5-2016

Amelioration of Chikungunya through Inhibition of the Inflammatory Response

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AMELIORATION OF CHIKUNGUNYA THROUGH INHIBITION OF THE INFLAMMATORY RESPONSE

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Animal Health and Disease

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UTAH STATE UNIVERSITY
Logan, Utah

2016

ABSTRACT

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by

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Utah State University, 2016

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Chikungunya (CHIK) is an emerging viral di7sease, which causes significant morbidity and mortality throughout tropical/subtropical areas of the world, including a recent outbreak in the Americas. Disease typically includes fever, rash, and arthritis. Joint involvement is generally self-limiting, but infection with Chikungunya virus (CHIKV) can lead to chronic debilitating arthritis that can last for months to years. With no vaccine and no licensed treatment, suitable animal models of CHIKV are needed to test intervention strategies. We developed a model of CHIK in DBA1/J mice that develop joint swelling, increase in inflammatory cytokines and splenomegaly in mice, which include important symptoms of disease seen in infected humans. We used this model to test the hypothesis that treatment

with immune-modulatory compounds would ameliorate disease. GP1681, which suppresses TNF-α, IL-1β, and IL-6, exacerbated CHIK as indicated by increased footpad swelling and viral load. Prophylactic treatment with mDEF201, an adenovirus-vectored interferon, reduced disease, including joint swelling, virus titers at the site of virus challenge and inflammatory cytokines (IL-6, MCP-1, MIP-1α, and RANTES), although efficacy waned as treatment initiation was extended beyond virus challenge. Methotrexate treatment was also effective at ameliorating joint swelling and other disease parameters. Actemra (ACT), an anti-IL-6 antibody, reduced IL-6 levels to baseline, although the resulting improvement in footpad swelling was not significant. Combination therapy with methotrexate and ACT resulted in reduced footpad swelling. Based on our results, immune modulators have potential for the treatment of CHIKV and some of the compounds tested might have potential for clinical developmental.

(100 pages)

PUBLIC ABSTRACT

Amelioration of Chikungunya through Inhibition of

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Ashley L. Dagley

CHIK is an emerging viral disease that is rapidly spreading around the world and causing significant illness in infected people. This virus is spread through the bite of an infected mosquito. Symptoms of disease include high fever, rash, joint pain and arthritis-like symptoms. This disease has recently been reported in the United States, mainly as a result of importation from vacationers to areas affected by this disease. The purpose of this research was to model the disease in mice in order to identify intervention strategies that reduce disease in the hope that it will be useful in the treatment of infected humans. To do this we characterized disease in an animal model that supported virus growth and displayed important signs of disease similar to those seen in infected human patients. Mice developed swollen footpads after inoculation of virus into the footpad. Microscopic examination also revealed signs of muscle disease.

The therapeutic interventions we chose to test in the mouse model included mDEF201, GP1681, an IL-6 antibody (ACT) and methotrexate. Disease was either improved, made worse, or was unaffected, depending on the treatment administered. We demonstrated activity of mDEF201 and methotrexate in improving disease in mice, while ACT showed a slight trend towards improvement and GP1681 made the disease worse. The model appeared to be suitable in identifying active compounds, but further research needs to be done to verify these benefits in a clinical setting.

ACKNOWLEDGMENTS

I would like to thank Dr. Justin Julander for his patience and persistence while I was working on this degree. He has taught me so much, from how to restrain a mouse to the dedication required to write a journal publication, and I can't thank him enough for taking me on as a graduate student. Dr. Kerry Rood was instrumental in my career path, if it wasn't for him I would be pacing the floors at some meat packing plant and I wouldn't have been introduced to Dr. Julander, so thank you Dr. Rood for taking the time to discuss my career path with me. Dr. Brian Gowen, as a committee member, and mentor I appreciate your calm, cool demeanor and willingness to work with me.

I wouldn't be the person I am without my wonderful husband, Trent Dagley, who always encouraged me to finish my degree, even when it seemed impossible. He also gave me our two wonderful boys, Taren and Trey, who probably slowed down the degree process but were worth every minute. I want to thank Tristan Dagley for putting up with my OCD; you're a great kid and I'm lucky to be your mom.

I want to thank everyone at the IAR who is working, was working or will work there someday because it is a great place to work and I have really

enjoyed my time there. All the IAR PIs have helped me with one issue or another that has improved me scholarly and/or personally, Dr. Craig Day, Dr. John Morrey, Dr. Dale Barnard, Dr. Donald Smee, and Dr. Bart Tarbet; thank you all, it has been a pleasure working with you. Thank you Neil Motter, Isaac Wong, and Deanna Larsen for teaching me so much about working in a lab environment, you have provided me with a wealth of helpful information. Thank you Dr. Aaron Olsen and the LARC staff, I couldn't have done my research without you. I also want to thank my long-time friend, Ashley Longmore, who gave me this crazy idea to do a master's degree, and also to thank her for finishing her degree because it gave me the motivation to finish as well.

Ashley L. Dagley

CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

Originally described in a 1955 epidemiology report, Chikungunya (CHIK) roughly translates in Makonde to "*that which bends up"* (Ross, 1956) in reference to the stooped posture developed as a result of the arthritic symptoms of the disease (Seijo et al., 2014). This typically self-limiting disease occurs after infection with Chikungunya virus (CHIKV), which is spread by mosquitoes and probably originated in Central/East Africa where it circulates in a sylvatic cycle between mosquitoes and nonhuman primates (NHPs).

The arthralgia associated with CHIK can be debilitating. Recent adaptive mutations of this virus (Wasonga et al., 2015) include improved host immune system evasion and adaptation to different mosquito vectors (Kumar et al., 2014). Such changes are linked to the rise in mortality rate from rare (Mavalankar et al., 2008) to 1/1000 (Solignat et al., 2009) that was associated with the La Reunion outbreak. CHIKV can rapidly spread through naïve populations, which has been shown with the La Reunion outbreak and recent spread through the Caribbean (Lum and Ng, 2015). With no vaccine and no licensed treatment there is a great need to conduct

research on CHIKV to increase our understanding of this disease and identify ways to prevent or treat infections.

The purpose of this research was to develop an animal model capable of replicating symptoms of CHIK in humans, which we accomplished by infecting DBA1/J mice in the footpad to induce joint swelling, myositis and tenosynovitis (Dagley et al., 2014). We have also shown the utility of this model by testing 4 different compounds (mDEF201, GP1681, methotrexate and anti-IL6), which show varying results from protection to exacerbation.

With CHIK observed more frequently in the United States, it has become an important focus for research, but sadly we are lacking a lot of information on natural infection, including the interplay between virus and host immunological processes during CHIK. The mechanisms underlying severe disease are poorly understood. This is especially important in regard to immune therapy. We hypothesize that modifying the immune response will ameliorate disease in CHIKV infected mice by limiting the immune-mediated pathology. We tested this hypothesis with several different strategies and the results of our studies demonstrate the complexities associated with immunomodulatory strategies.

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2. LITERATURE REVIEW

2.1 Overview of Chikungunya and associated symptoms

CHIKV is an alphavirus that was first identified in Africa in the 1950s where it was given its name that in Makonde means "*that which bends up* " (Ross, 1956). This virus has been identified in 80 countries across 5 continents and caused more than 6 million cases of human disease (Petitdemange et al., 2015). CHIKV has led to several outbreaks in Africa and Asia and has affected more than 3 million people in the Indian ocean zone, and led to the establishment of autochthonous disease cases in Europe during the 2005-2007 outbreak (Hoarau et al., 2010). In 2005-2006, 266,000 cases (1/3rd of the population) were reported in La Reunion Island. This outbreak was unprecedented as many of the cases exhibited atypical manifestations, including severe complications in adults (persistent arthralgia, arthritis, and neurological complications), encephalitis in newborns and increasing human morbidity (Hoarau et al., 2013). CHIK fever has also been documented in many areas including France, Italy, Australia and the USA, where international travelers have facilitated the introduction of the virus from endemic areas (Mahendradas et al., 2013).

CHIKV is spread to humans by the bite of an infected mosquito. The typical vector is *Aedes aegypti*, but in 2006 a mutation in the E1 envelope gene allowed more efficient transmission of the virus by *Aedes albopictus,* a mosquito species that has a wide distribution throughout the world, including the United States (Sourisseau et al., 2007). This mutation is postulated to have facilitated the replication and transmission of the virus in mosquitoes by reducing the cholesterol dependence of the virus (Mahendradas et al., 2013). This vector also associates in more peridomestic locations, which can allow the virus to cycle from human to mosquito to human due to high viremia (10^7 pfu/ml average) in infected individuals (Thiberville et al., 2013).

The *Alphaviruses* are divided into two groups; one that causes arthritis (CHIKV, Semliki Forest virus, O'Nyong Nyong virus, Ross River virus and Barmah Forest virus) and another that causes encephalitis (western, eastern, and Venezuelan equine encephalitis viruses) (Leung et al., 2011). These are enveloped, positive-sense, single-stranded RNA viruses that are approximately 12 kb in length. The genome of CHIKV codes for capsid protein (CP), 2 surface envelope glycoproteins (E1 and E2), 2 small peptides (E3 and 6k) and 4 nonstructural proteins (nsP 1-4) (Voss et al., 2010). The

CP is multifunctional and plays a crucial role in the assembly and budding phase (Goh et al., 2015). The replication cycle begins with the attachment of E1 to unknown cellular receptors, which leads to fusion of the viral particle to the host cell membrane. After virus entry, the positive-sense genomic RNA acts directly as a messenger RNA to produce nonstructural proteins, which synthesize the structural proteins required for viral encapsidation and budding (Gasque et al., 2015).

This viral infection is typically self-limiting and disease manifestations generally include fever, swollen joints/joint pain and rash. Arthralgia is reported in 87-98% of cases (Thiberville et al., 2013) but can progress to a debilitating chronic arthritis, similar to RA. Typically, joint pain is found in the ankles, wrists and phalanges (Simon et al., 2007). The incidence of arthritis accompanying CHIK can be devastating. For example, eighteen months after the 2006 outbreak in La Reunion, 60% of persons infected were still reporting joint pain (Gerardin et al., 2012). CHIK is endemic in many underprivileged and/or rural parts of Africa and India, which have documented a significant economic burden due to this disease (Gerardin et al., 2012). This study also emphasizes the importance of controlling the perinatal mother-to-child spread of virus infection, which can

lead to severe disease in infants born to infected mothers, including developmental delays or encephalopathy (Gerardin et al., 2014). Severe cases of CHIK may include neurologic or hepatic manifestations (Schuffenecker et al., 2006), and occur more often in immune compromised individuals (Das et al., 2010). Since late 2013, CHIKV has reached the shores of the Americas, causing more than a million cases of infection (Lum and Ng, 2015). Mosquito sampling conducted in Southern Mexico in 2014 yielded 32.3/1,000 mosquitos positive for CHIKV; an accompanying lack of herd immunity and poor vector control in various parts of the Americas will only lead to an increased spread throughout this area (Diaz-Gonzalez et al., 2015). With increased tourism, and world travel, recent outbreaks of CHIKV in Europe and the Americas make this virus one of critical research importance, especially since there is no vaccine and only supportive therapy for treatment.

