obtained from Toyama Chemical Company, Ltd. (Toyama, Japan). The compound was dissolved in 2.9% sodium bicarbonate buffer (Life Technologies, Carlsbad, CA). The T-705 dilution was filtered (0.2-micron filter) for sterility and stored in amber glass injection bottles fitted with a metal cap. Bottles were kept at 4ºC for the duration of the treatment schedule. The stability of the compound in solution was confirmed using HPLC analysis for up to 10 days.

4.2.4. Experiment Design

4.2.4.1. Characterization of BVI, IND, NGR, SEN and LR06 CHIKV Strains in DBA/1J Mice

Female DBA/1J mice were randomly assigned to groups of five animals. The mice were weighed daily for six days starting from the day of infection (Day 0) and percent change in weight was calculated.

Two characterization experiments were conducted. In the first experiment, mice were infected with two challenge doses, $10^{3.5}$ or $10^{6.5}$ $CCID_{50}$/0.1ml, of BVI, IND and NGR CHIKV strains. Two different doses of each strain were used to determine an effective infective dose that would result in foot pad swelling in mice. In the second, the same study design was used and mice were infected with the two challenge doses of BVI, NGR, SEN and LR06. A second characterization experiment was conducted since unexpected results from the first characterization experiment needed to be reaffirmed. Furthermore, it later became apparent that including SEN and LR06 strains was necessary for comparison. The viruses were prepared in minimal essential media (MEM).

Animals were anesthetized with isoflurane prior to subcutaneous (s.c.) injection in the footpad and hock of the right leg with a total volume of 0.1 ml of the diluted virus (0.05 ml each site). Animals were weighed and observed for morbidity and mortality daily for the experimental duration of 9 days. Serum was obtained via cheek bleeds on day 2 post infection to assess viremia. Footpad measurements were obtained daily for 9 days using a caliper. Percent increase in footpad swelling was assessed by comparing the right hind footpad, at the site of virus challenge, to the contralateral left hind footpad (Dagley and Julander, 2013).

4.2.4.2. Evaluation of the Efficacy of T-705 Against Robust CHIKV Strains in DBA/1J

Mice were randomly assigned to groups of 10 animals. Two challenge doses, $10^{3.5}$ $CCID_{50}$/0.1ml, of LR06, SEN and NGR strains were prepared in minimal essential media (MEM). Animals were anesthetized with isoflurane prior to subcutaneous (s.c.) injection in the footpad and hock of the right leg with a total volume of 0.1 ml of the diluted virus (0.05 ml each site).

T-705 was dissolved in 2.9% sodium bicarbonate buffer (Life Technologies, Carlsbad, CA) at a concentration of 38 mg/ml to deliver compound at a treatment dose of 400 mg/kg/d. In a separate experiment, the compound was dissolved in bicarbonate buffer at a concentration of 9.5 mg/ml in order to treat animals with a 100 mg/kg/d treatment dose. The route of treatment administration was via intraperitoneal (i.p.) injection. Mice were treated twice daily for 8 days beginning 4h prior to virus challenge. A group of infected animals was treated with a sodium bicarbonate vehicle along the same schedule as T-705 and served as a placebo treatment control. Animals were weighed and observed for morbidity and mortality daily for the experimental duration of 9 days. Footpads were measured using a caliper every day for 9 days to check for foot pad swelling. Percent increase in footpad swelling was measured as described above. Serum was obtained through cheek bleeds on day 1 and 2 post infection for titers. Five out of ten animals from both T-705 and vehicle groups were necropsied on 6 dpi to obtain samples for virus and cytokine titration.

4.2.5. Tissue Virus Titration

Right hind leg samples were weighed and homogenized in 1ml of MEM containing 0.05 mg/ml gentamicin. The homogenates were centrifuged for 10 min at 3,000 RPM. Serial log dilutions of the samples were plated in quadruplicate on 96-well plates seeded with Vero 76 cells. After an incubation period of 3 days at 37ºC and 5% CO_2, the plates were examined for cytopathic effect (CPE) under the microscope. Virus titers were determined by end point titration as described previously (Reed and Muench, 1938).

4.2.6. Cytokine Analysis

Cytokines were quantified using the Q-Plex™ Mouse Cytokine – Screen (16-Plex) kit (Quansys Biosciences in Logan, UT). The quantitative ELISA-based chemiluminescent assay allows the concurrent measurement of the levels of 16 cytokines including, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, MCP-1, IFNγ, TNFα, MIP-1α, GM-CSF and RANTES. Cytokine levels in footpad homogenates were quantified per manufacturer's instructions. Briefly, samples were diluted 1:5 and 1:25 in assay diluent, added to plates and read using a Quansys Q-View TM Imager with Q-View™ software. Concentrations obtained from the two dilutions were averaged during data analysis.

4.2.7. Statistical Analysis

Footpad swelling data were analyzed using two-way ANOVA. Statistical analyses of weight, viremia and tissue virus titer were performed using one-way

ANOVA using a Bonferroni group comparison (Prism 7, GraphPad Software, Inc.).

4.2.8. Ethics Statement

This study was conducted in accordance with the approval of the Institutional Animal Care and Use Committee of Utah State University dated February 8, 2017 (Protocol #2713). The work was done in the AAALAC-accredited Laboratory Animal Research Center of Utah State University.

4.3. Results

4.3.1. Characterization of Various CHIKV Strains in DBA/1J Mice

In the first characterization experiment, DBA/1J mice were infected with $10^{3.5}$ $CCID_{50}$ and $10^{6.5}$ $CCID_{50}$ of BVI, IND and NGR CHIKV strains. They were weighed daily and measured for footpad swelling for 9 days. Serum was also collected on the second day post infection for virus titer. No significant weight changes were observed in the mice (Fig. 10).

Mice infected with a lower ($10^{3.5}$ $CCID_{50}$) dose of the BVI strain had detectable virus titer 2 days-post infection, while those infected with the higher virus dose ($10^{6.5}$ $CCID_{50}$) did not (Fig. 11). This was not completely unexpected, as previous studies demonstrated earlier peaks in viremia in mice infected with higher virus challenge (unpublished data). Mice that were infected with either of the infective doses of the IND strain also did not have detectable viremia (Fig. 11). Thus, in a subsequent study, viremia was evaluated on day 1. Viremia was

detectable in mice infected with $10^{3.5}$ and $10^{6.5}$ $CCID_{50}$ challenge doses of NGR. Despite a 1,000-fold difference in virus challenge dose, both NGR groups resulted in serum virus titers that were not significantly different, demonstrating a phenotypic difference between this strain with those of the Asian clade.

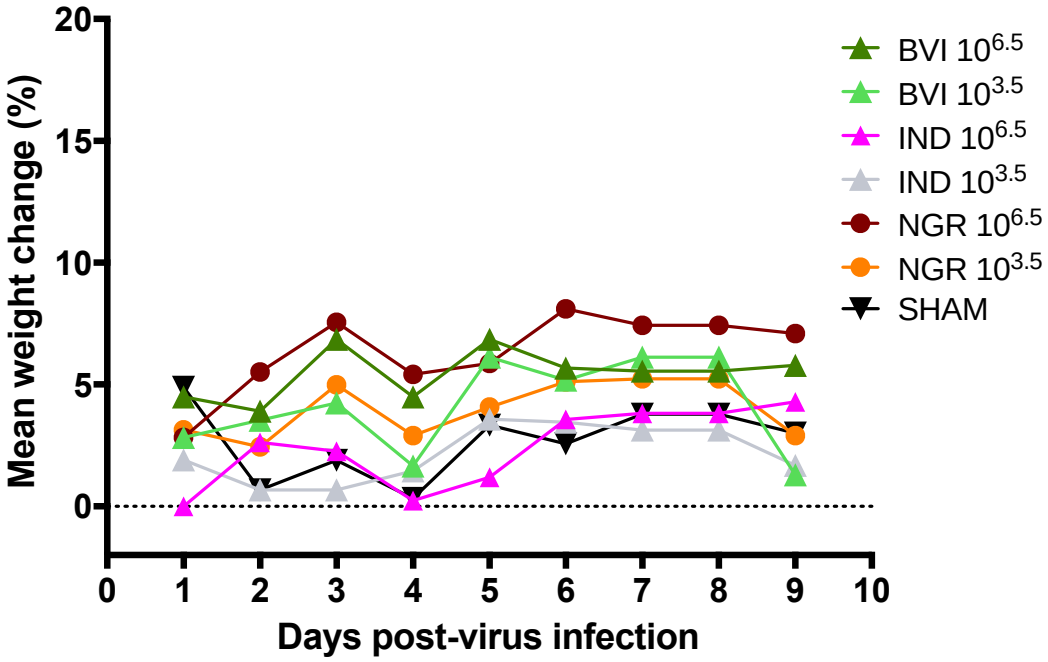


Fig. 10. Mean percent weight change of DBA/1J mice infected with two doses ($10^{3.5}$ $CCID_{50}$ & $10^{6.5}$ $CCID_{50}$) of BVI, IND and NGR CHIKV strains.

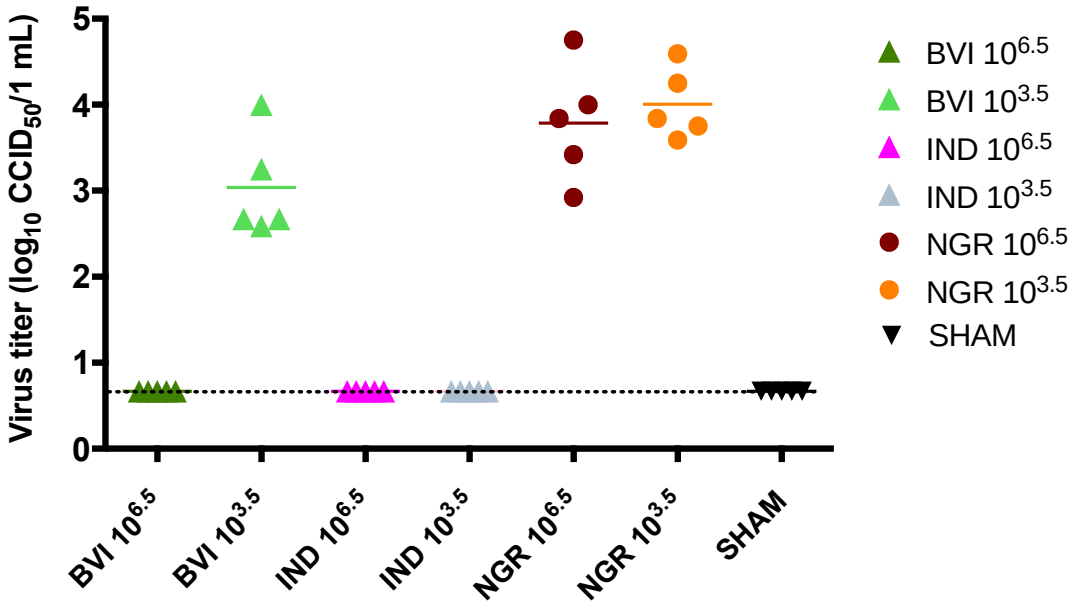


Fig. 11. Virus titer of serum collected 2 dpi from mice infected with two doses ($10^{3.5}$ $CCID_{50}$ & $10^{6.5}$ $CCID_{50}$) of BVI, IND and NGR.

Both Asian strains, BVI and IND, did not show significant footpad swelling in DBA/1J mice (Fig. 12, A and B). This was not expected since the DBA/1J mouse model has been identified as a useful model for replicating arthralgia observed during natural infection after inoculation with various CHIKV strains (Dagley et al., 2014).

The West African strain, NGR, caused significant footpad swelling on day 6 at the site of injection (Fig. 12C). Average swelling of the inoculated foot was 40% higher than the unchallenged contralateral foot (Fig. 12C, Table 5). There was no significant difference in footpad swelling among the groups of mice infected with two ($10^{3.5}$ and $10^{6.5}$ $CCID_{50}$) NGR challenge doses; indicating that swelling may not be dose dependent in this strain. Contrarily, dose-dependent

swelling has previously been observed in mice infected with the LR06 CHIKV strain, as shown in preliminary data (Fig. 1).

Although insignificant in this experiment, some footpad swelling was also observed on day 3 in mice infected with both challenge doses of NGR strain. This bimodal joint swelling profile has been previously observed in both DBA/1J (Fig. 1) and C57BL/6 mice infected with CHIKV (Gardner et al., 2010).