2.2 Immune response to Chikungunya

The bodies' innate immune response to CHIKV includes type-1 interferon (IFN) signaling through its receptor (IFN-I-R), which is present on most cells including natural killer (NK) cells. IFN has a strong anti-viral activity and activation of this protein through various pathways is the

primary response to virus infection. The interferon response promotes the transcription of genes for various antiviral proteins. These proteins interrupt steps in the viral life cycle, including virus penetration, and uncoating, transcription of viral mRNAs, synthesis of viral proteins, replication of viral genome, and assembly and release of progeny (Sen, 2001).

IFN works by activating the Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathways, stimulating production of IFN-stimulated genes (ISGs) of which there are 100s, that help establish a cellular antiviral state (Ivashkiv and Donlin, 2014). Regulation of this system is complex and often stochastic in that not all infected cells produce Type-1 IFN or express ISGs (Ivashkiv and Donlin, 2014). Type-1 IFNs also play a pivotal role during chronic infections, mediating the production, and function of inflammatory cytokines and suppressing the production of pathogenic cell types. This immunosuppressive state is thought to limit host toxicity, and morbidity during chronic infections, allowing for coexistence of host and pathogen (Ivashkiv and Donlin, 2014).

NK cells respond to infection with the help of cytokines, including interleukin (IL)-12 and IL-18, which then results in the killing of infected cells. NK cells mediate their antiviral effects through at least three different

mechanisms: (1) the release of immunoregulatory cytokines, particularly IFN-γ which enhance the innate immune response and help to shape the subsequent adaptive immune response, (2) the production of cytolytic granules for lysis of infected cells, and (3) the induction of target-cell death through cell surface receptors (Petitdemange et al., 2015). In CHIKV infection NK cells do not produce as much IFN-γ as they do with other infections, which is due to an immune evasion mechanism of the virus linked to modulation of $CD56^{bright}$ NK-cell subset (Petitdemange et al., 2015).

When infection by a pathogen is detected by antigen presenting cells, they secrete IL-12 and IL-18 that in turn stimulates the production of IFN-γ, a type II IFN (Schroder et al., 2004). IFN- γ also works through the JAK/STAT pathway. The C57BL/6 mouse strain produces a significantly higher amount of IFN- γ as compared with the commonly used BALB/c strain, leading to a greater resistance to bacteria and viruses in C57BL/6 mice (Schroder et al., 2004). IFN- γ induces a specific feedback inhibitor of itself, the suppression of cytokine signaling (SOCS)-1 protein, which associates with JAK to interfere with tyrosine kinase activity, and inhibiting further downstream stimulation of IFN-γ production (Schroder et al., 2004).

Although NK cells are typically beneficial in the acute phase of infection, they can also be harmful by maintaining an inflammatory environment (Long and Heise, 2015). Reduced joint pathology is also associated with limited NK cell activity (Teo et al., 2015). One of CHIKV's nonstructural proteins can also inhibit transcription in vertebrate cells, although the mechanism for this is unknown (Akhrymuk et al., 2012). Another immune evasion mechanism is a targeted block of late synthesis of antiviral mRNA transcripts, which are strongly induced by CHIKV and accumulate but are not synthesized into proteins, this is thought to occur through blocking global cellular protein synthesis (White et al., 2011). Monocytes are one of the main producers of IFN-α, and concentrations of IFN-α generally correlate with plasmatic viral load (Petitdemange et al., 2015), further underscoring the importance of a strong innate immune response, including IFN, in the control of acute CHIKV infection. People with an attenuated innate response, such as pregnant women, the very young, and very old, are more susceptible to CHIKV infection (Wauquier et al., 2011).

Monocytes, and macrophages have been suggested to be a cellular vehicle for virus dissemination as well as being a reservoir for persistent

CHIKV infection (Hoarau et al., 2010; Labadie et al., 2010). IFN-γ and IL-12 act synergistically to promote innate immune cell activation and both are increased in the majority of CHIKV patients (Ng et al., 2009). Since CHIKV can infect these cells, stimulation of their production results in an increased amount of susceptible cells that can increase viral titers, and enhance dissemination.

Elevated cytokines and growth factors are observed in humans and animal models infected with CHIKV. Growth factors are usually produced in response to injury and viral infections, such as CHIKV. Induction of cellular damage by the virus may lead to secretion of these factors. The increase of IL-1β, IL-6, and decrease in RANTES are associated with severe CHIKV infection; HGF, FGF-basic, and VEGF were also produced at high levels during infection (Ng et al., 2009). These cytokines are meant to protect the body from infection but can also cause more damage by eliciting an overactive immune response. There is a fine balance that must be maintained in the body to allow for activation during infection but down-regulation once the infection has passed. When this balance is not maintained it can lead to immune dysfunction and may include autoimmunity. A good example of this phenomenon, and a disease that

shares many features with CHIK, is rheumatoid arthritis (RA) where the immune response is targeted to self-proteins on bone and cartilage in response to cytokine dysregulation. Features of RA include increase in TNF-α, IL-1β, IL-6, and/or IL-17 (Yin et al., 2015), many of which are also elevated during CHIKV infection. A review by Teng identified a correlation between CHIKV load with arthralgia and an immune mediated signature dominated by pro-inflammatory cytokines (Teng et al., 2015).

Several cases of CHIK related, RA-like illnesses have been reported (10-20%), where the virus is observed in synovial macrophages but without erosion of the cartilage and bone observed in autoimmune RA (Hoarau et al., 2013). CHIK is similar to RA in that there is a common pattern of leukocyte infiltrate, cytokine production, and complement activation (Burt et al., 2012). Some cases of both show bony erosions, joint space narrowing, and test positive for rheumatoid factor and/or anti-CCP (antibodies used to diagnose RA) (Bouquillard and Combe, 2009).

2.3 Chikungunya Models of Infection

Models used for CHIK research include zebrafish, various species of mice, and non-human primates (NHPs). The complex interactions of the immune system with CHIKV make each model useful for studying different

aspects of infection. Small animal models are important for initial studies to identify options for therapeutic intervention.

A zebrafish animal model has been developed and used to visualize CHIKV infection and innate host response to infection (Palha et al., 2013). Fish are a useful research model because they are easy to manage and have an innate and adaptive immune response like that of mammals; but for the first month of life larval stage, zebrafish rely solely on innate immune responses (Langevin et al., 2013). Live imaging is also possible and easily done in fish. The antiviral compound, Suramin, was tested in fish and was shown to have some activity against CHIKV through binding to the E1/E2 glycoprotein to block viral entry and transmission (Ho et al., 2015).

Mice deficient in interferon receptors (i.e., AG129 or A129 mice) develop rapid mortality due to a lack of type 1 interferon signaling during infection (Couderc et al., 2008). Susceptibility to CHIKV is also age dependent in mice, suckling mice at 6 days old succumb to viral infection because they are lacking an adequate immune response mechanisms while at 9 days 50% survive infection (Couderc et al., 2008). Adult mice generally survive CHIKV infection. These models emphasize the importance of the interferon response and a developed immune response to resilience to CHIK

and give insight into tissue and cell tropism of the virus. Fibroblasts have been identified as the main cell type targeted by CHIKV in mice (Couderc et al., 2008).

In 2010 Gardner et al. found that young C57BL/6j mice were susceptible to CHIKV infection when infected in the footpad. This model produces measurable footpad swelling, myositis, and tenosynovitis (Gardner et al., 2010). Mice are by no means a perfect model for studying human viral infections and differ substantially in some mechanisms of pathogenesis and immune response, which may limit in some way their predictive value for pharmaceutical studies (Louz et al., 2013).

Recently Seymour et al. (2015) developed a persistent infection model to mimic chronic infection often seen in humans. Footpad measurements, viremia, viral load in tissues, and histological findings are used as parameters. This model is suitable for testing vaccines. It is in recombinant activating gene 1 (RAG1-/-) knockout mice, deficient in both T and B lymphocytes, and models infection of the immune compromised populations in CHIKV-endemic countries with a high prevalence of HIV and undernourishment. This model was used to demonstrate the occurrence of tissue damage in the absence of an adaptive immune response and to study

vaccine safety. Since this model is in immune-compromised mice, it has limited applicability in other studies with CHIKV where immune competence is important (Seymour et al., 2015).

In our research, we have found the DBA/1J mouse strain to have utility in preclinical testing of therapies that target both viral replication and the associated joint disease (Dagley et al., 2014). Infection of DBA/1J mice via subcutaneous injection into the footpad results in the development of high viral titers, joint swelling, and muscle degeneration. This model works with different strains of CHIKV including S27 (African strain) and LR2006 OPY1 (La Reunion 2006 emergent strain). Differential response of this mouse strain to different virus strains has been demonstrated and the S27 strain of the virus required approximately 1,000-fold higher titers in order to induce a similar disease as compared with the LR2006 OPY1 strain. Details on the development and use of this model are included in the following chapter.

A hamster model was recently developed that produces consistent infections showing high viral titer, inflammatory lesions involving skeletal muscle, fascia, and tendon sheaths of multiple limbs following subcutaneous infection (Bosco-Lauth et al., 2015). Hamsters make excellent models for

other arboviruses including West Nile virus (WNV) and Japanese Encephalitis virus (JEV) (Bosco-Lauth et al., 2011), because they are outbred, easily managed when compared to some models, and have an intact immune system.

CHIKV is maintained in a sylvatic infection cycle within populations of non-human primates (NHPs) and NHPs are routinely used as sentinels to alert health departments of the presence of CHIKV. NHPs have been used to model persistent infection as well and infected macrophages were found to remain in the liver and lymph nodes up to 6 months following infection (Labadie et al., 2010). Primate models are important in identifying and characterizing therapeutic or prophylactic strategies to treat CHIK infection prior to clinical trials in people (Labadie et al., 2010). NHPs have an intact immune system and mimic many of the viral, clinical, and pathological infection seen in humans, making them necessary in preclinical evaluation of vaccines and therapeutics, as well as for the discovery of mechanisms of CHIKV pathogenesis (Broeckel et al., 2015).

With all the animal models (Table 2-1) available, we are still severely lacking many important aspects of CHIKV pathogenesis, and most importantly there is no treatment and no vaccine available.

Table 2-1
Summary of CHIKV animal models, including utility, pros and cons of each discussed.

Animal	Strain	Utility	PRO	CON
Zebrafish		Visualize CHIKV infection, innate host response to infection, Suramin testing	Easily managed, cost effective	Far removed from humans
Mouse	AG129	Inefficient IFN signaling increases susceptibility	Pathogenesis of viral infection in highly susceptible immunocompromised populations	Immunocompromised
Mouse	C57BL/6	Antiviral and vaccine testing	Symptoms similar to humans	Differ from humans in pathogenesis and immune response
Mouse	DBA/1J	Antiviral and vaccine testing, mDEF201 testing	Consistent infection, high systemic viral titers, easily managed	Differ from humans in pathogenesis and immune response
Mouse	RAG -/-	Study innate immune response	Persistent infection	Immunocompromised
Hamster	Golden Syrian	Antiviral and vaccine testing, pathogenesis of CHIKV	Intact immune system, model used in other alphavirus testing, outbred model	Lacking some human aspects of disease
Nonhuman Primate (NHP)	Rhesus Macaque	CHIKV pathogenesis, antiviral and vaccine pre-clinical testing	Demonstrates chronic infection, many joints affected by viral inoculation	Expensive, few facilities available to manage BSL-3 conditions

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3. PROTECTION AGAINST CHIKUNGUNYA VIRUS INDUCED ARTHRALGIA FOLLOWING PROPHYLACTIC TREATMENT WITH ADENOVIRUS VECTORED INTERFERON (MDEF201)[1]

3.1 Abstract

Recent outbreaks of Chikungunya virus (CHIKV) infection have resulted in millions of cases of disease with significant morbidity. No approved antiviral treatments exist for the prevention or treatment of this viral disease. Infection with CHIKV results in a high rate of symptomatic disease that primarily includes a debilitating arthralgia. To model this cardinal disease manifestation, adult DBA/1J mice were challenged with CHIKV by footpad injection. Viremia and hind limb virus titers increased ~100-fold while spleen virus increased >1,000-fold within 1 day post-virus infection (dpi). Footpad swelling was measured over a 10-day period, with peak swelling observed between 6 and 7 dpi. Histology of the hind leg at the site-of-virus challenge showed evidence of myositis and synovitis starting on 5 dpi. Cytokine profiling of the hind limb at the site of inoculation revealed a biphasic inflammatory response represented by an increase in IL-6, MCP-1,

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IFN-γ, MIP-1α, RANTES, and IL-17. To investigate the prophylactic capacity of IFN, mice were treated with mDEF201, an adenovirus-vectored IFN-α. Intranasal administration of a single 10^7 pfu/ml dose of mDEF201 administered 21 days to 24 h prior to infection, significantly reduced footpad swelling, virus titers in the hind leg and spleen, and several inflammatory cytokines. Efficacy was not observed when treatment was initiated 24 h after virus challenge. This arthralgia model of CHIKV recapitulates relevant disease features commonly observed in human disease making it applicable to preclinical testing of therapies that target both viral replication and the associated joint disease.

3.2 Introduction

CHIKV virus (CHIKV) is associated with significant morbidity and mortality worldwide. Historically, the primary vector for spread of CHIKV was *Aedes aegypti,* although recent adaptive mutations within the virus have resulted in a more efficient infection of *Aedes albopictus* (Tsetsarkin et al., 2006). This has resulted in outbreaks outside the historical range, making this emerging virus a major public health concern.

The rapid emergence of CHIKV and the millions of cases of disease underscore the importance of the development of countermeasures to

prevent or treat the symptoms of CHIKV. Fever and arthralgia are common symptoms of CHIKV infection, although a small number (5-18%) of infected people, especially those below age 25, are asymptomatic (Dupuis-Maguiraga et al., 2012). Mortality as a result of CHIKV infection is rare and is generally associated with underlying health issues (Thiberville et al., 2013). Rheumatic manifestations typically affect the extremities, primarily including ankles, wrists and phalanges (Kennedy et al., 1980). A study conducted after the La Reunion Island outbreak identified persistent arthritis in over half of the participants 15 months after acute infection, with 43% of those with persistent arthritis significantly impaired in carrying out daily or household activities (Sissoko et al., 2009). As the rheumatic disease is debilitating and very common in persons infected with CHIKV, it would be important to prevent or treat this aspect of the disease.

An animal model that replicates human arthralgia is essential for the development of countermeasures for the prevention and treatment of disease associated with CHIKV infection. Macaques have been used to study CHIKV, and this model mimics many acute symptoms seen in humans such as fever, rash, and high viral titers but displays inconsistent swelling of the

joints and lacks the joint and muscle pathology typically observed after infection with CHIKV (Higgs and Ziegler, 2010).

Various mouse models have been developed which model different aspects of the disease. The C57BL/6 neonatal mouse model includes relevant disease manifestations such as joint swelling. There is also consistency between disease seen in children less than one year of age and this model, with virus being found in similar organs and a strong age-dependent correlation with severity of disease (Couderc et al., 2008). Interferon receptor knockout mice were highly susceptible to infection with CHIKV and rapidly succumbed to infection (Couderc et al., 2008), which serves to model severe infection and mortality that is occasionally seen. Infection of these immunocompromised mice results in dissemination of the virus to all tissue types, including the central nervous system (Partidos et al., 2011). This pathology may have relevance in light of recently reported neurologic complications of encephalopathy in newborns and meningitis and encephalitis in older children and adults (Arpino et al., 2009). Inoculation of the footpad of 14-day old C57BL/6 mice resulted in swelling, arthritis, tenosynovitis, and myositis (Morrison et al., 2011), but a neonatal model has limitations, especially in regard to testing vaccines. Others have developed a

model of footpad swelling and joint involvement in adult C57BL/6 by injecting virus into the footpad (Gardner et al., 2010).

A replication-deficient adenovirus type 5 vector containing a gene for mouse IFN-α, mDEF201, is administered intranasally where it enters nasal epithelial cells and expresses the transgene (Julander et al., 2011). This results in the production of systemic levels of IFN several hours after treatment (Wu et al., 2007). With non-lethal infections such as CHIKV, type I interferon (IFN) plays a role in coordinating early antiviral immune response (Havenar-Daughton et al., 2006), and plays a central role in controlling CHIKV infection, acting indirectly as an antiviral through the induction of IFN-stimulated genes that inhibit viral replication (Schilte et al., 2010). The nonstructural proteins (nsPs) of CHIKV antagonize the IFN response as infection progresses, so the timing of IFN intervention is critical (Fros et al., 2010).

This manuscript details the establishment of an adult mouse model of CHIKV arthritis and swelling. Footpad swelling, virus replication, and cytokine profiles are used as parameters for the characterization of the antiviral effect of mDEF201 against CHIKV. These results demonstrate the utility of this model for use in antiviral studies.

3.3 Materials and Methods

3.3.1 Cells

C6/36 cells were obtained from ATCC (Manassas, VA) and were used for virus propagation. These cells were grown in RPMI at 28˚C with 5% fetal bovine serum (FBS). Vero 76 cells, maintained in minimal essential medium (MEM) supplemented with 10% FBS, were also obtained from ATCC and were used in viral titer assays. Media was obtained from Hyclone Laboratories, Logan, UT.

3.3.2 Virus

CHIKV strain S27 (VR-64) was obtained from ATCC. Stock virus was prepared by harvesting supernatant from C6/36 cells 2 days after inoculation.

3.3.3 Animals.

Seven-week old DBA/1J mice were obtained from Jackson Laboratory. Mice were quarantined for 48 hours prior to infection.

3.3.4 Test Materials

A stock of mDEF201 was provided by Defyrus (Toronto, ON, Canada), which had a titer of 1.45 X 10^9 pfu/ml (construction of vector

described fully in Wu et al, 2007). An empty Ad-5 vector was also provided by Defyrus, which was used as a negative control.

3.3.5 Experimental Design

For model characterization studies, DBA/1J mice were randomly assigned to groups of 10 animals. Mice were challenged under isoflorane anesthesia by footpad and hock injection with 10^8 $CCID_{50}$/0.1 ml of virus (0.05 ml at each site) diluted in MEM. On days 1-10, 14, and 21, 4 mice were necropsied and serum, hind legs, and spleen were collected, weighed and prepared for virus titration, and cytokine profiling. Animals were also weighed throughout the study to determine the effect of infection on weight change.

For treatment studies, mice were randomly distributed into groups of 10 to 15 animals and were treated with mDEF201, empty vector or saline. Sham infected controls were also included to monitor potential toxicity of treatment. In the first study, animals were treated with a range of mDEF201 doses. At 2 dpi, 5 animals per group were necropsied and spleens and hind limbs were harvested, weighed, and the virus titer was determined. Virus titer and cytokine levels were determined from hind limb homogenates. The remaining animals were weighed on days 0, 2, 4, 6, 8, & 10. The

dorsoventral thickness of the footpad at the site of virus challenge was measured with a digital caliper and the percent increase, as compared to the contralateral foot, was calculated.

In subsequent studies, a dose of 10^7 of mDEF201 was administered intranasally at various times prior to or after virus challenge to determine the effect of extended prophylaxis or therapeutic treatment on CHIKV infection. Footpad increase in all animals was measured on 6 dpi as described above. Tissues were then harvested from a subset of animals on 6 dpi with virus and cytokine titers determined as described below.

3.3.6 Tissue virus titer determination

Samples were homogenized and diluted in 1 ml MEM, containing 0.05 mg/ml gentamicin. The homogenate was centrifuged for 10 min at 2300 RCF added to Vero 76 cells in triplicate and examined for cytopathic effect (CPE) on 3 dpi. Virus titers were determined by end point titration as described previously (Reed and Muench, 1938).

3.3.7 Cytokine analysis

Homogenized leg samples were evaluated for proinflammatory cytokine/chemokine levels using the Quansys Q-plex™ Mouse Cytokine Array (Quansys Biosciences, Logan, UT). The Quansys array quantifies

levels of murine IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL12p70, IL-17, MCP-1, IFNγ, TNFα, MIP-1α, GMCSF and RANTES. Samples were diluted in sample diluent at 1:5 and 1:20 concentrations. A standard curve for each antigen was generated by serial dilution of antigen standards from 1:3 to 1:729. Standards were plated in duplicate on opposite sides of the plate and samples were plated at two dilutions. Manufacture's instructions were followed for adding substrate, incubating and washing. Plates were quantified on a Quansys Q-View™ Imager with Q-View™ software.

3.3.8 Histopathology

Right and left hind legs were collected at 6 and 12 h, and days 1-10, 14 and 21 post-infection. Samples were fixed in 10% formalin and sent for analysis to the Utah State University Veterinary Diagnostic Laboratory. Samples were paraffin-embedded and sectioned for histopathologic evaluation. Slides were stained with hematoxylin and eosin.

3.3.9 Statistical analysis

Statistical analyses were done using one-way ANOVA using a Bonferroni group comparison (Prism 5, GraphPad Software, Inc).

3.4 Results

3.4.1 Characterization of CHIKV infection of DBA/1 mice

Several mouse strains, including C57BL/6, AG129, CAST/EiJ and DBA/1J, were inoculated with CHIKV and disease signs, including footpad swelling, differences in behavior, weight change and mortality, were observed. The DBA/1J strain was selected based on the development of robust footpad swelling, availability and use in arthritis research (Chiba et al., 2012, Eros et al., 2009 and Nishida et al., 2002). To characterize CHIKV replication and disease in this mouse strain, DBA/1J mice were inoculated with S27 CHIKV in the rear hind footpad and footpad swelling, virus titer of various tissues, cytokine levels, and weight change were quantified. Significant swelling of the footpad, ankle and hock at the site of virus challenge was observed as early as 6 dpi (Fig. 1A). The contralateral footpad was normal and served as a control for measuring percent increase in footpad thickness. Swelling was time-dependent and showed a dose responsive increase depending on virus challenge dose (data not shown). Significant swelling ($P < 0.001$) of the footpad was initially observed on 6 dpi, with peak swelling on 7 dpi. Footpad thickness returned to baseline levels by 9 dpi. Average virus titer in the right hind leg at the site of

inoculation was approximately 6 $\log_{10}$ $CCID_{50}$/g at 6 h after virus challenge (Fig. 1B), which was due to the footpad inoculation at this site. Titers increased approximately 100-fold to just below 8 $\log_{10}$ $CCID_{50}$/g at 24 h after virus challenge. The right hind leg virus titers persisted at an average titer of 6–7 $\log_{10}$ $CCID_{50}$/g until 6 dpi, at which point they began to return to baseline levels and were below the limit of detection on 8 dpi. In contrast, no virus was observed in the left hind leg contralateral to the injection site.