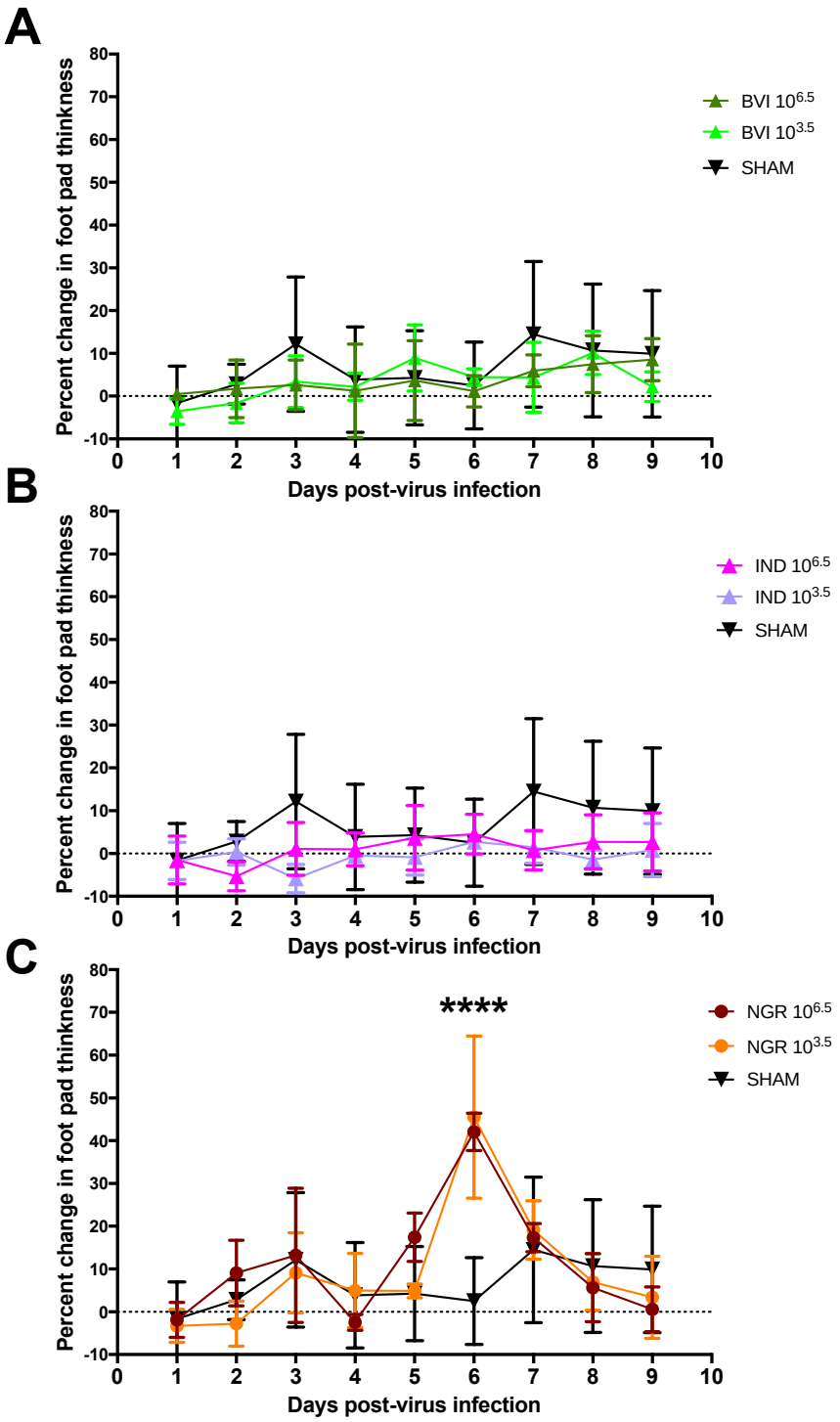


Fig. 12. Percent change in footpad swelling of DBA/1J mice infected with two doses ($10^{6.5}$ $CCID_{50}$ and $10^{3.5}$ $CCID_{50}$) of (A) BVI, (B) SEN and (C) NGR CHIKV strains.

Table 5
Serum virus titers and percent increase in footpad swelling in DBA/1J mice infected with two doses of BVI, IND and NGR.

Strain	Virus Dose	Log_{10} serum virus titer[a]	Footpad swelling (%) (n) [b]
British Virgin Islands (BVI)	$10^{6.5}$ $CCID_{50}$	<0.7 ± 0.0	1.2 ± 3.7
	$10^{3.5}$ $CCID_{50}$	3.0 ± 0.6	4.5 ± 1.9
Indonesia (IND)	$10^{6.7}$ $CCID_{50}$	<0.7 ± 0.0	4.5 ± 4.6
	$10^{3.7}$ $CCID_{50}$	<0.7 ± 0.0	2.7 ± 0.6
Nigeria (NGR)	$10^{6.5}$ $CCID_{50}$	3.7 ± 0.7	42.0 ± 4.4
	$10^{3.5}$ $CCID_{50}$	3.7 ± 0.7	45.5 ± 19.0
Sham	N/A	<0.7 ± 0.0	2.5 ± 10.0

[a]Serum was collected 2 dpi to determine virus titer.
[b]Percent increase in footpad as compared with the contralateral foot on 6 dpi.

A second characterization study was conducted to compare disease phenotype against a previously established LR06 strain, measure day 1 viremia and confirm absence of footpad swelling in DBA/1J mice infected with BVI. DBA/1J mice in this experiment were infected with BVI, LR06, NGR, and also SEN. As expected no mortality or significant differences in weight change was observed in mice infected with these CHIKV strains (Fig. 13).

Significant increase in serum virus titer was observed in the low challenge dose groups on day 2 as compared with day 1 in mice that were infected with BVI and SEN strains (Fig. 14). High challenge dose groups of the SEN and NGR strain maintained relatively similar titers between day 1 and 2, while titers of high challenge groups of BVI and NGR decreased on day 2. (Fig. 14)

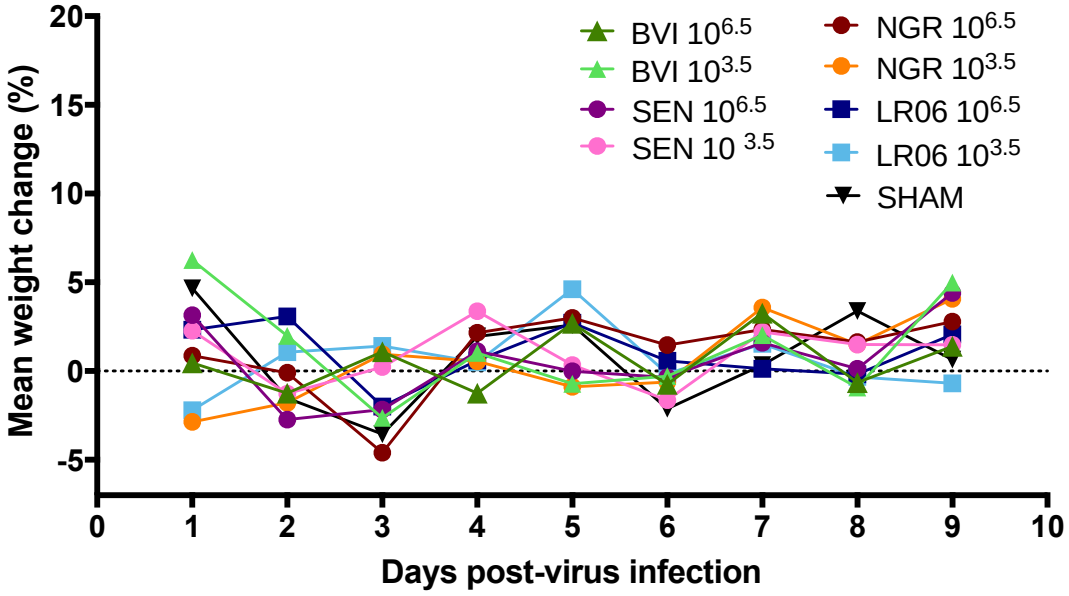


Fig. 13. Mean percent weight change of DBA/1J mice infected with two doses ($10^{3.5}$ $CCID_{50}$ & $10^{6.5}$ $CCID_{50}$) of BVI, SEN, NGR AND LR06 CHIKV strains.

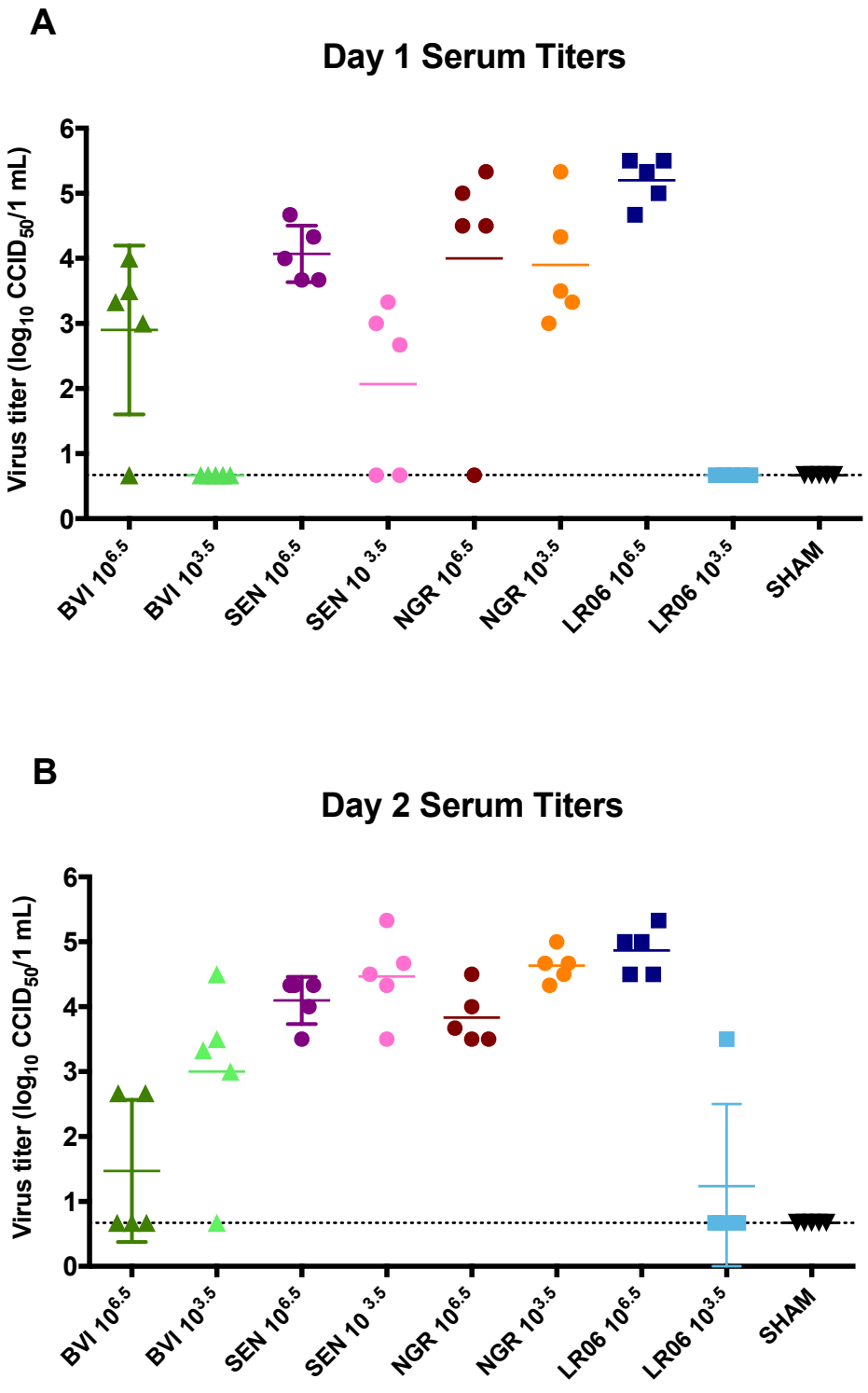


Fig. 14. Virus titer of serum collected 1 (A) and 2 (B) dpi from mice infected with two doses ($10^{6.5}$ and $10^{3.5}$ $CCID_{50}$) of BVI, SEN, NGR and LR06.

Consistent with the previous characterization experiment, groups infected with both challenge doses of BVI ($CCID_{50}$ $10^{6.5}$ and $10^{3.5}$), did not show significant footpad swelling (Fig. 15A). This further suggests that the DBA/1J mouse model may not be suitable for efficacy investigations involving Asian strains. Although reduction of virus titer could be used to evaluate the efficacy of antiviral agents. Overt arthralgia, however, could not be utilized in such studies.

The day of peak footpad swelling observed between groups infected with two challenge doses of SEN strain ($10^{6.5}$ and $10^{3.5}$ $CCID_{50}$) differed from the previous study (Fig. 15B). Delayed swelling was observed at in mice infected with the lower challenge dose. While swelling in the high challenge dose group peaked on day 6, the low dose group showed peak footpad swelling on day 7. A similar phenomenon was observed in the groups infected with two different infective doses of NGR (Fig. 15C). Nevertheless, the level of joint swelling in this second experiment was consistent with the first (Fig. 12 and 15). DBA/1J mice can be used to demonstrate swelling and viremia after infection with both SEN and NGR, and thus can be used for evaluating antiviral compounds and vaccines against those strains.

Mice infected with the higher dose of LR06 ($10^{6.5}$ $CCID_{50}$) showed a peak footpad swelling on day 6 (Fig. 15D), which is consistent with previous results (Fig. 1). LR06 causes relatively more severe joint swelling in mice as compared with other CHIKV strains. The 80% percent increase in swelling observed in the mice that received the high challenge infection is also consistent with the previous preliminary study (Fig. 1, Table 6).