Virus titer in the spleen was detected as early as 12 h after virus challenge. Spleen titers peaked at around 7 $\log_{10}$ $CCID_{50}$/g tissue, which was >1000-fold increase in virus above the level of detection in that organ (Fig. 1B). Virus was reduced below the limits of detection by 4 dpi. A similar increase in serum virus was also observed, although a more rapid clearance of viremia was observed with virus titer reduction to undetectable levels a day earlier on 3 dpi as compared with spleen titers (Fig. 1B). No virus was detected in any of the tissues assayed from sham-infected animals (data not shown).

Splenomegaly was observed in mice infected with CHIKV as early as 2 dpi, with spleen size continuing to increase through 10 dpi (Fig. 1C). Weight change of CHIKV-infected mice did not differ significantly from

that of sham-infected animals (data not shown).

Histologic analysis of the right hind limbs demonstrated cellulitis, myositis, tendinitis, and teno and arthrosynovitis as early as 5 dpi, which continued over the course of the infection (Fig. 2). No significant histological lesions were seen on the left hind limb (Fig. 2A and C). Several proinflammatory cytokines were significantly elevated in tissue homogenate of the limb inoculated with CHIKV. There appeared to be a biphasic increase in cytokine levels in the hind leg with an initial peak in IL-6, MCP-1, IFN-γ, MIP-1α, RANTES, and IL-17 around 1 dpi with a subsequent peak in levels 5–7 dpi (Fig. 3). Other cytokines, including IL-1α, IL-1β, IL-2, IL-3, IL-5, IL-10, IL-12, TNF-α, and GM-CSF did not appear to be affected by CHIKV infection of mice as compared with control mice (data not shown). Cytokine levels in the serum of infected animals were not significantly altered after CHIKV infection (data not shown). There did appear to be some slight increase in serum IL-6, MCP-1, MIP-1α, and RANTES on 2 dpi, which corresponded with the initial peak observed in the hind limb at the site of virus inoculation, but the second peak around 8 dpi was only observed with IL-6 and MCP-1 (Table 1), suggesting a more localized effect at the site of inoculation. Cytokine titers in the spleen were similar to those in the

serum, with the exception of RANTES levels that were elevated 20–87-fold in the spleen homogenates as compared with serum on 2 and 9 dpi (Table 1). Sham-infected animals also had higher levels of RANTES in the spleen as compared with serum, suggesting higher baseline levels in this tissue.

3.4.2 Treatment of CHIKV with mDEF201

To determine the effect of mDEF201 treatment on the development of disease after CHIKV infection, animals were treated with a single intranasal instillation 24 h prior to virus challenge. Evaluation parameters used to determine mDEF201 efficacy included percent footpad swelling as compared with the contralateral foot, virus titers in the hind leg and spleen and cytokine levels in the hind leg.

Footpad swelling was significantly ($P < 0.001$) reduced on 7 dpi in animals treated with 10^7 pfu/ml of mDEF201 as compared with placebo- and empty vector-treated controls and was comparable with sham-infected controls (Fig. 4A). A significant ($P < 0.001$) reduction of viral load was observed on 2 dpi in hind limbs and spleens of animals treated with 10^7 pfu/ml of mDEF201 as compared with placebo- or empty vector-treated animals (Fig. 4B and C, respectively). Average titers in these tissues were reduced approximately 2 $\log_{10}$ $CCID_{50}$/g.

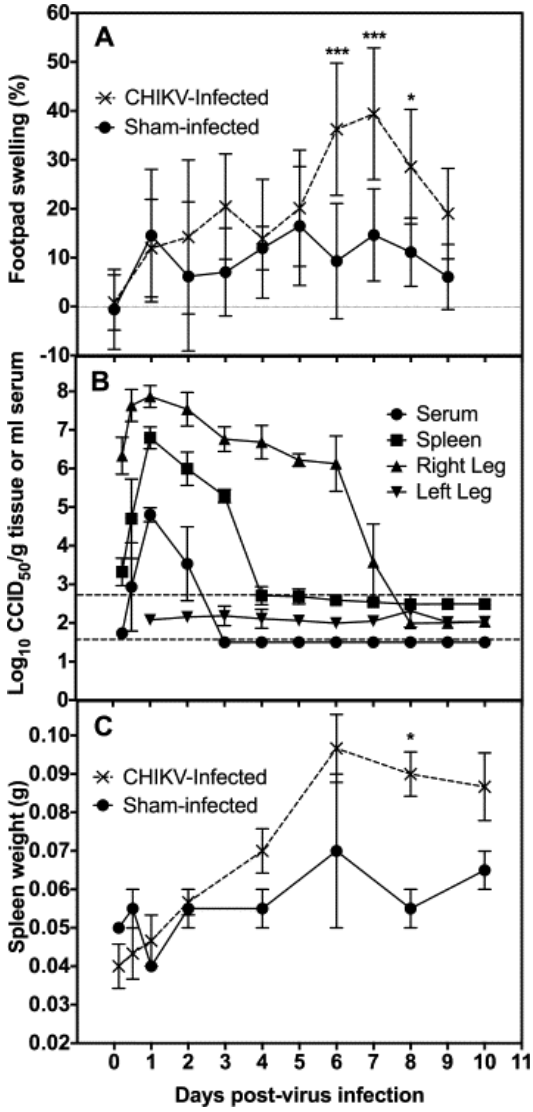

Fig. 3-1. Disease parameters of mice infected with the S27 strain of CHIKV. (A) The effect of CHIKV infection on the time course of footpad swelling of DBA/1J mice at the site of virus challenge when compared to contralateral footpad. (B) Time-course of virus in serum, spleen, right hind leg, and left hind leg of DBA/1J mice infected with CHIKV. Viral titer is plotted as 50% cell culture infectious dose ($CCID_{50}$) per gram of tissue or ml of serum. Upper dashed line is the average limit of detection for tissue homogenates and lower line is for serum. (C) Comparison of Sham infected and CHIKV infected spleen weights in grams throughout course of infection ($^{***}P < 0.001$, $^{*}P < 0.05$, as compared with sham-infected controls).

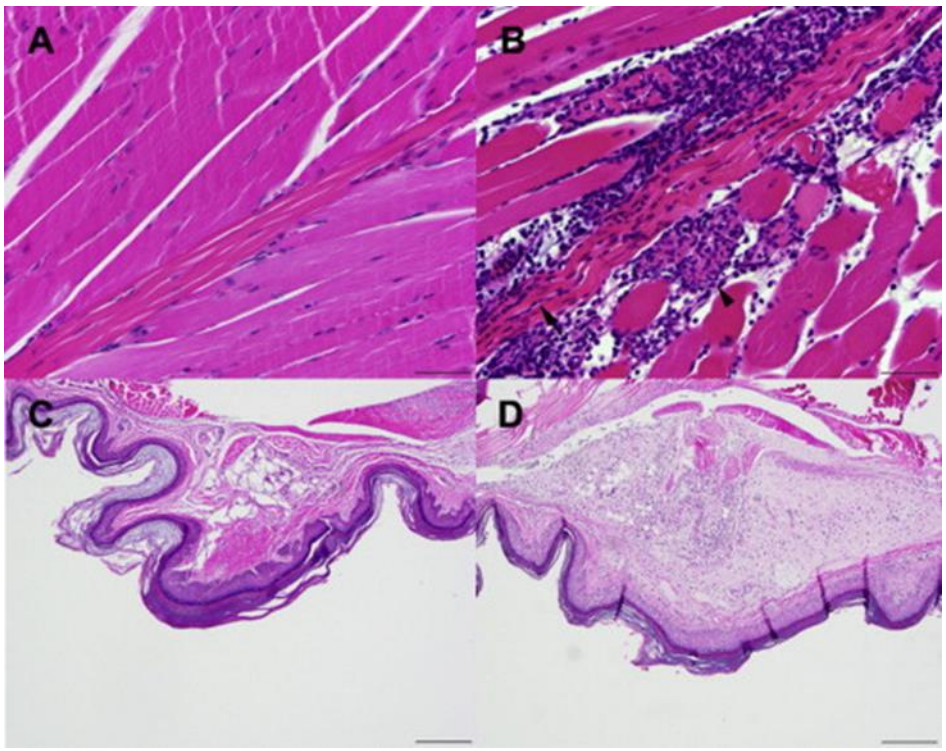

Fig. 3-2. Hematoxylin and eosin stained sections of skeletal muscle (A and B) and footpads (C and D) show pathologic changes after CHIKV infection. (A) Normal skeletal muscle taken 5 dpi from control mice. (B) Myositis (arrow head) and tendinitis (arrow) are apparent in the skeletal muscle of a CHIKV-infection mouse taken on 5 dpi (400× magnification of skeletal muscle, bar = 50 μm). (C) Normal footpad section from a mouse on 7 days after mock infection. (D) Cellulitis due to mixed inflammatory cell infiltration is observed on 7 dpi in footpad sections taken 7 dpi from mice challenged with CHIKV (200× magnification of footpad sections, bar = 200 μm).

Table 3-1
Serum and spleen levels (pg/ml) of selected cytokines of DBA/1J mice challenged with CHIKV. Virus challenge did not appear to cause an increase in cytokines in these tissues as compared with cytokine levels at the site of virus challenge.

		2 dpi				9 dpi			
Tissue	Challenge	IL-6	MCP-1	MIP-1a	RANTES	IL-6	MCP-1	MIP-1a	RANTES
Serum	CHIKV	6.6 ± 9.4[a]	223 ± 147	10 ± 0.8	50 ± 20	10.4	383 ± 503	14 ± 0.8	35 ± 1.8
	Sham	bdl[b]	25	9.9 ± 2.0	31 ± 0.8	bdl[b]	26 ± 0.4	16 ± 4.4	34 ± 1.1
Spleen	CHIKV	bdl[b]	107 ± 41	219 ± 181	4351 ± 661	9.9 ± 1.6	29 ± 4.3	20 ± 9.1	669 ± 7.7
	Sham	bdl[b]	26	28	1056	bdl[b]	26	23	1959

a Mean ± standard deviation for 2 samples is shown. Values with no SD listed are from single data points.
b Below detection limit

Footpad swelling and tissue titers in groups treated with lower doses of mDEF201 were similar to those parameters of saline- or empty vector-treated controls and had no observed effect on disease (Fig. 4A–C).

Reduced levels of key cytokines, including IL-6, MCP-1, MIP-1α, and RANTES, were observed on 2 dpi in the virus-inoculated hind limb of mice treated with the highest dose of mDEF201 as compared with saline treatment (Fig. 4D–G, respectively). These reductions were significant ($P < 0.05$) for MCP-1 and RANTES (Fig. 4E and G, respectively). In a subsequent study, cytokine levels from hind leg samples taken 6 dpi from animals treated with 10^7 mDEF201 or with saline were quantified and showed a similar trend towards reduction of IL-6, MCP-1, and MIP-1α (Fig. 4H–J, respectively), as well as a significant ($P < 0.001$) reduction in RANTES (Fig. 4K).