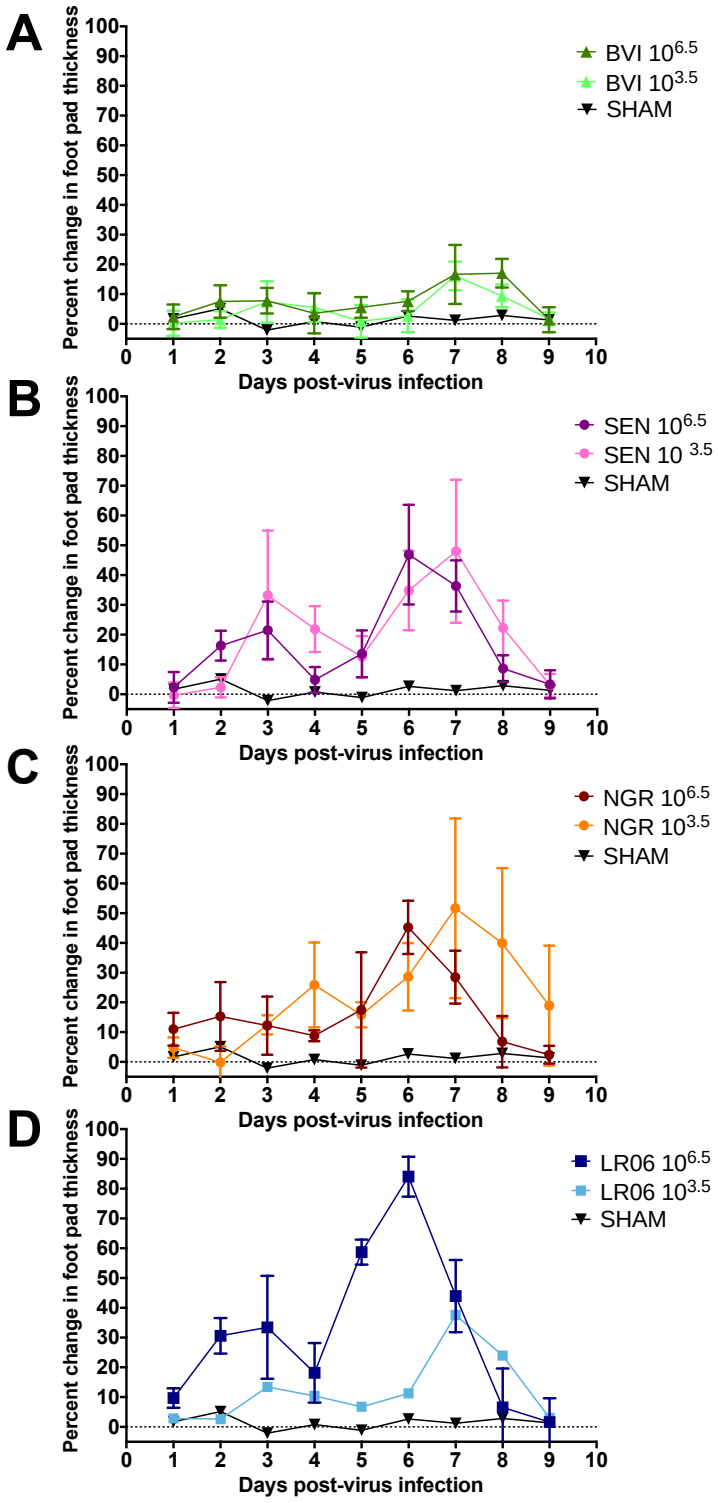


Fig. 15. Percent change in footpad swelling of DBA/1J mice infected with $10^{6.5}$ and $10^{3.5}$ $CCID_{50}$ doses of (A) BVI, (B) SEN, (C) NGR and (D) LR06 CHIKV strains.

Table 6
Serum virus titer and percent increase in footpad swelling in DBA/1J mice infected with two doses of BVI, IND and NGR.

Strain	Virus Dose	Log_{10} serum Day 1 titer	Log_{10} serum Day 2 titer	Footpad swelling (%) (n) [a]
British Virgin Islands (BVI)	$10^{6.5}$ $CCID_{50}$	2.1 ± 0.8	1.1 ± 0.6	16.6 ± 9.9*
	$10^{3.5}$ $CCID_{50}$	<0.7 ± 0.0	2.2 ± 1.0	16.1 ± 4.8*
Senegal (SEN)	$10^{6.5}$ $CCID_{50}$	3.1 ± 0.4	3.1 ± 0.4	46.9 ± 16.7¥
	$10^{3.5}$ $CCID_{50}$	1.5 ± 0.8	3.5 ± 0.7	48.0 ± 24.1*
Nigeria (NGR)	$10^{6.5}$ $CCID_{50}$	3.2 ± 1.5	2.8 ± 0.4	45.2 ± 8.9¥
	$10^{3.5}$ $CCID_{50}$	2.9 ± 0.9	3.6 ± 0.3	51.6 ± 30.2*
La Reunion (LR06)	$10^{6.5}$ $CCID_{50}$	4.2 ± 0.4	3.9 ± 0.4	84.0 ± 6.7¥
	$10^{3.5}$ $CCID_{50}$	<0.7 ± 0.0	1.0 ± 0.8	37.6 ± 7.5*
Sham	N/A	<0.7 ± 0.0	<0.7 ± 0.0	1.2 ± 2.8*

[a]Percent increase in peak footpad swelling as compared with the contralateral foot. Peak swelling was observed on 6 dpi¥ or 7 dpi*

4.3.2. Evaluation of the Efficacy of T-705 Against Various Strains in DBA/1J Mice

The effect of T-705 was evaluated against strains that caused significant swelling in DBA/1J mice. Consistent with characterization experiments, minimal weight change and mortality was observed in DBA/1J mice infected with LR06, SEN and NGR CHIKV strains and treated with 400mg/kg/d or 100mg/kg/d of T-705 (Fig. 16).

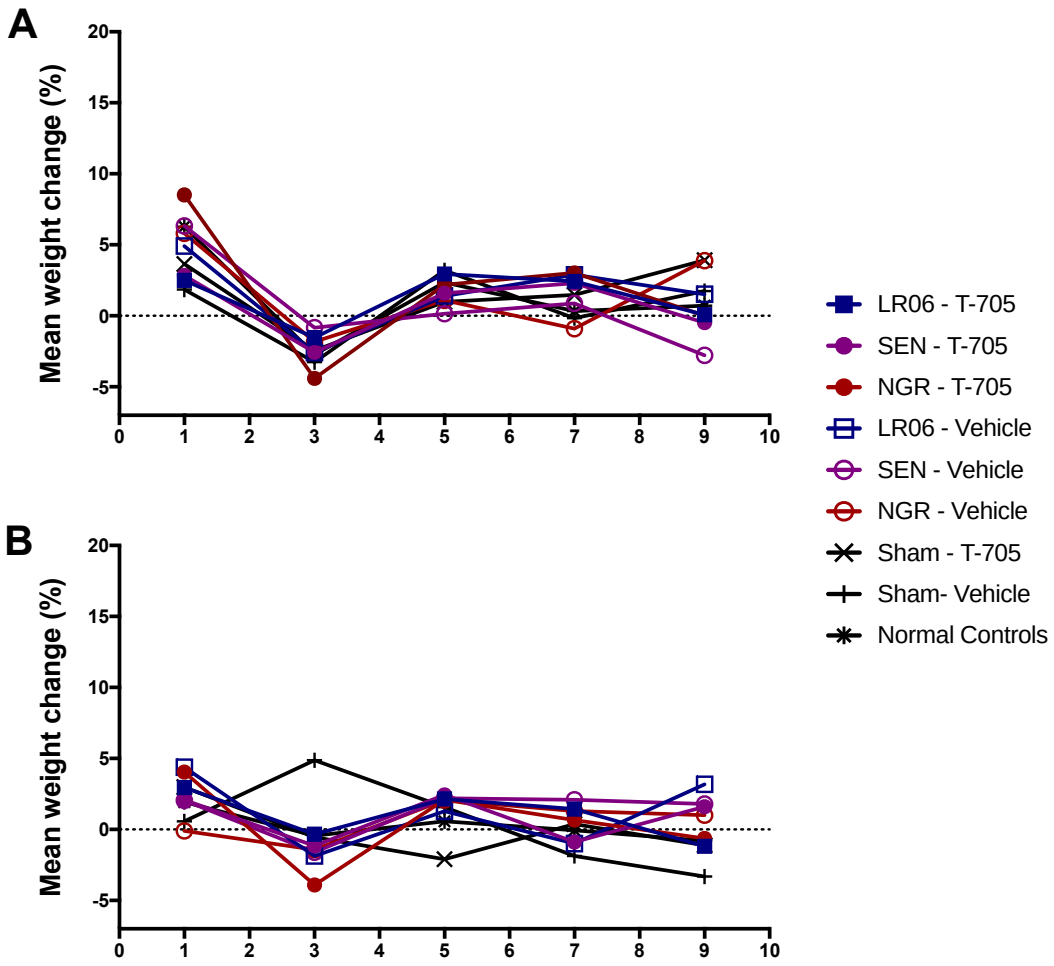


Fig. 16. Mean percent weight change of DBA/1J mice infected with LR06, SEN and NGR CHIKV strains and treated with (A) 400mg/kg/d and (B) 100mg/kg/d of T-705.

Treatment with 400 mg/kg/d of T-705 significantly (****P<0.0001) reduced viremia to baseline levels on day 2 (Fig. 17A). The virus titer reduction was uniform across mice infected with all three CHIKV strains. Similar to the 400mg/kg/d dose of T-705, the 100mg/kg/d T-705 dosage also significantly (****P<0.0001) reduced viremia in serum collected 2 days-post infection (Fig. 17B).

The 400mg/kg/d T-705 treatment reduced virus titers at the site of infection (right hind leg, RHL), but the reduction in virus titer varied from strain to strain and was not reduced to baseline (Fig. 18A). Both treated groups of SEN and NGR had an average RHL titer of 3.6 ± 1.1 and 3.3 ± 1.6, respectively (Table 7). These, and previous results, suggest that there is a phenotypic difference in disease after inoculation of DBA/1J mice with West African clade viruses that is more difficult to overcome as compared with strains from other clades. The low T-705 dose did not result in the significant reduction of virus titers at the site of infection (right hind leg) (Fig. 18B, Table 8), despite the significant reduction in viremia reported above (Fig. 17B).

High dose treatment with T-705 was effective in significantly (****$P<0.001$) reducing footpad swelling to baseline levels in mice infected with LR06, SEN and NGR strains (Fig. 19). A dose of 100mg/kg/d of T-705, however, did not significantly reduce footpad swelling in mice infected with these strains. As shown in Figure 20, the low dose of T-705 treatment resulted in a delayed peak in footpad swelling compared to mice treated with vehicle. Significant reduced footpad swelling was only observed on day 6 in mice infected with LR06 and NGR strains (Fig. 20 A and C).

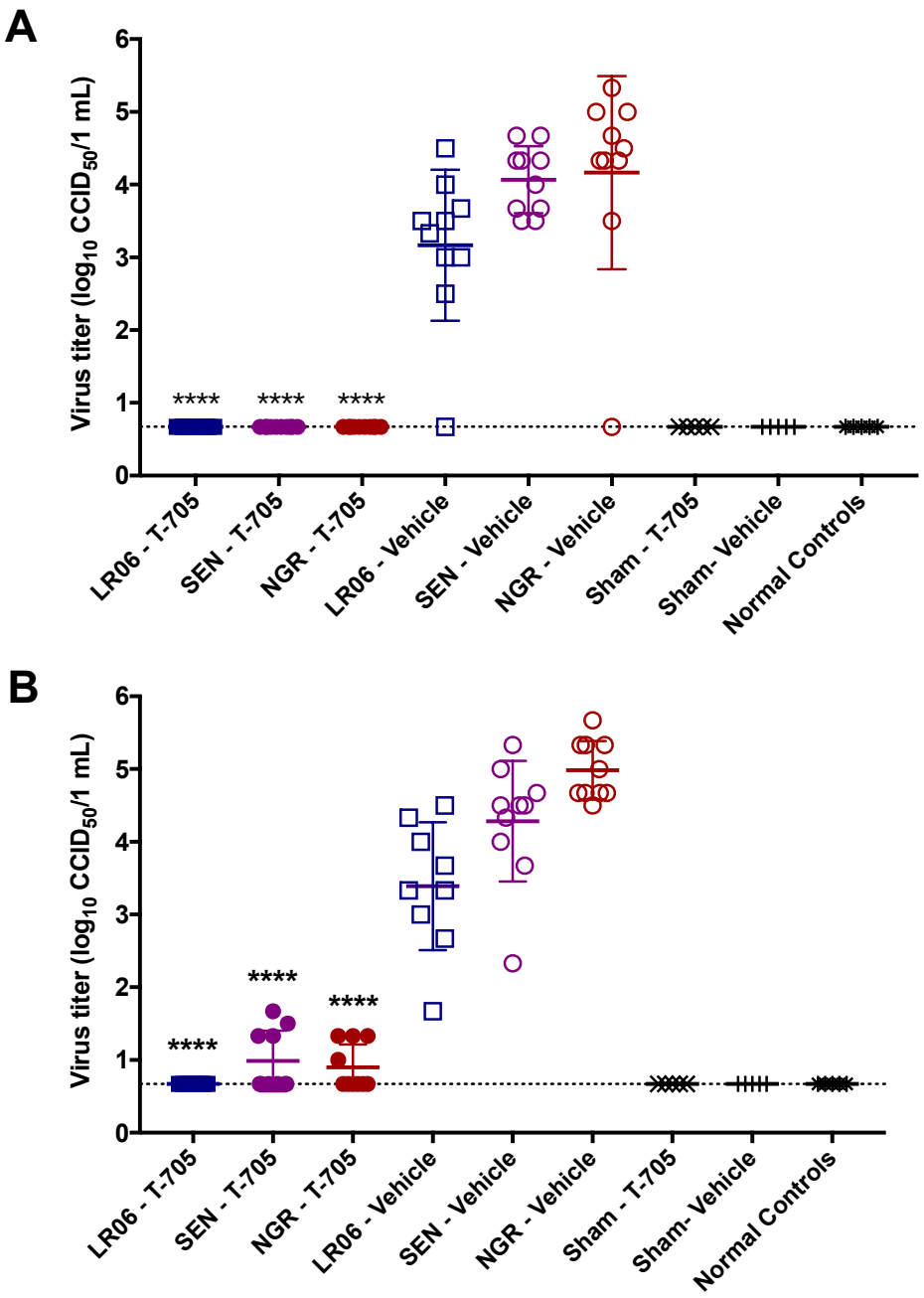


Fig. 17. Virus titer of serum collected on day 2 post-infection from mice infected with LR06, SEN and NGR CHIKV strains and treated with (A) 400mg/kg/d and (B) 100mg/kg/d of T-705. (****P<0.0001, as compared to vehicle treatment)

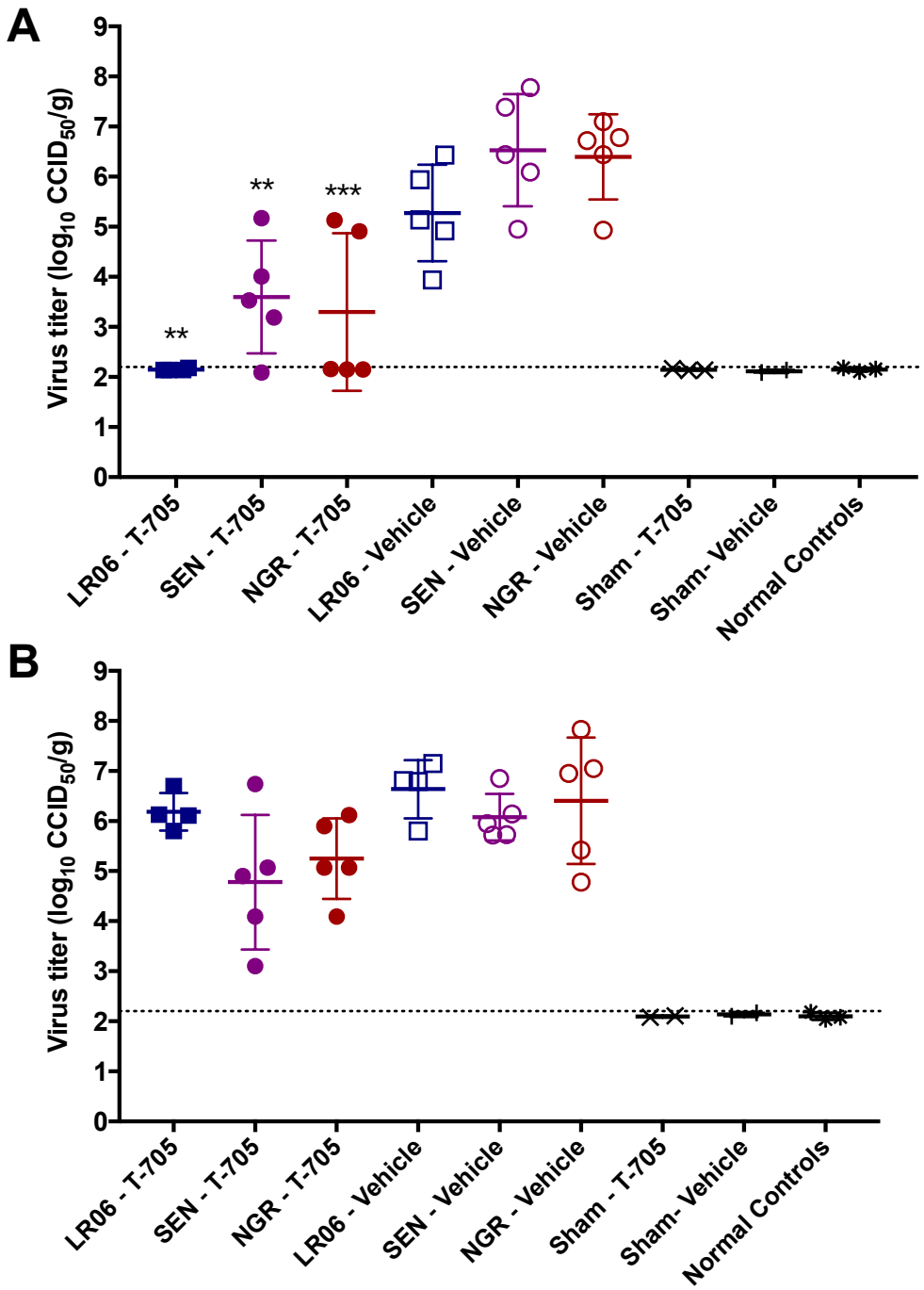


Fig. 18. Virus titer of right-hind leg collected on day 6 post-infection from mice infected with LR06, SEN and NGR CHIKV strains and treated with (A) 400mg/kg/d and (B) 100mg/kg/d of T-705 and vehicle. (***P<0.001; **P<0.01, as compared to vehicle treatment)

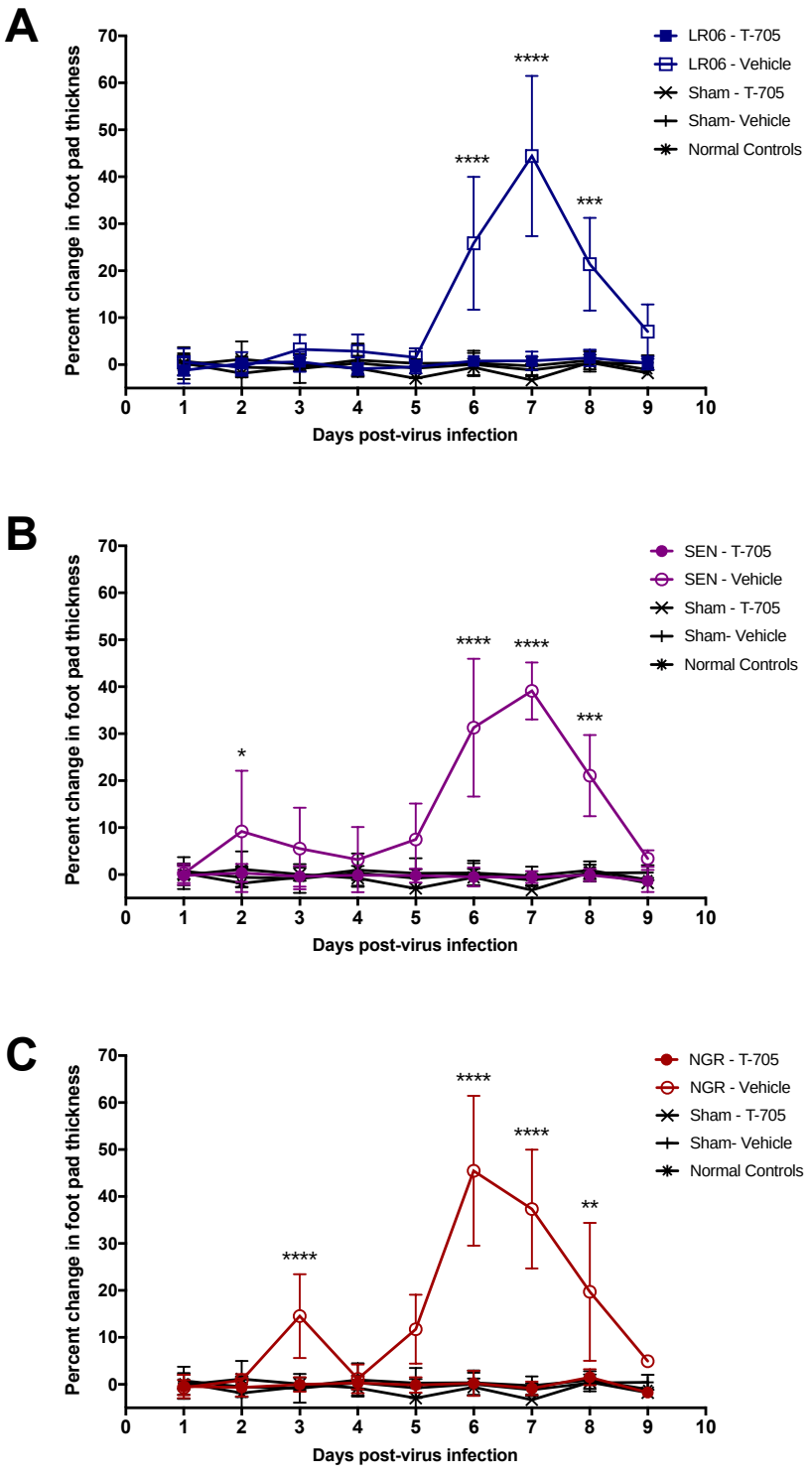


Fig. 19. Percent change in footpad swelling of DBA/1J mice infected with SEN, NGR and LR06 CHIKV strains and treated with 400mg/kg/d of T-705. (****P<0.0001, ***P<0.001; **P<0.01; *P<0.05, as compared to vehicle)

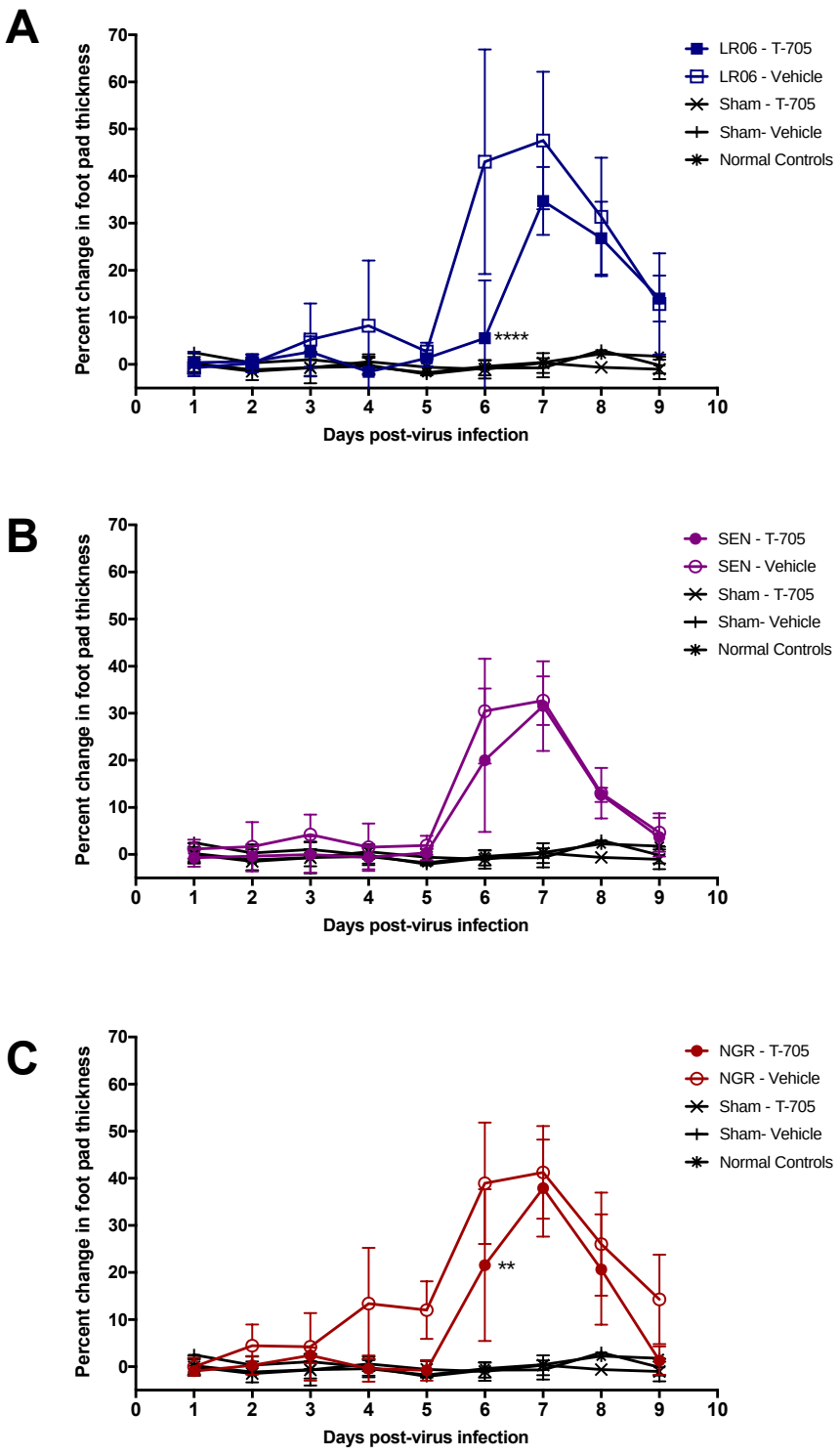


Fig. 20. Percent change in footpad swelling of DBA/1J mice infected with SEN, NGR and LR06 CHIKV strains and treated with 100mg/kg/d of T-705. (****P<0.0001, **P<0.01, as compared to vehicle.)