To determine the effect of treatment with mDEF201 after virus challenge, 10^7 pfu was administered 24, 48 or 72 h post-inoculation. Administration of mDEF201 at these time points failed to significantly impact footpad thickness, virus titer or reduction of cytokine levels in the hind limb (data not shown). As a positive control to this study, mDEF201 was administered at 24 h prior to virus challenge, which reduced footpad thickness as in previous studies. Virus titers measured in hind limb samples

taken on 6 dpi, however, were not significantly reduced (data not shown), despite a previously observed reduction of virus titer on 2 dpi after treatment with DEF201 given along the same schedule (Fig. 4B).

Extended prophylaxis evaluation was conducted to test the efficacy of mDEF201 when administered as early as 21 days prior to infection. Swelling of the footpad was significantly reduced (*P*-value <0.001) in animals treated one day prior to virus challenge (Fig. 5A), as seen in previous studies. A single treatment with mDEF201 on 7, 14, or 21 days prior to virus challenge prevented footpad swelling at the site of virus inoculation (right hind leg). Treatment at these time points resulted in footpad thickness similar that observed for sham-infected, treated controls, and were significantly ($P < 0.001$) lower than that of infected animals treated with empty vector or with placebo (Fig. 5A).

Treatment of mice with mDEF201 1 or 7 days prior to virus challenge had no effect on reducing virus replication in the hind leg (at the site of virus challenge) as measured on 6 dpi (Fig. 5B). However, mice that were treated 14 or 21 days prior to virus challenge had significantly lower ($P < 0.01$) virus titers than animals treated with empty vector or placebo.

Cytokine levels were measured from hind leg homogenates of the inoculated limb (also used for virus titration), which were taken from a subset of animals on 6 dpi. In general, treatment with mDEF201 at all time points prior to virus challenge resulted in significant reduction ($P < 0.001$) in IL-6, MCP-1, MIP-1α, and RANTES when measured 6 dpi (Fig. 5C–F, respectively). One exception was the lack of significant reduction of IL-6 in animals treated one day prior to virus challenge (not shown), which was consistent with previous results (Fig. 4H).

3.5 Discussion

Infection of DBA/1J mice with CHIKV mirrors several disease signs observed in humans, including arthralgia, high acute viral titers and increased cytokine levels (Couderc and Lecuit, 2009). This non-lethal model focuses primarily on the arthritic disease manifestations that occur most commonly in infected individuals. The primary clinical and economic burden of human CHIKV disease is arthralgia, which renders infected individuals unable to work and perform other necessary activities, which in a setting of limited resources, can be a large drain to the financial wellbeing of hundreds of thousands of people. Treatment or prevention of arthralgia in

infected individuals, therefore, would be very important to reducing the burden of this viral disease.

Increased levels of several cytokines, accompanied by swelling and severe tissue damage, were observed in mice infected with CHIKV 6-7 dpi. Infected patients have elevated IL-6, GM-CSF and MCP-1 during infection (Chirathaworn et al., 2013) (Lohachanakul et al., 2012), further supporting the relevance of this mouse model to human infection. In addition, mDEF201 prophylactic treatment alleviated cytokine elevation and footpad swelling. The immune response to infection, including recruitment of macrophages by MCP-1 and activation of these cells by IL-6 within the joint, plays an important role in the immunopathogenesis associated with CHIKV infection and is a common mechanism of disease of various viral arthritides (Suhrbier and Mahalingam, 2009). It is likely that the arthrogenic immune response in CHIKV infection is excessive and may be targeted as an immunopathology, as evidenced herein by progressive clearance of the virus as the joint swelling and cytokine response mounts. This must be further delineated, but future studies will focus on targeting specific aspects of the immune response to determine the effect of immunotherapy as a treatment for arthralgia associated with CHIKV infection.

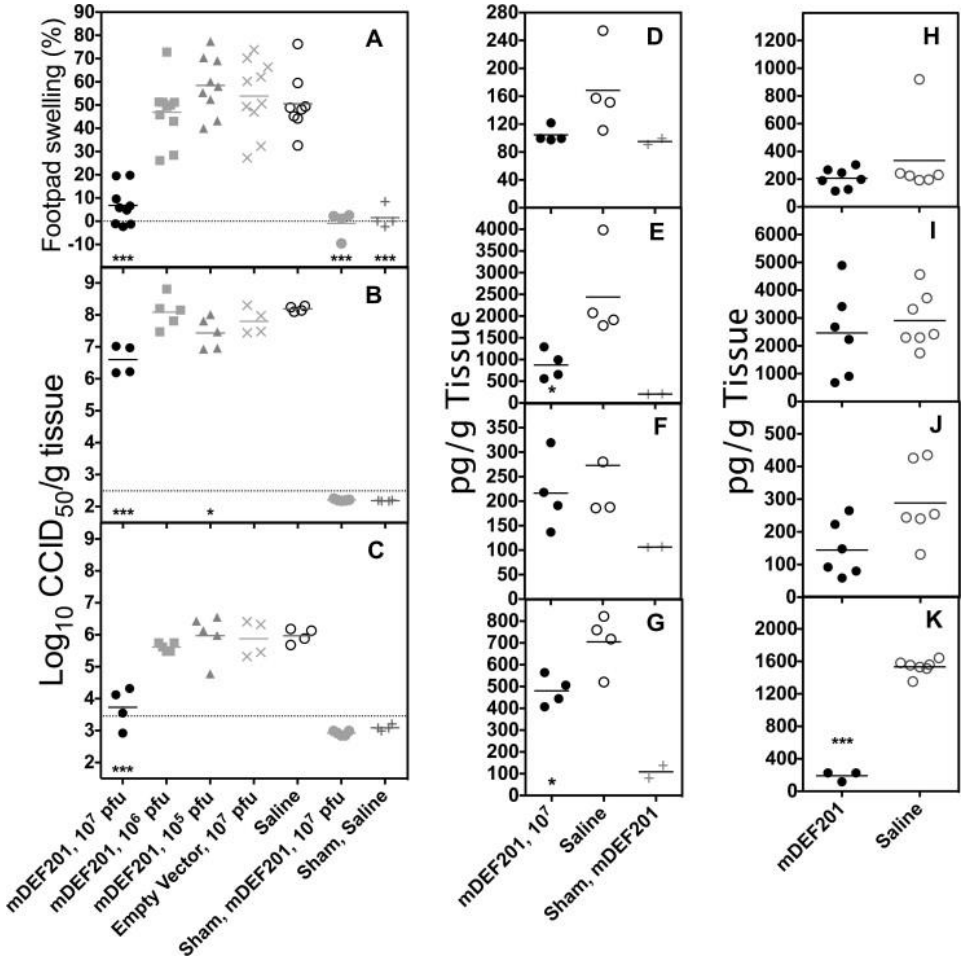

Fig. 3-3. The effect of mDEF201 administered intranasally 24 h prior to virus challenge at doses of 10^7, 10^6, or 10^5 pfu/animal. (A) The percent increase in footpad swelling at the site of virus challenge was significantly reduced as compared with the contralateral footpad on 7 days post infection (dpi) in animals treated with the highest dose of mDEF201. Virus titer of (B) the inoculated limbs and (C) spleen of mDEF201-treated or control mice on 2 dpi. Significant reduction in virus titer was observed at the highest dose of mDEF201. Cytokines including (D) IL-6, (E) MCP-1, (F) MIP-1α, (G) RANTES, all cytokines show a trend towards reduction with mDEF201 treatment. Cytokine reduction was verified in a subsequent study and a similar trend towards reduction of (H) IL-6, (I) MCP-1, (J) MIP-1α, and (K) RANTES was observed in the hind limb on 6 dpi after prophylactic treatment with 10^7 pfu of mDEF201.

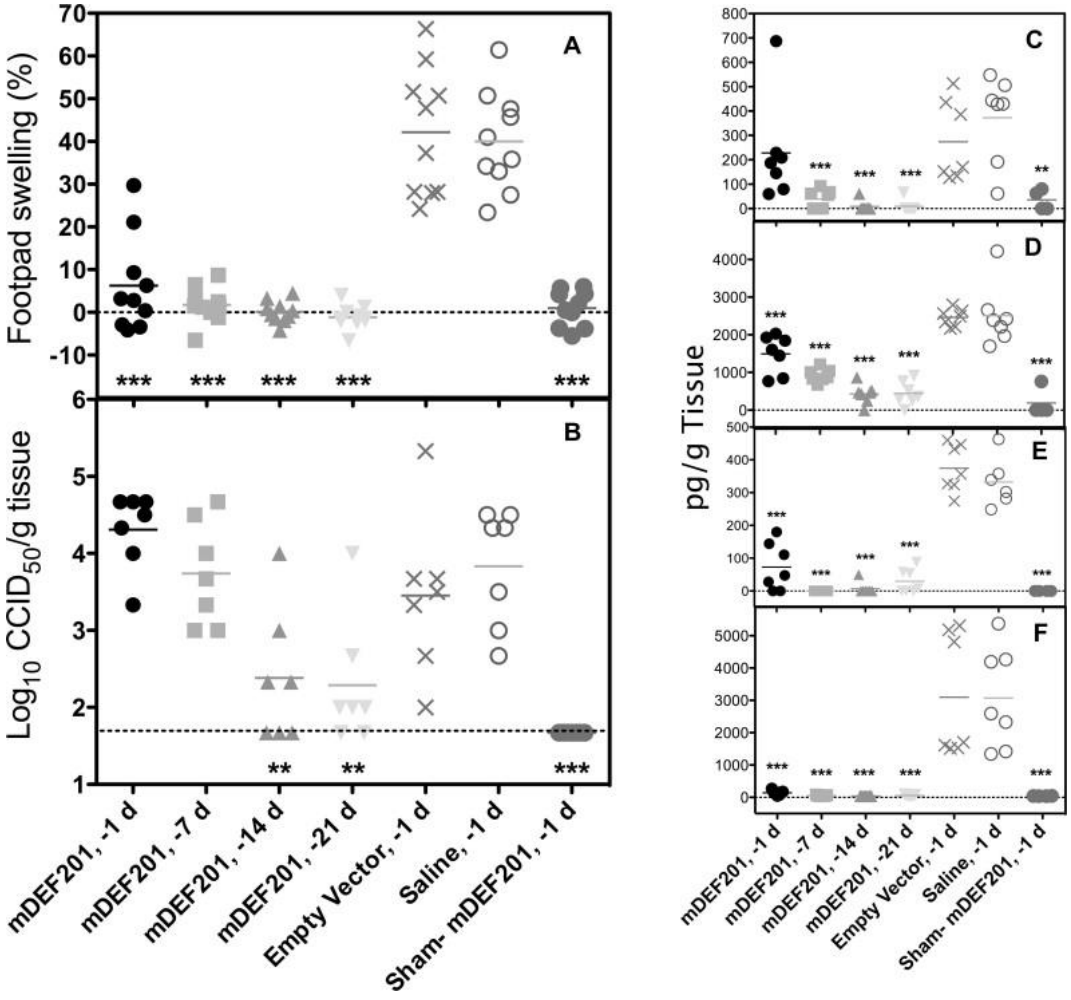

Fig. 3-4. The effect of prophylactic mDEF201 treatment administered i.n. on 21, 14, 7, or 1 day prior to virus challenge at a dose of 10^7 pfu. (A) Footpad swelling was significantly reduced on 6 days post infection in all treatment groups when compared with empty vector and saline treated control groups. (B) Virus titer in the hind limb homogenates on 6 days post infection were reduced in earlier treatment groups and (C) IL-6, (D) MCP-1, (E) MIP-1α and (F) RANTES were generally reduced on 6 days post infection.