Of the 16 cytokines screened in right hind leg tissues collected on day 6, only 4, MCP-1, RANTES, IFNγ and MIP-1α, were significantly increased with CHIKV infection compared to controls. The other cytokines analyzed were not significantly elevated in infected mice, compared with uninfected mice. MCP-1 and RANTES were significantly lowered by T-705 treatment in mice infected with LR06, SEN and NGR strains (Fig. 21 A & C).

Levels of IFNγ and MIP-1α were not significantly elevated in mice infected with the LR06 strain compared to uninfected control mice, but a trend toward reduced cytokines in treated animals was observed. Mice infected with NGR also did not show significant elevation of MIP-1α (Fig. 21D). However, mice infected with the SEN strain showed significant increase in both IFNγ and MIP-1α cytokines that were significantly reduced by T-705 treatment (Fig. 21 B & D).

Both MCP-1 and RANTES were significantly elevated in untreated mice infected with SEN and NGR CHIKV strains, and subsequently reduced by 100mg/kg/d of T-705 treatment (Fig. 22). This treatment also resulted in reduced IFNγ in mice infected with NGR and IFNγ and MIP-1α in mice infected with SEN. Cytokine reduction profile of mice treated with 100mg/kg/d of T-705 is very similar to mice treated with 400mg/kg/d of T-705 (Fig. 21 and 21).

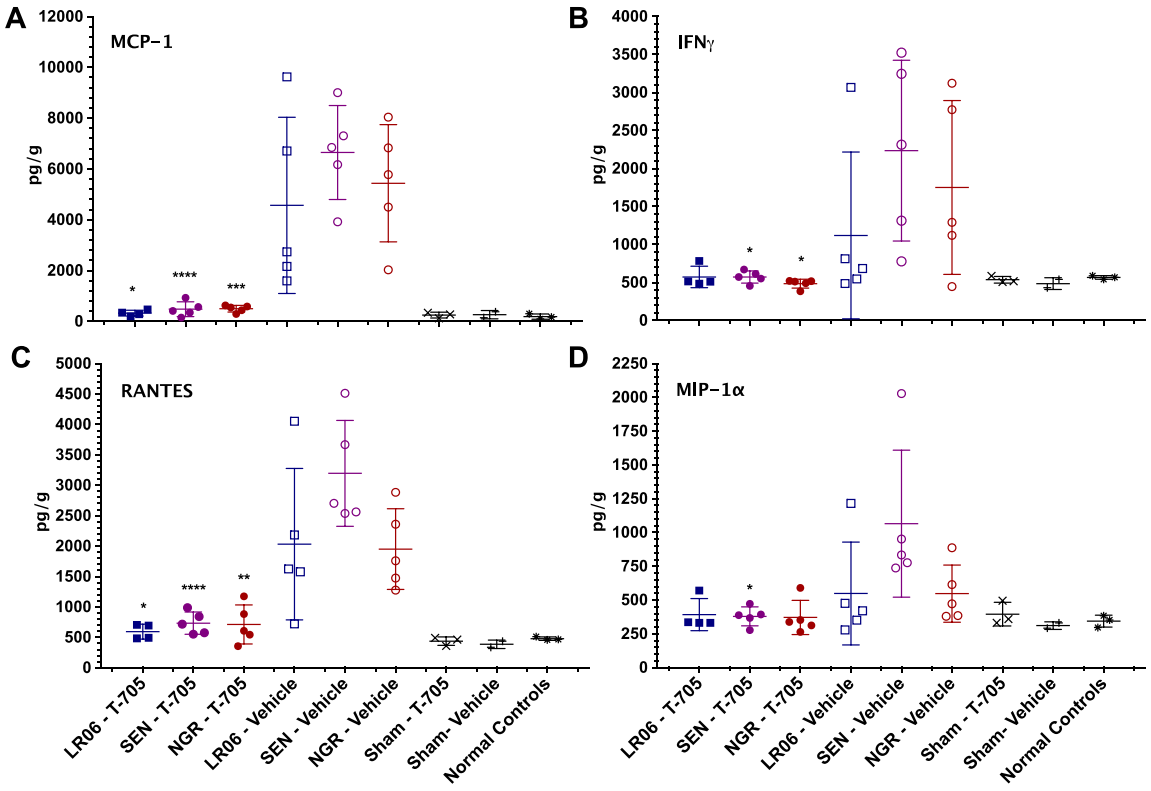


Fig. 21. The effect of 400mg/kg/d T-705 treatment on day 6 cytokine profile of the hind leg of mice infected with $10^{3.5}$ $CCID_{50}$ of LR06, SEN and NGR. (****P<0.0001, ***P<0.001, **P<0.01, *P<0.05, as compared with vehicle treatment).

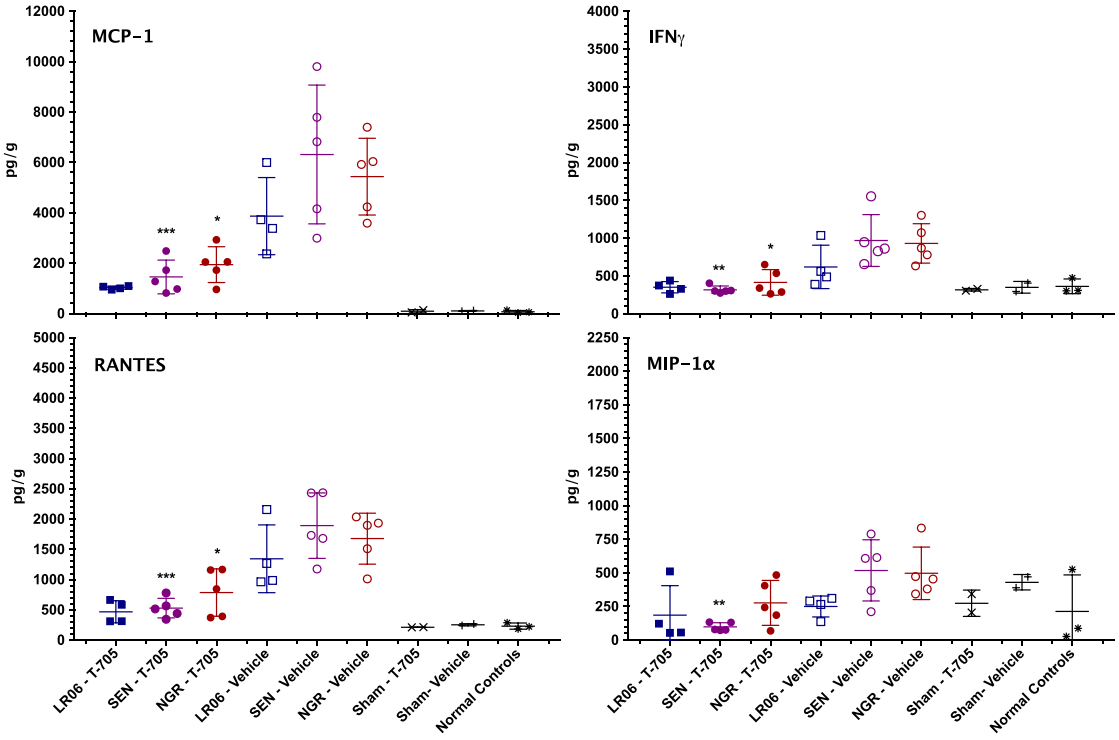


Fig. 22. The effect of 100mg/kg/d of T-705 treatment on cytokine profile of the hind leg at the site of virus inoculation in CHIKV-infected mice on 6 dpi. (****P<0.0001, ***P<0.001, **P<0.01, *P<0.05, as compared with vehicle treatment).

Table 7
The effect of 400mg/kg/d dose treatment of T-705 on serum virus titer, right hind leg virus titer and percent increase in footpad swelling in DBA/1J mice infected with two doses of LR06, SEN and NGR.

Treatment	Strain	Log_{10} serum Day 2 titer[a] ± SD	Log_{10} right hind leg Day 6 titer[b] ± SD	Footpad swelling (%)[c]
T-705	LR06	<0.7 ± 0.0	2.2 ± 0.0	0.8 ± 2.0[£]
T-705	SEN	<0.7 ± 0.0	3.6 ± 1.1	-0.5 ± 1.3[£]
T-705	NGR	<0.7 ± 0.0	3.3 ± 1.6	-0.2 ± 2.7[¥]
Vehicle	LR06	3.2 ± 1.1	5.3 ± 1.0	44.4 ± 17.1[£]
Vehicle	SEN	4.1 ± 0.5	6.5 ± 1.1	39.1 ± 6.1[£]
Vehicle	NGR	4.2 ± 1.3	6.4 ± 0.9	45.5 ± 16.0[¥]
T-705	N/A	<0.7 ± 0.0	2.2 ± 0.0	-0.6 ± 1.8[¥]
Vehicle	N/A	<0.7 ± 0.0	2.1 ± 0.0	-1.1 ± 1.0[¥]
N/A	N/A	<0.7 ± 0.0	2.2 ± 0.0	0.3 ± 1.6[¥]

[a]Serum collected 2 dpi to determine virus titer.
[b]Right hind leg collected 6 dpi to determine virus titer.
[c]Percent increase in peak footpad swelling as compared with the contralateral foot. Peak swelling was observed on 6 dpi[¥] or 7 dpi[£].

Table 8
The effect of 100mg/kg/day of T-705 on serum virus titer, right hind leg virus titer and percent increase in footpad swelling in DBA/1J mice infected with two doses of LR06, SEN and NGR.

Treatment	Strain	Log_{10} serum Day 2 titer[a] ± SD	Log_{10} right hind leg Day 6 titer[b] ± SD	Footpad swelling (%)[c]
T-705	LR06	<0.7 ± 0.0	6.2 ± 0.4	34.7 ± 7.2
T-705	SEN	1.0 ± 0.4	4.8 ± 1.4	31.5 ± 9.5
T-705	NGR	0.8 ± 0.3	5.3 ± 0.8	37.9 ± 10.3
Vehicle	LR06	3.4 ± 0.9	6.6 ± 0.6	47.6 ± 14.6
Vehicle	SEN	4.3 ± 0.8	6.1 ± 0.5	32.7 ± 6.2
Vehicle	NGR	5.0 ± 0.4	6.4 ± 1.3	41.3 ± 9.8
T-705	N/A	<0.7 ± 0.0	2.1 ± 0.0	0.3 ± 2.1
Vehicle	N/A	<0.7 ± 0.0	2.1 ± 0.1	-0.7 ± 2.0
N/A	N/A	<0.7 ± 0.0	2.1 ± 0.1	0.4 ± 0.0

[a]Serum collected 2 dpi to determine virus titer.
[b]Right hind leg collected 6 dpi to determine virus titer.
[c]Percent increase in peak footpad swelling as compared with the contralateral foot. Peak swelling was observed on 7 dpi

4.4. Discussion

The characterization of various CHIKV strains in DBA/1J mice demonstrated that the strains display different levels of infectivity and disease phenotype. Both Asian strains, BVI and IND, did not result in footpad swelling. However, the West African strains, SEN and NGR, resulted in significant footpad swelling with peak swelling observed on days 6 and 7 for $10^{6.5}$ and $10^{3.5}$ $CCID_{50}$ challenge groups respectively (Fig. 12 and 15).

Although mice infected with the Asian strains did not display footpad swelling, the strains could potentially be passaged in mice in order to produce adapted strains that might cause rheumatic disease. According to Gardner et al, infection with an Asian strain from Thailand (isolate AF15561), resulted in inconsistent viremia and resulted in no disease symptoms in C57BL/6 mice until it was serially passaged in mice 9 times (Gardner et al., 2010).

Percentage increase in footpad swelling also varied among the strains. The LR06 strain resulted in the highest 80% increase in footpad swelling, while the NGR and SEN strains resulted in 40-50% increase. Footpad swelling was dose dependent in mice infected with the LR06 strain, while mice infected with SEN and NGR with two different challenge doses ($10^{6.5}$ $CCID_{50}$ and $10^{3.5}$ $CCID_{50}$) showed similar footpad swelling that weren't significantly different. The day of peak swelling was delayed in lower challenge dosage groups.

Since infection with BVI and NGR CHIKV did not result in footpad swelling in DBA/1J mice, evaluation of T-705 efficacy against the two Asian strains was not conducted in the DBA/1J mouse model. However, efficacy evaluation experiments revealed that 400mg/kg/d of T-705 significantly reduced footpad swelling and virus titers to baseline levels in mice infected with SEN, NGR and LR06. Since these strains cause more robust disease and replicate at a more rapid rate, it stands to reason that Asian clade strains would be effectively treated with T-705. Treatment with this compound also reduced levels of pro-inflammatory cytokines, MCP-1, RANTES, IFNγ and MIP-1α. Footpad swelling is caused by the infiltration of immune cells, primarily monocytes/macrophages, into

infected synovial tissue and the accumulation of inflammatory cytokines (Gardner et al., 2010). Thus, it was anticipated that the reduction of footpad swelling would also have a concomitant decrease in cytokine levels.

Although treatment with 100mg/kg/d of T-705 reduced viremia and cytokine levels in mice, it did not significantly reduce foot pad swelling or virus titer in the right hind leg at the site of virus challenge. We find the observed decrease in cytokine levels in the absence of decreased footpad swelling to be contradictory. This may be due to the fact that cytokine levels were measured on day 6 where we observe some reduction of footpad swelling due to T-705 treatment specifically for that day (Fig. 21). Low dose T-705 treatment did not have any effect on virus titer at the site of infection. The right hind leg virus titers of mice treated with 100mg/kg/d of T-705 were not different from mice treated with the vehicle. Thus, there appeared to be a stronger correlation between footpad swelling and virus titer in this study.

Virus titer reduction in the serum but not the right hind leg could be explained by the fact that bio-distribution of T-705 to tissues is limited in mice when the drug is administered in low concentrations. Synovial joints of infected limbs in mice develop high virus titers. Although macrophages are critical in clearing of virus from tissues, they are also important targets of CHIKV that carry the virus at and towards site of infection. Infected macrophages harbored in synovial joints have been implicated with causing chronic persistence of CHIKV patients (Hoarau et al., 2010). Thus, if blood flow is compromised in arthritic joints due to inflammation and increased synovial fluid, significant virus titer

reduction in infected joints may require higher concentrations of antiviral compounds (Haywood and Walsh, 2001).

In the DBA/1J model, difference in response to both treatment doses of T-705 was not observed between the different strains of CHIKV. Footpad swelling was uniformly reduced in mice infected with SEN, NGR and LR06 in the 400mg/kg/d of T-705 treatment experiment. 100mg/kg/d of T-705 also failed to improve disease across all strains. The one parameter that suggested difference in response to treatment was day 2 serum titer of mice treated with 100mg/kg/d of T-705.

CHAPTER 5

ANTIVIRAL EFFECT OF T-705 AGAINST DIFFERENT STRAINS OF CHIKV IN A LETHAL MOUSE MODEL

5.1. Introduction

Chikungunya virus (CHIKV) is an alphavirus that causes Chikungunya disease, which is mainly characterized by acute febrile arthralgia. The virus emerged in 2004 and has since been spreading extensively throughout the world. Within the last decade, it has caused a series of outbreaks resulting in over 3.3 million cases (Powers, 2015). The various strains of CHIKV currently found around the world have been grouped into three phylogenetic groups/clades, namely, West African, Asian and East/Central/South African (ECSA).

Currently, there are no approved antiviral drugs and vaccines available for CHIKV. Therefore, identifying compounds that are efficacious against multiple strains of CHIKV belonging to the different phylogenetic groups is critical. Recently, T-705, a purine analog that has exhibited broad activity against multiple RNA viruses has been identified as an ideal candidate for treating CHIKV (Delang et al., 2014).

There are several different types of animal models utilized for evaluating antiviral efficacy against CHIKV *in vivo* (Haese et al., 2016). Infection of AG129 mice, deficient in interferon α/β and γ receptors, results in lethal disease, as these mice are highly susceptible to CHIKV (Couderc et al., 2008). As they lack a critical antiviral immune response, they succumb to virus infection within 4 – 7

days. This also makes them useful to test effective therapeutics and vaccines as well as for studying the role of type I IFN system in CHIKV pathogenesis.

Herein, we describe the disease phenotype of AG129 mice infected with various strains of CHIKV belonging to different phylogenetic groups. The efficacy of T-705 is then evaluated against certain strains of CHIKV to determine if there is a difference in the efficacy of treatment against virus strains with differing disease severity.

5.2. Materials and Methods

5.2.1. Animals

Male and female AG129 mice with an average weight of 22g were obtained from an in-house breeding colony at the Institute for Antiviral Research. The mice used were between 8 – 10 weeks of age. The mice were randomly assigned to experimental groups and individually marked with ear tags. The mice were weighed daily for six days starting from the day of infection (Day 0) and percent change in weight was calculated.

5.2.2. Test Compound

T-705 was obtained from Toyama Chemical Company, Ltd. (Toyama, Japan). T-705 was dissolved in 2.9% sodium bicarbonate buffer (Life Technologies, Carlsbad, CA) at a concentration of 38 mg/ml to deliver compound at a treatment dose of 400 mg/kg/d. In a separate experiment, the compound

was dissolved in bicarbonate buffer at a concentration of 9.5 mg/ml in order to treat animals with a 100 mg/kg/d treatment dose.

5.2.3. Virus and Cells

Chikungunya virus isolates from British Virgin Islands (BVI, R99659 TVP 20811), Nigeria (NGR, IbH 35 TVP-1337), Tanzania (S27) and Reunion Island (LR06, LR2006-OPYI) were obtained from Robert Tesh (UTMB, WRCEVA). Virus stocks were prepared by passaging twice in C6/36 cells. The passage 2 of the BVI, NGR, S27 and LR06 virus stocks used had titers of 10^8, $10^{9.67}$ and $10^{8.5}$ and 10^9 50% cell cultures infectious doses ($CCID_{50}$)/1ml, respectively. For titration and plaque neutralization assays the African green monkey kidney cell line, Vero 76, was used. The cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in minimal essential medium (MEM) containing 10% fetal bovine serum (FBS) (GE Healthcare HyClone Laboratories, Logan, UT).

5.2.4. Viral Titer Assay

The virus titers in tissues or plasma were assayed using the end point dilution assay where serial log dilutions of samples were made and added to Vero 76 cells on 96-well plates. After three days of incubation, cytopathic effect (CPE) was used to identify the end-point of infection. Four replicates were used to calculate the 50% cell culture infectious doses ($CCID_{50}$) per mL of plasma or

gram of tissues. The assay had a lower limit of detection for virus at 0.7 log_{10} $CCID_{50}$/mL serum.

5.2.5. CHIKV Plaque Reduction Neutralization (PRNT) Assay

$PRNT_{50}$ assay was used to quantify antibody levels of serum as previously described (Warter et al., 2011). Briefly, the assay was conducted using 12-well plates seeded with 4 x 10^5 Vero-76 cells/mL. Serum samples were diluted 1:2 starting with a 1/10 dilution. To inactivate any virus present in the serum, the samples were incubated for 30 minutes at 56°C. Equal volumes of diluted CHIKV, titrated to produce a countable number of plaques, was added to the sample dilutions. A resulting volume of 250 µL was added to a single well of a 12-well plate. The plates were incubated at 37°C and 5% CO2 for one hour to allow for virus attachment, after which 1mL of 1.7% methylcellulose (4000 cps, Sigma-Aldrich), overlay was added. Three days later, cells were stained by the addition of crystal violet dye containing 10% formalin for 30 minutes. Plates were then thoroughly washed and allowed to dry before plaques were counted. The inverse of the dilution that caused a 50% reduction in plaque number was reported as the plaque reduction neutralization titer.

5.2.6. Experiment Design

5.2.6.1. Characterization of BVI, NGR and LR06 CHIKV Strains in AG129 Mice

AG129 mice of both genders were randomly assigned to cages of 4 - 5 animals per group. Two virus doses of $10^{1.5}$ and $10^{2.5}$ $CCID_{50}$/0.1ml of BVI, NGR, S27 and LR06 strains were prepared in MEM. The high virus challenge groups of each strain contained five mice while each low challenge dose groups contained four mice. Animals were anesthetized with isoflurane prior to subcutaneous (s.c.) injection in the footpad and the hock of the right leg with a total inoculum volume of 0.1 ml of the diluted virus (0.05 ml each site). Survival was monitored twice a day for morbidity and mortality for the experimental period of 10 days. Weight change was monitored from 0-5 dpi. Serum was obtained through cheek bleeds on day 2 post infection for viral titers.

Serum was also obtained on day 14 after the course of the experiment via cheek bleeds for a Plaque Reduction Neutralization Test (PRNT). This was done to confirm that effective infections were administered in the mice that survived at the end of the experiment.

5.2.6.2. Evaluation of the Efficacy of T-705 Against SEN, NGR and LR06 CHIKV Strains

Male and female AG129 mice were randomly assigned to cages of seven animals per group. $10^{2.5}$ $CCID_{50}$/0.1ml of BVI, NGR, S27 and LR06 CHIKV strains were prepared in MEM.

Treatment with a dose of 400 mg/kg/d of T-705 began four hours prior to virus infection and was administered twice a day for six days via intraperitoneal (i.p.) injection. Animals were anesthetized with isoflurane prior to subcutaneous (s.c.) injection in the footpad and the hock of the right leg with a total inoculum volume of 0.1 ml of the diluted virus (0.05 ml each site).

Survival was monitored twice a day for morbidity and mortality for the experimental period of 10 days. Weight change was monitored from 0-5 dpi. Serum was obtained through cheek bleeds on day 2 post infection for viral titers.

5.2.7. Statistical Analysis

The Wilcoxon log-rank test was used to analyze the survival data of mice. Statistical analyses of weight, tissue virus and antibody titer were performed using one-way ANOVA using a Bonferroni group comparison (Prism 7, GraphPad Software, Inc).

5.2.8 Ethics Statement

This study was conducted in accordance with the approval of the Institutional Animal Care and Use Committee of Utah State University dated February 8, 2017 (Protocol #2713). The work was done in the AAALAC-accredited Laboratory Animal Research Center of Utah State University.

5.3. Results

5.3.1. Characterization of Various CHIKV Strains in AG129 Mice

To characterize the disease phenotype of different strains of CHIKV belonging to the three clades in a lethal model, AG129 mice were inoculated with BVI, NGR, and LR06 strains. Survival, weight change and viremia were determined.

Weight change observed over the course of the study was minimal (less than 6%) (Fig. 23). The uniform decline within the first two days can be likely attributed to excessive handling due to infection and beginning of treatment regimens. There was no significant difference between mice infected with two different challenge doses of each strain. Weight decreased sharply in mice infected with BVI just prior to death (Fig. 23). Less weight change was observed

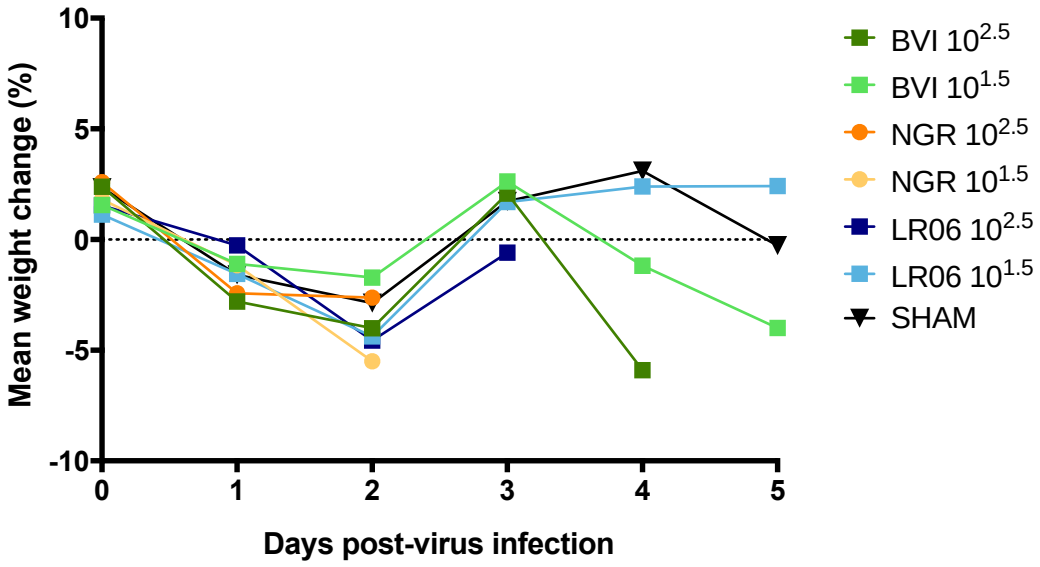


Fig. 23. Mean percent weight change of DBA/1J mice infected with two doses ($10^{1.5}$ and $10^{2.5}$ $CCID_{50}$) of BVI, NGR and LR06 CHIKV strains.

after infection with NGR, as these mice succumbed to virus infection very early in the study. There was a dose-dependent increase in viremia observed in AG129 mice infected with BVI and LR06 CHIKV (Fig. 24). Higher dose challenge resulted in a significantly higher serum titer. Virus titers for the high ($10^{2.5}$ $CCID_{50}$) NGR challenge group are not shown because the mice died on the day of serum collection. Serum titers measured in mice infected with BVI or LR06 were not significantly different from each other. However, the titer of the low dose challenge group of mice infected with NGR was significantly ($P<0.0001$) higher than the mice infected with either ($10^{2.5}$ or $10^{2.5}$) doses of BVI and LR06 strains.