The DBA/1J mouse model appears to be a more relevant model compared to other CHIKV models. Infection of C57BL/6 mice with the S27 East African isolate of CHIKV was also conducted in our lab in parallel with infection of DBA/1J mice, but no significant swelling was observed. Despite the utility observed with the C57BL/6 model in other labs, including use in vaccine studies (Wang et al., 2011), the DBA/1J, mice appear to be more sensitive to infection with CHIKV. In addition to infection of DBA/1J and C57BL/6 mice, we also challenged AG129 mice in parallel (data not shown). Similar results, including rapid mortality, were observed as previously reported (Partidos et al., 2011). The CAST/EiJ mouse has previously been shown to be susceptible to infection with monkeypox, a virus that does not cause disease in BALB/c, C57BL/6, and other commonly used inbred strains (Americo et al., 2010). For this reason, we included CAST/EiJ mice in our mouse strain screen to determine if they would be suitable for modeling CHIKV disease. Significant swelling of the foot and hock was observed at the site of virus injection after footpad inoculation, which was similar to swelling observed in DBA/1J mice. The CAST/EiJ mice, however, are limited in availability and were abandoned in favor of the more readily available DBA/1J strain.

Neonatal mouse models of CHIKV are also commonly used. Intradermal infection of 12-day-old mice results in morbidity and mortality (Couderc et al., 2008), representing severe disease phenotype. Virus is detected in the muscle, joint, skin and brain tissue of these mice, which is similar to rare cases of encephalitic disease of patients infected with CHIKV (Gerardin et al., 2008; Ramful et al., 2007; Robin et al., 2008). While virus was very rarely detected in the brains of infected DBA/1J mice (data not shown), detectable titers were observed in various tissues including joints, spleen, and serum. Use of adult DBA/1J mice allows for virus interaction with a developed immune system and may better model aspects of human disease that arise as a result of immunopathogenesis, which may be different in neonatal mice.

Another potential benefit of the DBA/1J model is the consistency of swelling among animals. All infected mice had fairly consistent swelling, as compared with inconsistent swelling of joints and rash observed in infected macaques in a recent study (Chen et al., 2010). In addition, no evidence of arthritis was observed in the macaques, as compared with histopathology consistent with arthritis in DBA/1J mice after CHIKV infection. Notably, no

rash is detected in these mice, despite being a common disease sign associated with CHIKV infection in man (Schmidt-Chanasit et al., 2012).

The main limitation of CHIKV infection in DBA/1J mice is the lack of the development of a persistent chronic arthralgia that is observed in human patients (Jaffar-Bandjee and Gasque, 2012). Measurable swelling was observed between 5 and 8 days after virus inoculation, while joint pain and swelling in infected patients can be observed for weeks to months, and may even persist for years in some individuals (Couturier et al., 2012). The few days of swelling, however, does provide a measureable parameter of disease that can be used to determine the effect of antiviral or host-directed therapeutics on the amelioration of this important disease manifestation. No animal models have been shown to develop chronic arthralgia, and the basis for the persistence of this disease parameter in man has yet to be elucidated.

Another potential limitation of this model is the lack of mortality associated with CHIKV infection at a relatively high virus challenge. Despite the lack of mortality in this model, other mouse strains, such as AG129 mice deficient in IFN receptors, may be used to further characterize the effect of antiviral therapies against more severe disease manifestations. A study conducted during the 2005-2006 Reunion outbreak identified 610

atypical cases where 546 of these cases had underlying medical conditions and 65 patients died (Economopoulou et al., 2009). This aspect of underlying medical conditions shows the potential for CHIKV to affect immune compromised individuals. Nevertheless, arthralgia, the primary disease manifestation in this mouse model, is still the most prominent and debilitating aspect overall of CHIKV disease in man and should be a focus for the development of therapeutics.

Prophylaxis with mDEF201 may have clinical potential in endemic areas of CHIKV disease. In the event of an outbreak of CHIKV, it would be useful to have a single dose prophylactic agent that could provide protection over several weeks to those at risk of infection. Future studies will be conducted to determine the pre-exposure prophylactic window for mDEF201 utilizing the DBA/1J model.

Studies in the DBA/1J model demonstrate a rapid course of virus replication and clearance, suggesting a limited treatment window for antiviral compounds targeting the lifecycle of the virus. Treatment with DEF201 results in the production of consensus IFN and a decrease in disease when given prior to virus challenge. These studies demonstrate prophylaxis with a safe and effective mediator of antiviral response is effective in

preventing or ameliorating disease as a result of CHIKV infection. In addition, vaccine development would likely be an effective means of disease prevention within endemic regions.

Acknowledgements

We thank Isaac Wong, Joseph Hagloch, Brett Hurst, Makda Gebre, and Shelby Wilcox for expert technical assistance. This work was supported by funding from the Virology Branch, DMID, NIAID, NIH (HHSN272201000039I/HHSN27200004/A21).

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4. IMMUNE MODULATION AND EXACERBATION IN A MOUSE MODEL OF CHIKUNGUNYA VIRUS

4.1 Abstract

Chikungunya virus (CHIKV) has been identified in 80 countries across 5 continents and has caused more than 6 million cases worldwide (Petitdemange et al., 2015). This viral infection is usually self-limiting and typically results in fever, swollen joints/joint pain and rash, but can lead to debilitating chronic arthritis. Using a mouse model of CHIKV-induced swelling, we tested the efficacy of three immunomodulatory compounds. Methotrexate (MTX) significantly reduced swelling, splenomegaly, and cytokine levels. Treatment with GP1681 significantly increased swelling. Actemra (ACT) (Anti-IL6 receptor antibody) reduced IL-6 to baseline and significantly reduced swelling. We also tested a combination treatment of ACT with MTX, which had a slight additive effect on disease improvement. As expected, these immune modulators had no effect on viral titer. These studies demonstrate the potential for improvement or exacerbation of CHIK disease after treatment with compounds that target host immunity.

4.2 Introduction

CHIKV is an alphavirus, which was identified in the 1950s in Africa. The name Chikungunya roughly translates to “that which bends up”. CHIKV is spread by mosquitoes, typically *Aedes aegypti* and more recently *Aedes albopictus* (Wasonga et al., 2015). The immune system plays a pivotal role on how CHIKV will affect the body. In some cases the virus is a self-limiting disease with febrile symptoms, swollen joints and a rash but may progress to a chronic, debilitating arthritis. There is no vaccine or licensed treatment for this disease.

Methotrexate (MTX) is a JAK/STAT pathway inhibitor (Thomas et al., 2015). The JAK/STAT pathway is involved in signal transduction after cytokine release and binding to immune cells and is also important in haematopoiesis, immunity and inflammation. Having been clinically approved for over 35 years, it is most commonly used at low doses to treat inflammatory diseases such as rheumatoid arthritis (RA) and Crohn’s disease (Cronstein, 2005). Methotrexate was selected for testing because of its potential to reduce the inflammation associated with CHIK infections; by inhibiting the JAK/STAT pathway, the downstream effects of hypercytokinemia could potentially be reduced.

Antiplatelet, vasodilating Prostaglandin 2 (PGI2), causes a significant increase in the cAMP production and can attenuate inflammation via suppressing TNF-α, IL-1β, and IL-6 production in lungs, human monocytes, and endothelial cells (Vicil and Erdogan, 2015). GP1681 sodium, a chemically stable PGI2 analogue, has been shown to possess a similar pharmacological profile to PGI2 (Vicil and Erdogan, 2015). TNF-α, IL-1β, and IL-6 play keys roles during CHIK infection, GP1681 was chosen to see the outcome of simultaneously suppressing all 3 during initial infection.

Tocilizumab (Actemra (ACT), RoActemra) is a humanized monoclonal antibody that acts as an interleukin-6 receptor antagonist (Dhillon, 2014). ACT is given to patients with RA and has shown positive results in reducing joint swelling and associated pain. The ACT label indicates that treatment can also be effectively administered in combination with methotrexate. IL-6 is elevated during initial infection with CHIKV, ACT was chosen to suppress this immune response.

The purpose of this study is to evaluate these three immunomodulatory agents in a mouse model of CHIK. These immune mediators were tested using a DBA/1J mouse model, which develops splenomegaly, footpad swelling and viral titers when challenged with

CHIKV subcutaneously in the footpad and can be used as parameters of disease to determine efficacy of the treatments (Dagley et al., 2014). We hypothesize that specifically targeting key mediators of host immunity that are hyperregulated during CHIKV infection will ameliorate disease in the absence of direct inhibition of virus.

4.3 Materials and methods

4.3.1 Cells

C6/36 cells were obtained from ATCC (Manassas, VA) and were used for virus propagation. These cells were grown in RPMI at 28˚C with 5% fetal bovine serum (FBS). Vero 76 cells, maintained in minimal essential medium (MEM) supplemented with 10% FBS, were also obtained from ATCC and were used in viral titer assays. Media was obtained from Hyclone Laboratories, Logan, UT.

4.3.2 Virus.

The Reunion Island isolate LR2006-OPY1 (LR06) was obtained from Robert Tesh (UTMB, WRCEVA) and propagated in C6/36 cells. The LR06 CHIKV stock has a titer of $10^{9.5}$ 50% cell cultures infectious doses ($CCID_{50}$)/ml.

4.3.3 Animals

Seven-week old DBA/1J mice were obtained from Jackson Laboratory. Mice were quarantined for at least 48 hours prior to infection.

4.3.4 Test Materials

Actemra (ACT, anti-IL-6r mAb) was purchased by the attending veterinarian from Logan Regional Hospital (Logan, UT) as a pre-formulated solution. Dilutions were made in sterile saline and solutions were stored at 4˚C during the experiment. GP1681, along with an inactive control, was provided by Gemmus Pharma (San Francisco, CA) in a ready to use formulation. Methotrexate was purchased from Sigma-Aldrich (St. Louis, MO) as a powder and prepared in sterile saline at various doses.

4.3.5 Experimental Design

Mice were randomly assigned to groups of 10 animals. A $10^{4.5}$ $CCID_{50}$/0.1 ml concentration of LR06 was prepared in minimal essential media. Animals were anesthetized with isoflurane prior to subcutaneous (s.c.) injection in the footpad and hock with a total volume of 0.1 ml of the diluted virus (0.05 ml in each site).

At 6 dpi, 7 animals per group were necropsied and spleens and hind limbs were harvested, weighed, and the virus titer was determined. Virus titer and

cytokine levels were determined from hind limb homogenates. The remaining animals were weighed every other day until Day 14. The dorsoventral thickness of the footpad at the site of virus challenge was measured with a digital caliper and the percent increase as compared to the contralateral foot was calculated.

GP1681, vehicle, and inactive GP1681 were administered bid for 10 days beginning 4 h after virus challenge. Animals were treated via the intraperitoneal (i.p.) route. ACT was given 4 h after virus challenge and on 4 dpi. Animals were treated via the intraperitoneal (i.p.) route. An isotype control Ab was also administered along the same treatment schedule as a negative control. Methotrexate was given once a day from -24 h to 6 dpi. Animals were treated via the subcutaneous (s.c.) route. Phosphate buffered saline (PBS) was also administered along the same treatment schedule and was included as a negative control.