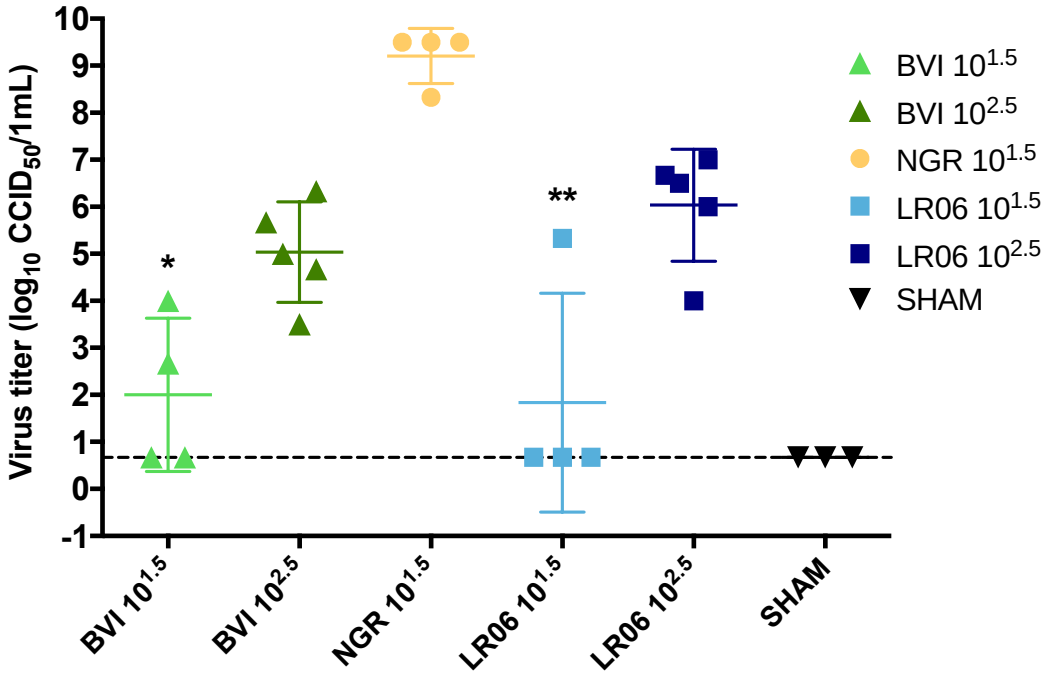


Fig. 24. Virus titer of serum collected 2 dpi from mice infected with two doses ($10^{1.5}$ and $10^{2.5}$ $CCID_{50}$) of BVI and LR06, and mice infected with 1.5 $CCID_{50}$ NGR. (**P<0.01; *P<0.05, as compared to vehicle treatment)

Although DBA/1J mice did not show observable clinical signs of disease when infected with Asian strains (BVI or IND), AG129 mice succumbed to disease after infection with BVI (Fig. 25A). This demonstrates the utility of this mouse strain to evaluate antiviral compounds against Asian strains. The BVI strain was the least robust strain tested in this study with the latest mean day-to-death (Fig. 25, Table 9). As can be seen in Figure 25, 75% (3 out of 4) of the mice infected with the low LR06 challenge dose survived infection. The three mice that survived were the same mice that also did not show detectable serum virus titer on day 2 (Fig. 24). It is most likely that the mice did not receive a sufficient virus challenge to cause a lethal infection.

The high serum virus titer combined with early average mean day-to-death observed in mice infected with NGR suggest that this strain is significantly more robust than BVI and LR06 (Fig. 25, Table 9).

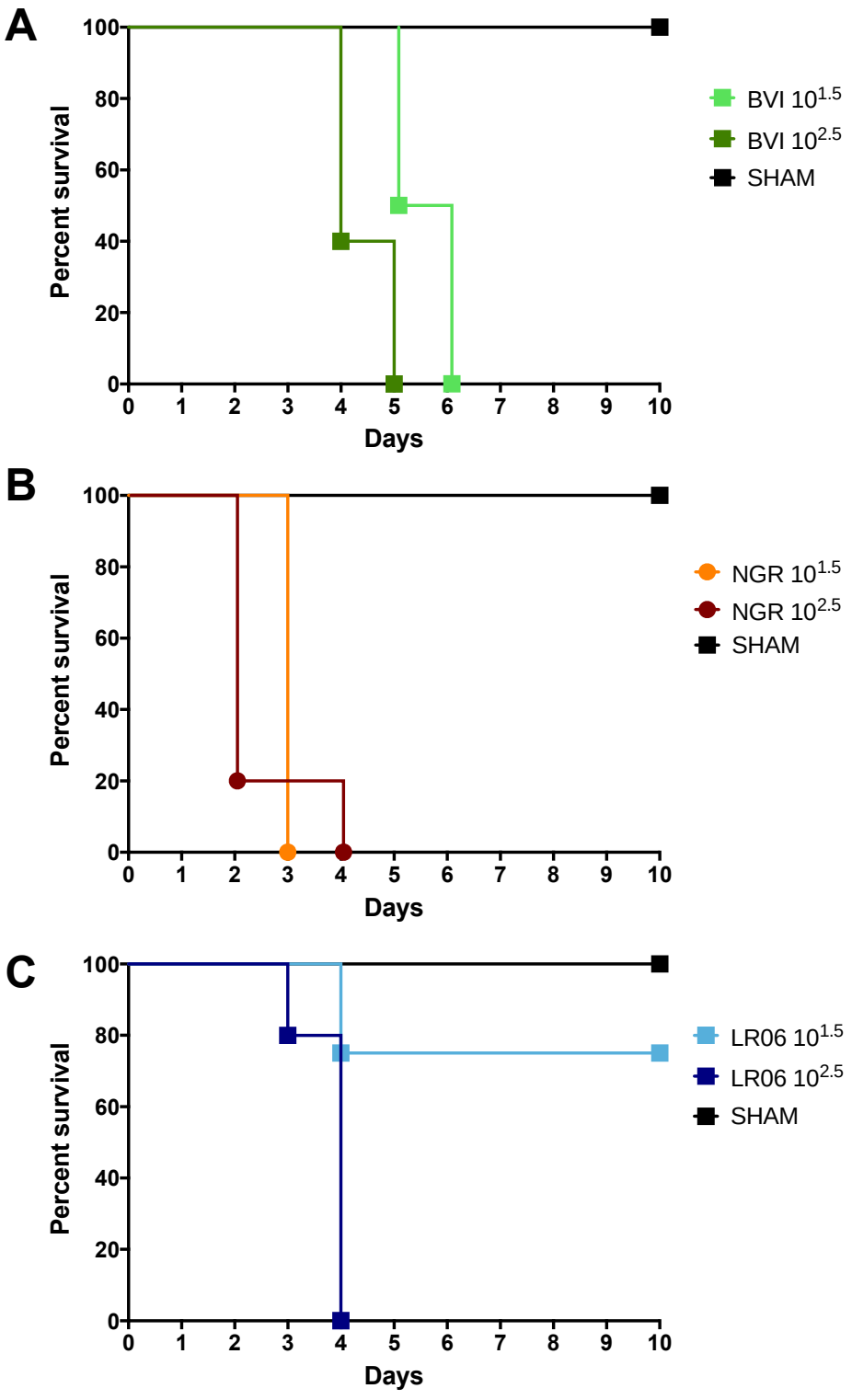


Fig. 25. Survival of AG129 mice challenged with $10^{1.5}$ and $10^{2.5}$ $CCID_{50}$ doses of (A) BVI, (B) NGR and (C) LR06 CHIKV strains.

Table 9
Serum titer & mortality of AG129 mice infected with BVI, NGR, and LR06 CHIKV strains.

Strain	Infective $CCID_{50}$	Log_{10} serum virus titer[a] ± SD	Survival (alive/total)	Mean day-to-death ± SD
British Virgin Islands (BVI)	$10^{1.5}$	2.0 ± 1.6	0/4	5.5 ± 0.6
	$10^{2.5}$	5.1 ± 1.1	0/5	4.4 ± 0.6
Nigeria (NGR)	$10^{1.5}$	9.2 ± 0.6	0/4	3.0 ± 0.0
	$10^{2.5}$	N/A	0/5	2.2 ± 0.5
La Reunion (LR06)	$10^{1.5}$	5.3	3/4	4.0
	$10^{2.5}$	6.0 ± 1.2	0/5	3.8 ± 0.5
Sham	N/A	<0.7 ± 0.0	3/3	>10.0 ± 0.0

[a]Serum was collected 2 dpi to determine virus titer.

5.3.2. Evaluation of the Efficacy of T-705 Against Selected CHIKV Strains in the AG129 Mouse Model

Unlike the characterization study, to evaluate differences in response to T-705 treatment, LR06 was substituted with the S27 strain. Since T-705 was effective in the treatment of the S27 strain in a preliminary study (unpublished data), while treatment of LR06 was not effective, we selected the S27 strain for use as a positive control in this study.

Minimal weight change was observed on most days in mice infected with S27, BVI and NGR CHIKV strains and treated with 400 mg/kg/d of T-705 (Fig. 27). However, untreated mice infected with the S27 strain failed to gain weight on day 2 compared to the other mice in the experiment.

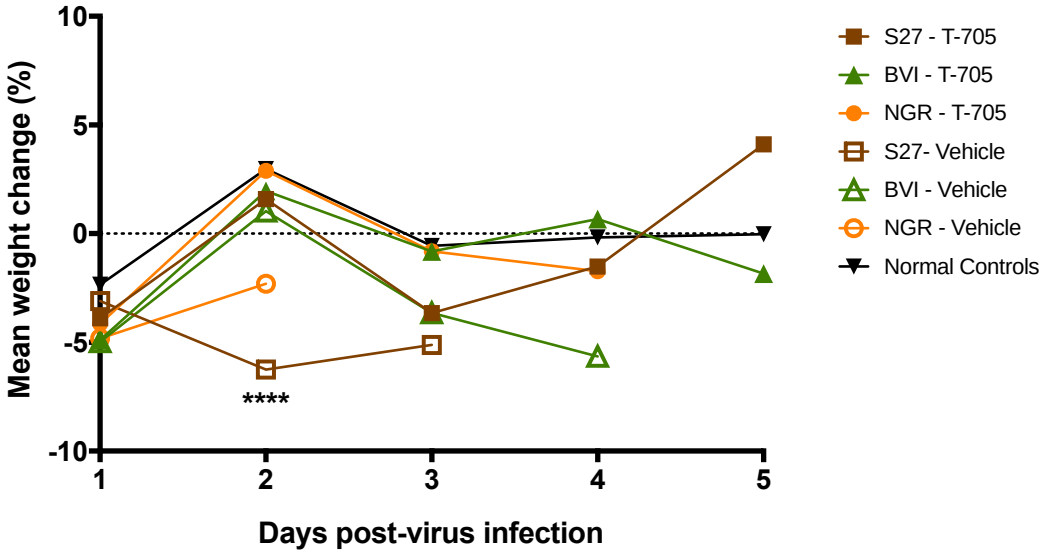


Fig. 26. Mean percent weight change of AG129 mice infected with $10^{2.5}$ $CCID_{50}$ of BVI, NGR and LR06 CHIKV strains and treated with 400 mg/kg/d of T-705. (****P<0.0001, vehicle compared to T-705 treatment)

Treatment with T-705 significantly (P<0.001) lowered virus titers in mice infected with all three strains as compared with untreated mice (Fig. 27). There was a fairly uniform decrease in viremia, with a decrease in titer around 10^6 $CCID_{50}$. NGR still proves to be the most robust strain, as mice infected with the strain and treated with T-705 still showed the highest average virus titer 3.4 $\log_{10}$ $CCID_{50}$ (Fig. 27, Table 10). Mice infected with BVI and treated with T-705 had viremia reduced to the low level of detection, with the lowest average virus titer of 0.8 $\log_{10}$ $CCID_{50}$ (Table 10).

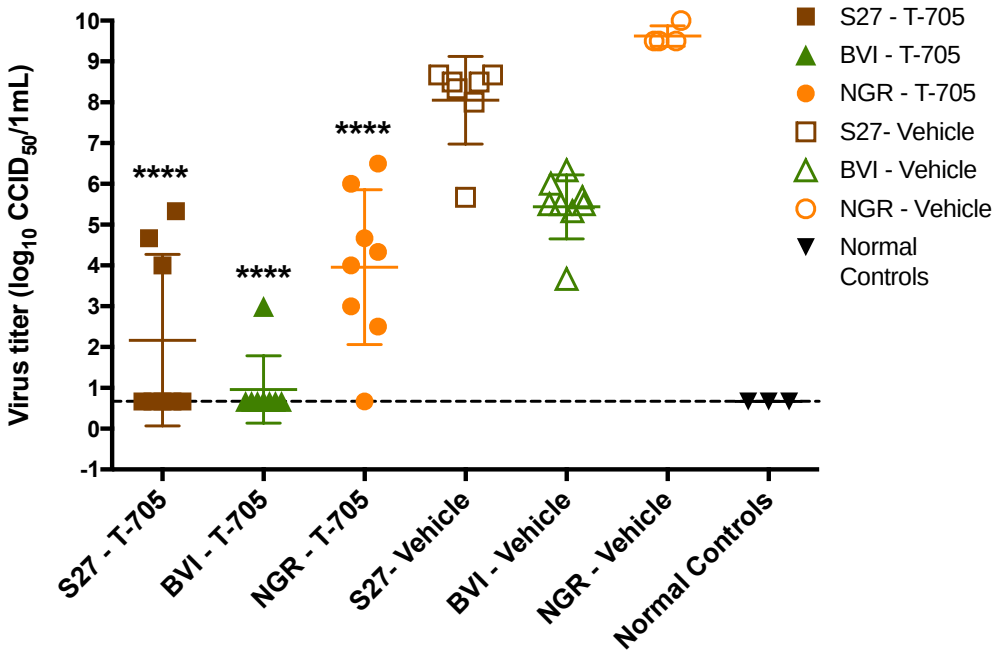


Fig. 27. Virus titer of serum collected 2 dpi from mice infected with S27, BVI and NGR CHIKV strains and treated with 400 mg/kg/d of T-705.
(****P<0.0001, as compared to vehicle treatment)

Antiviral treatment with T-705 significantly improved survival in mice infected with BVI and S27 strains but not with the NGR strain (Fig. 28). There was no statistical difference between survival data of treated and untreated mice infected with NGR. In groups of mice infected with S27 and BVI and treated with T-705, 4/8 and 5/8 mice survived, respectively (Table 10). However, all treated mice infected with the NGR strain succumbed to disease.