4.3.6 Tissue virus titration

Samples were homogenized and diluted in 1 ml MEM, containing 0.05 mg/ml gentamicin. The homogenate was centrifuged for 10 min at 2300 RCF 10-fold dilutions were prepared and added to Vero 76 cells in triplicate and examined for cytopathic effect (CPE) on 3 dpi. Virus titers were

determined by end point titration as described previously (Reed and Muench, 1938).

4.3.7 Cytokine analysis

Homogenized leg samples were evaluated for proinflammatory cytokine/chemokine levels using the Q-plex™ Mouse Cytokine Array (Quansys Biosciences, Logan, UT). The Quansys array quantifies levels of murine IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL12p70, IL-17, MCP-1, IFNγ, TNFα, MIP-1α, GMCSF and RANTES. Samples were diluted in sample diluent at 1:5 and 1:20 concentrations. A standard curve for each antigen was generated by serial dilution of antigen standards from 1:3 to 1:729. Standards were plated in duplicate on opposite sides of the plate and samples were plated at two dilutions. Manufactures instructions were followed for adding substrate, incubating and washing. Plates were quantified on a Quansys Q-View ™ Imager with Q-View ™ software.

4.3.8 Statistical analysis

Statistical analyses were done using one-way ANOVA using a Bonferroni group comparison (Prism 5, GraphPad Software, Inc).

4.3.9 Ethics statement

This study was conducted in accordance with the approval of the Institutional Animal Care and Use Committee of Utah State University under the approved protocol numbers 1526 and 2339. The work was done in the AAALAC-accredited and PHS Animal Welfare Assurance-approved Laboratory Animal Research Center of Utah State University.

4.4 Results

4.4.1 Methotrexate dose response

At doses of 4, 2, and 1 mg/kg/d of MTX, footpad swelling was significantly ($P<0.001$) reduced as compared with placebo-treated controls (Figure 4-1). Treatment with the lowest dose of 0.5 mg/kg/d was also effective in significantly ($P<0.05$) reducing swelling, although not to the extent of higher doses. Despite significant reduction, and although some individual animals were reduced to baseline, infected animals treated with MTX had measureable swelling at the site of virus inoculation between 0 and 75% as compared with the contralateral foot. There were no significant differences in swelling between the 4, 2, and 1 mg/kg/d MTX groups (Figure 1). A significant ($P<0.001$) increase in virus titer was observed in groups

treated with 4 or 2 mg/kg/d of MTX, while lower doses caused a non-significant increase (Figure 4-2). A trend towards a reduction in spleen size was also observed in animals treated with the three highest doses of MTX.

4.4.2 Effect of Actemra on CHIKV infection

A significant reduction of swelling was observed in animals treated with ACT, at 40 mg/ml (Figure 4-3). A reduction of IL-6 to baseline levels was observed (Figure 4-4), although as expected, viral titer was not affected (Figure 4-5).

4.4.3 Treatment with GP1681 exacerbates disease

GP1681 treatment resulted in a significant ($P<0.001$) increase in footpad swelling (Figure 4-6). Interestingly, the lower dose resulted in a greater increase in footpad swelling, although the difference between the two treatment groups was not significant. The highest dose of GP1681 resulted in a significant increase in virus titer on 6 dpi at the site of virus challenge, although no increase in titer was observed in mice treated with the lower dose (Figure 4-7).

No significant increases or decreases were observed in key cytokines (data not shown), although MCP-1 was increased in animals treated with GP1681. Levels of IFN-γ were reduced to baseline in animals treated with

the highest dose of GP1681, while increased levels were seen in animals treated with the lower dose of GP1681.

4.4.4 The effect of Methotrexate and Actemra Combination

Combination of methotrexate (2 mg/kg) and ACT (20 mg/ml) reduced the swelling to levels that were slightly lower, although not significantly so, as compared with monotherapy treatment. Treatment did not have a synergistic effect in reducing footpad thickness (Figure 4-8). No appreciable swelling was observed in sham-infected toxicity controls treated with either compound.

As expected, virus titer in the hind foot at the site of virus inoculation was not affected by monotherapy treatment with MTX or ACT (Figure 4-9, Table 2). There was also no significant reduction in cytokine levels observed with combination treatment (Figure 4-10).

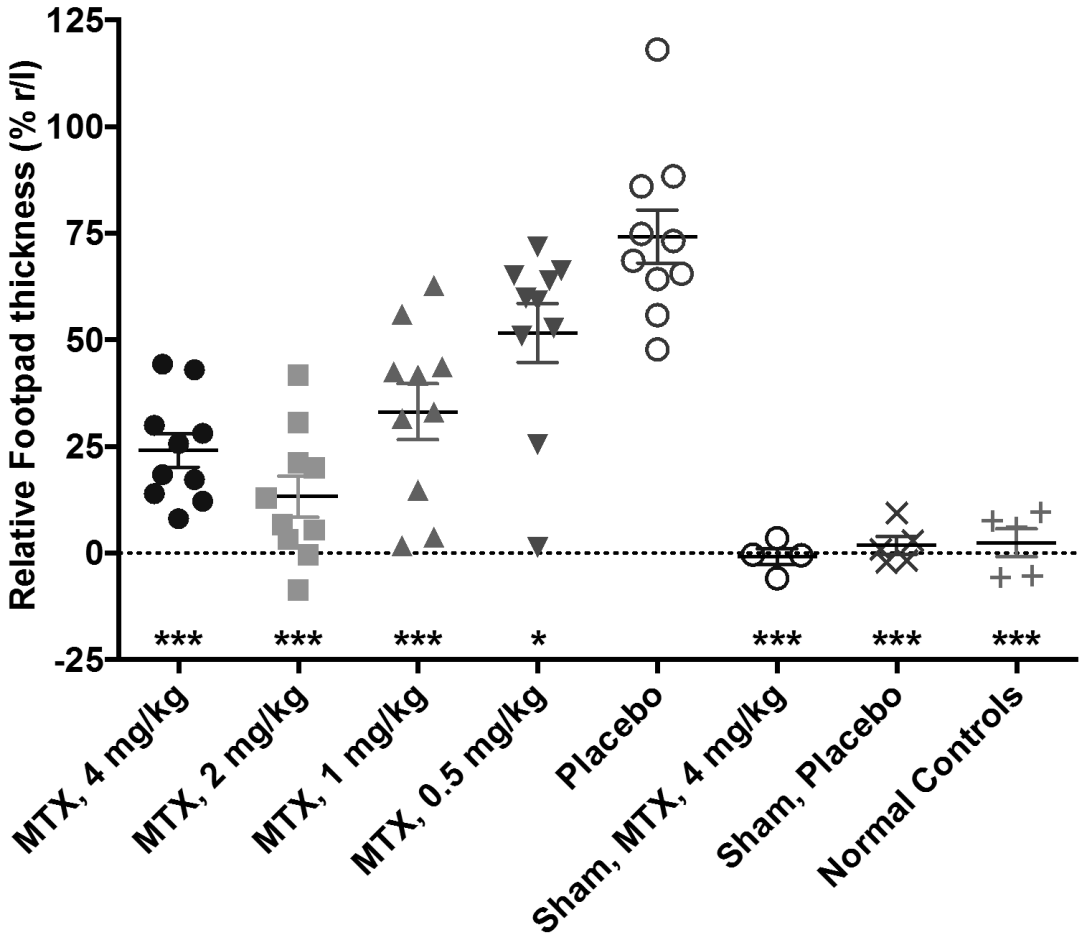

Fig. 4-1. The effect of methotrexate (MTX) administered subcutaneously starting at -1 through 6 dpi at doses of 4, 2, 1 and 0.5 mg/kg on footpad swelling. Treatment with MTX significantly improves footpad swelling on 6 dpi in DBA/1J mice infected with CHIKV. (***P<0.001, *P<0.05, as compared with placebo treatment)

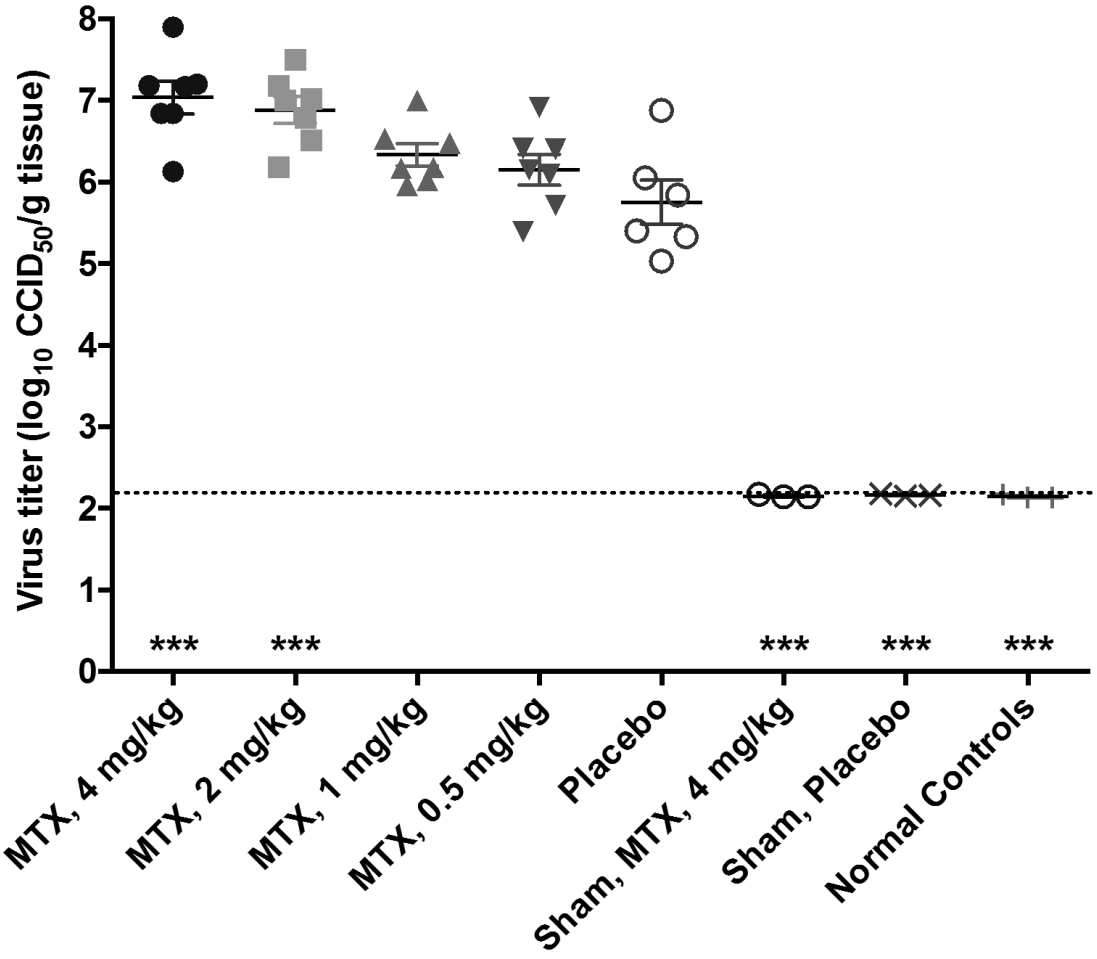

Fig. 4-2. The effect of methotrexate (MTX) administered subcutaneously starting at -1 through 6 dpi at doses of 4, 2, 1 and 0.5 mg/kg on virus titer of hind limb. Virus titer at the site of inoculation is not reduced after MTX treatment. Higher doses of MTX result in significantly increased virus titers. (***P<0.001, as compared with placebo treatment)