To confirm that the survival of mice in the study was due to T-705 treatment after successful infection with CHIKV, a Plaque Reduction Neutralization Test (PRNT) was conducted using serum acquired from the mice at day 14 post infection.

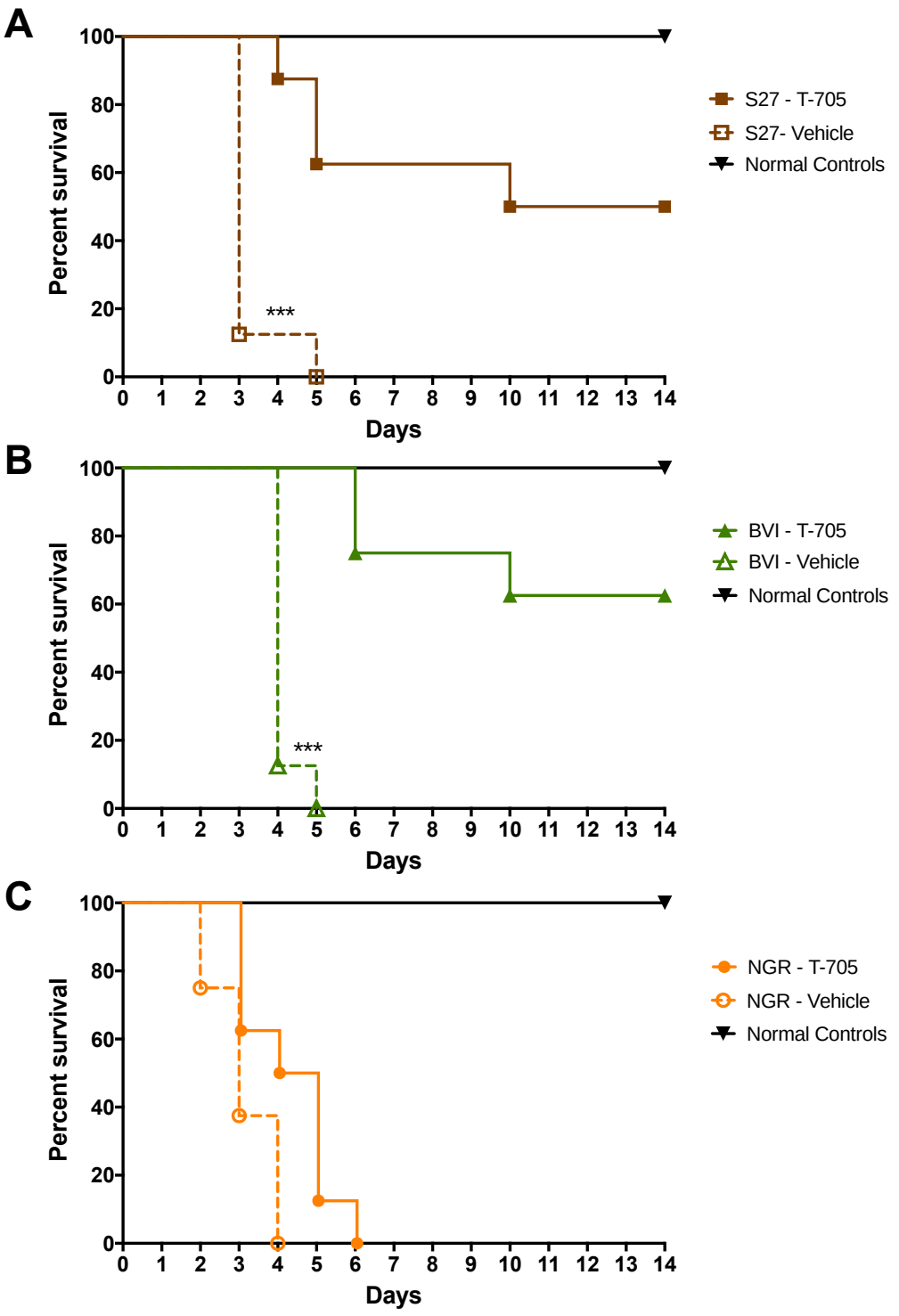


Fig. 28. Survival of AG129 mice challenged with (A) S27, (B) BVI and (C) NGR CHIKV strains and treated with 400 mg/kg/d of T-705. (***P<0.001 as compared to vehicle treatment).

Levels of neutralizing antibody present in mice treated with T-705 that survived S27 and BVI infections were $10^{4.1}$ and $10^{3.5}$ $PRNT_{50}$, respectively (Fig. 29). In addition to day 2 serum titers, this confirms the infection of the mice with S27 and BVI CHIKV strains and that successful immune response was established.

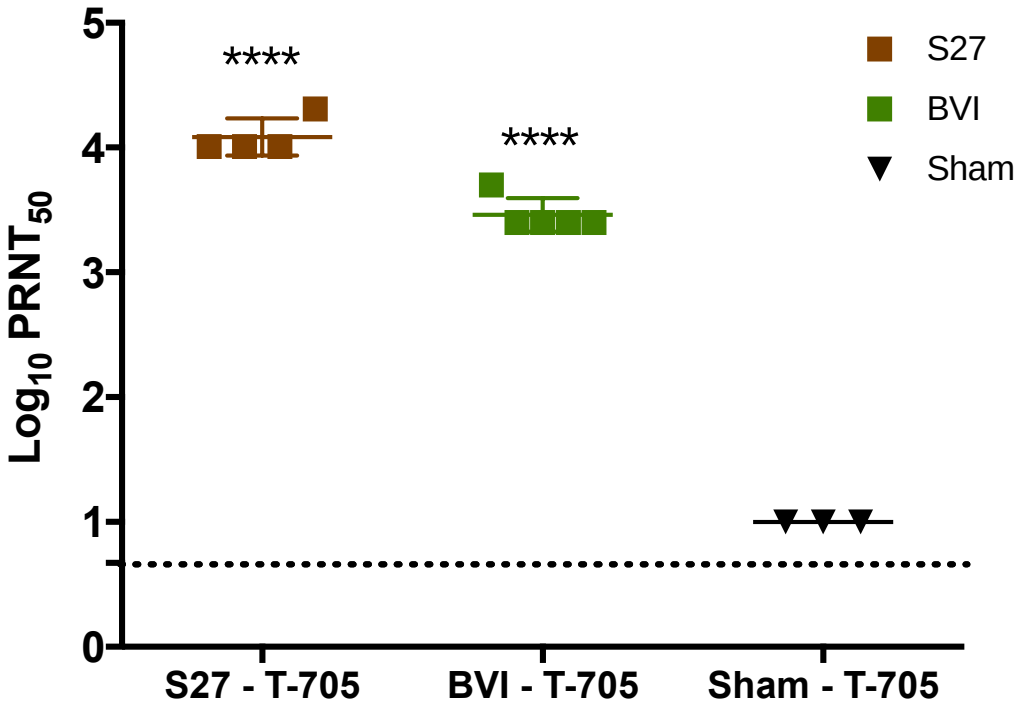


Fig. 29. Neutralizing antibody in mice treated with 400 mg/kg/d of T-705 and infected with S27 and BVI CHIKV strains. (****P<0.0001, as compared with sham infection).

Table 10
Serum titer & mortality of AG129 mice infected with S27, BVI and NGR CHIKV strains.

Treatment	Strain	Log_{10} serum virus titer[a] ± SD	Survival (alive/total)	Mean day-to-death ± SD
T-705	S27	2.1 ± 2.0	4/8	6.0 ± 2.7
T-705	BVI	0.8 ± 0.5	5/8	7.3 ± 2.3
T-705	NGR	3.4 ± 2.0	0/8	4.3 ± 1.2
Vehicle	S27	7.9 ± 1.4	0/8	3.1 ± 0.4
Vehicle	BVI	5.4 ± 0.8	0/8	4.1 ± 0.4
Vehicle	NGR	8.5 ± 0.0	0/8	2.9 ± 0.4
N/A	N/A	0.7 ± 0.0	3/3	>14.0 ± 0.0

[a]Serum was collected 2 dpi to determine virus titer.

5.4. Discussion

T-705 is an antiviral candidate that has broad activity against a wide range of RNA viruses. It has been tested in previous studies against the S27 and LR06 strains (Ahola et al., 2015). In the present study, the efficacy of T-705 was evaluated against three CHIKV strains belonging to different phylogenetic clades in the lethal AG129 mouse model.

T-705 significantly reduced viremia in AG129 mice that were infected with BVI, NGR and S27 CHIKV strains. T-705 also significantly improved survival of mice infected with the BVI and S27 strains. However, the most interesting finding in this study was that T-705 failed to significantly improve survival of mice infected with the West African strain, NGR. The NGR strain also replicated in the

AG129 mice more rapidly resulting in higher viremia compared to BVI and S27. This complements higher replication kinetics observed in *in vitro* experiments, establishing the strain's robustness.

Genetic contributions to phenotypic robustness observed after infection with the NGR strain have yet to be determined. Genetic mutations have been shown to be associated with increasing CHIKV virulence. The LR06 strain is associated with an E1: V226A mutation that allowed it to adopt to a new mosquito vector, *A. albopictus*, with a greater geographical range of infection than the original vector, *A. aegypti* (Tsetsarkin et al., 2007). Recently, Agarwal et al (2016), have identified two more mutations other than the E1: V266A mutation that also increase LR06 infectivity (Agarwal et al., 2016). The two mutations, E1:K211E and E2:V264A, are reported to increase infectivity, dissemination and transmission of CHIKV by 13, 15 and 16 folds, respectively (Agarwal et al., 2016).

Infection with the NGR strain of CHIKV resulted in a rapidly lethal and robust infection, although it is currently unknown if any genetic sequences contribute to this disease phenotype. A mutation that results in an amino acid difference has already been described in the BVI strain. The BVI strain has a mutation at the E2:113 position where the original valine amino acid is changed to an alanine (Lanciotti and Lambert, 2016). Although this mutation in the structural E2 protein could provide potential advantages in cell tropism and infectivity, according to our experiments, these mutations in the BVI strain do not appear to provide an enhanced disease phenotype in the AG129 mouse model.

In fact, BVI has been shown to be weaker than the other strains we investigated. In our experiments, T-705 treatment reduced viremia in AG129 mice infected with BVI to almost baseline levels. Mice infected with BVI also had the latest mean-day-to-death. In comparison, the NGR strain did not respond to T-705 treatment and resulted in lethality of all treated mice with the earliest mean-to-death.

Therefore, in the more stringent AG129 animal model, differences in disease phenotype and response to T-705 can be observed among the different strains of Chikungunya. Although CHIKV does not cause death in human clinical cases, the AG129 model can be a useful tool in evaluating potential drugs against various strains of CHIKV to establish global efficacy.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

Chikungunya virus has become a major public health concern since its reemergence in 2004 (Handler et al., 2017). Since there are no approved antiviral drugs or vaccines to treat or prevent CHIKV, it is of critical importance to identify potential candidates that can be used against the virus. T-705 has previously shown efficacy against CHIKV (Delang et al., 2014). Therefore, it is important to evaluate its range of efficacy against various strains of the virus.

In our studies, the responses of BVI, IND, SEN, NGR, S27 and LR06 CHIKV strains to T-705 treatment were investigated *in vitro* and *in vivo*. Our results in cell culture indicated that T-705 had some slight differences in efficacy, with a pattern towards greater efficacy against Asian lineage CHIKV strains. The strains also displayed slight differences in replication curves in RD cells.

Similarly, mice infected with different strains of CHIKV exhibited different disease phenotypes. DBA/1J mice infected with LR06 CHIKV showed an approximately 80% increase in footpad swelling. Mice infected with the S27, NGR and SEN strains showed 40 - 50% increase in footpad swelling. Both LR06 and S27 are grouped under the ECSA clade, while NGR and SEN belong to the West African clade. The two Asian clades, BVI and IND, did not cause significant measurable foot pad swelling in DBA/1J mice, demonstrating a difference between clades in regard to disease phenotype. Infection with another Asian strain from Thailand (isolate AF15561) also failed to cause footpad swelling in

C57BL/6 mice, even after adaption of the virus by passaging it nine times in mice (Gardner et al., 2010), further confirming our work in DBA/1J mice.

A similar differential response to T-705 treatment was observed *in vivo*. In the DBA/1J model, 400mg/kg/d of T-705 uniformly reduced footpad swelling in mice infected with NGR, SEN and LR06. Treatment with 100 mg/kg/d of T-705 reduced footpad swelling, significantly in mice infected with LR06 and SEN, on day 6, but footpad swelling increased thereafter in treated mice. Interestingly, viremia was reduced to baseline levels in mice infected with LR06 and treated with 100mg/kg/d of T-705, but not in mice infected with NGR and SEN despite significant reductions.

In a lethal model of CHIKV infection in AG129 mice, treatment with T-705 (400mg/kg/d) improved survival in mice infected with BVI and S27 strains, but failed to improve survival in mice infected with NGR. Since NGR and SEN showed similar disease profiles in DBA/1J mice, it would be likely that T-705 would also have no effect on the survival of AG129 mice infected with SEN. This should be investigated in future studies.

Finally, an important step forward to understanding differences in disease phenotype and response to antiviral treatment among CHIKV strains would be to conduct genotypic analysis. Previously, mutations in CHIKV have been associated with increased infectivity and adaption to a new vector (Agarwal et al., 2016). However, the effect of mutations in causing differential response to antiviral treatment needs to be researched and further investigated.

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