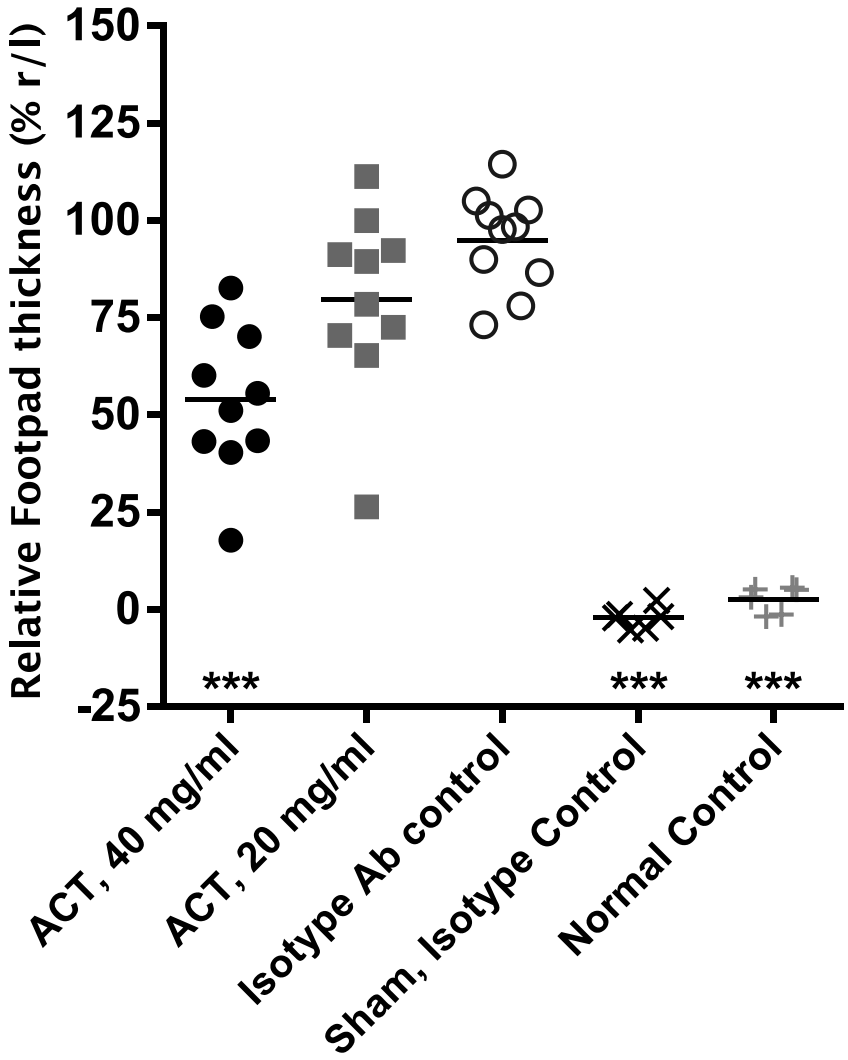

Fig. 4-3. The effect of 40 and 20 mg/ml of ACT treated intraperitoneally at 4 hrs and 4 dpi on percent increase in footpad thickness at the site of virus inoculation as compared with the contralateral footpad in CHIKV-infected mice. (***P<0.001, as compared with placebo treatment)

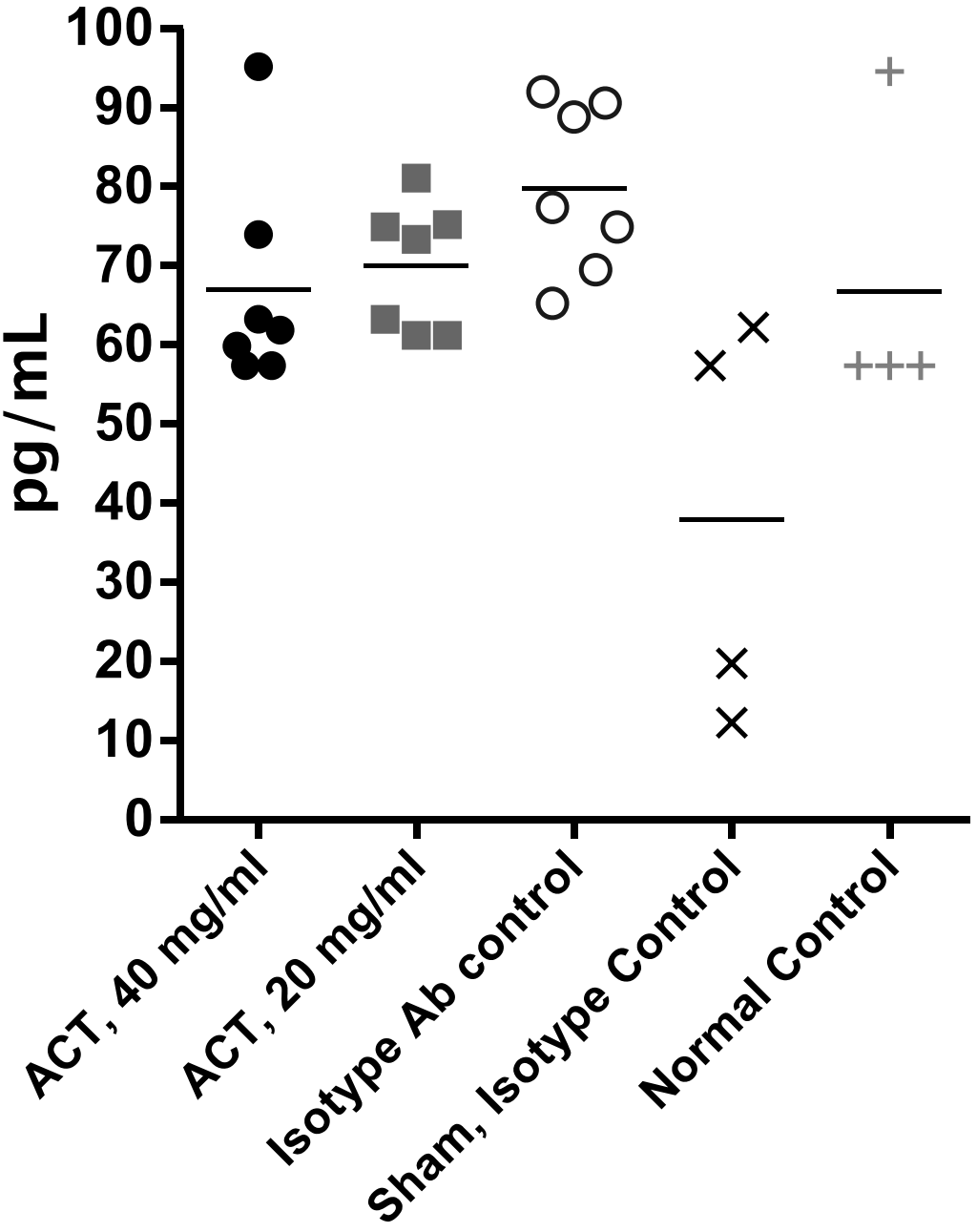

Fig. 4-4. The effect of 40 and 20 mg/ml of ACT treated intraperitoneally at 4 hrs and 4 dpi on IL-6. Hind limb cytokine analysis showing reduction of IL-6 to baseline levels at 40 and 20 mg/ml.

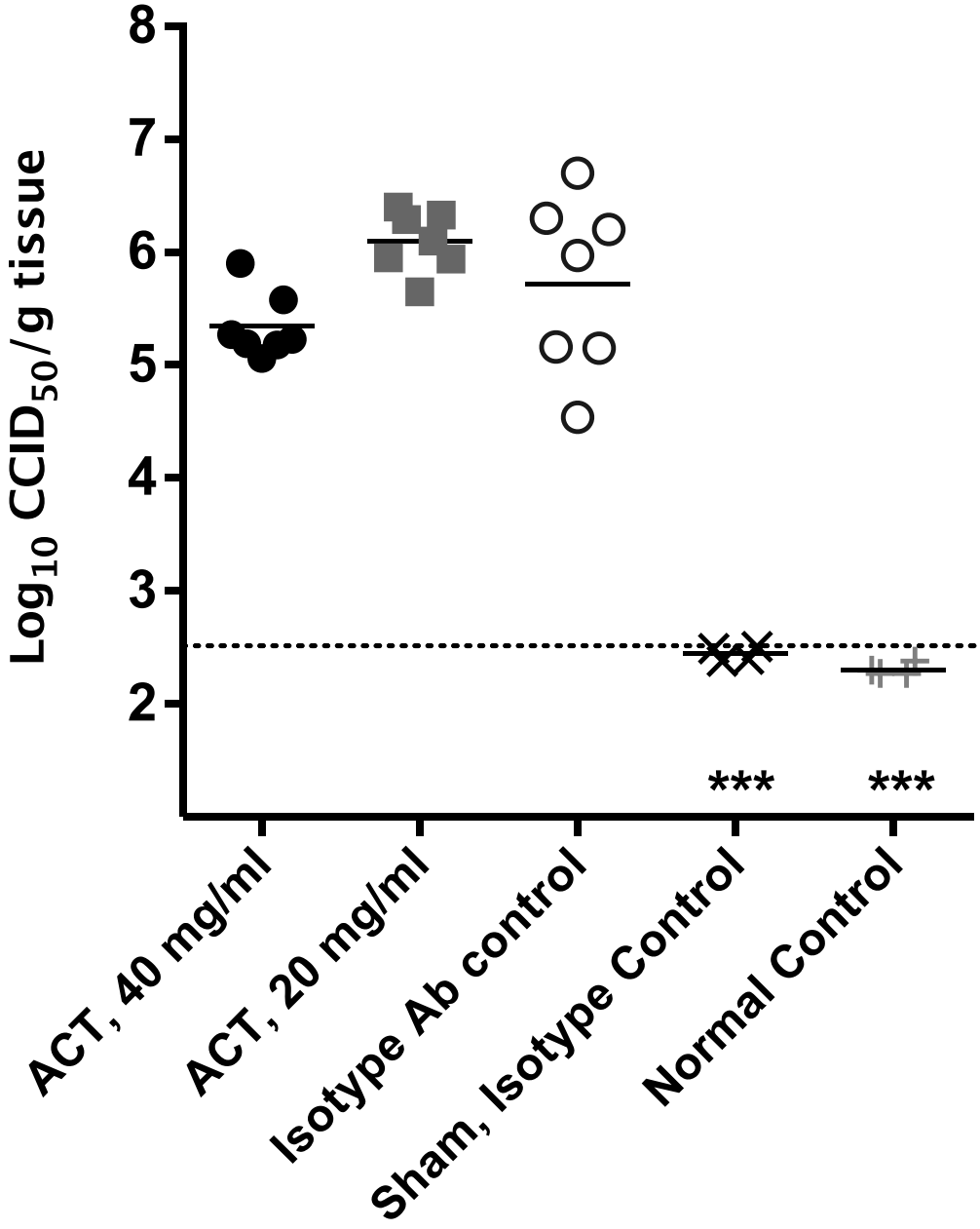

Fig. 4-5. The effect of 40 and 20 mg/ml of ACT administered intraperitoneally at 4 hrs and 4 dpi on virus titer. Right Hind limb viral titers are not significantly impacted.

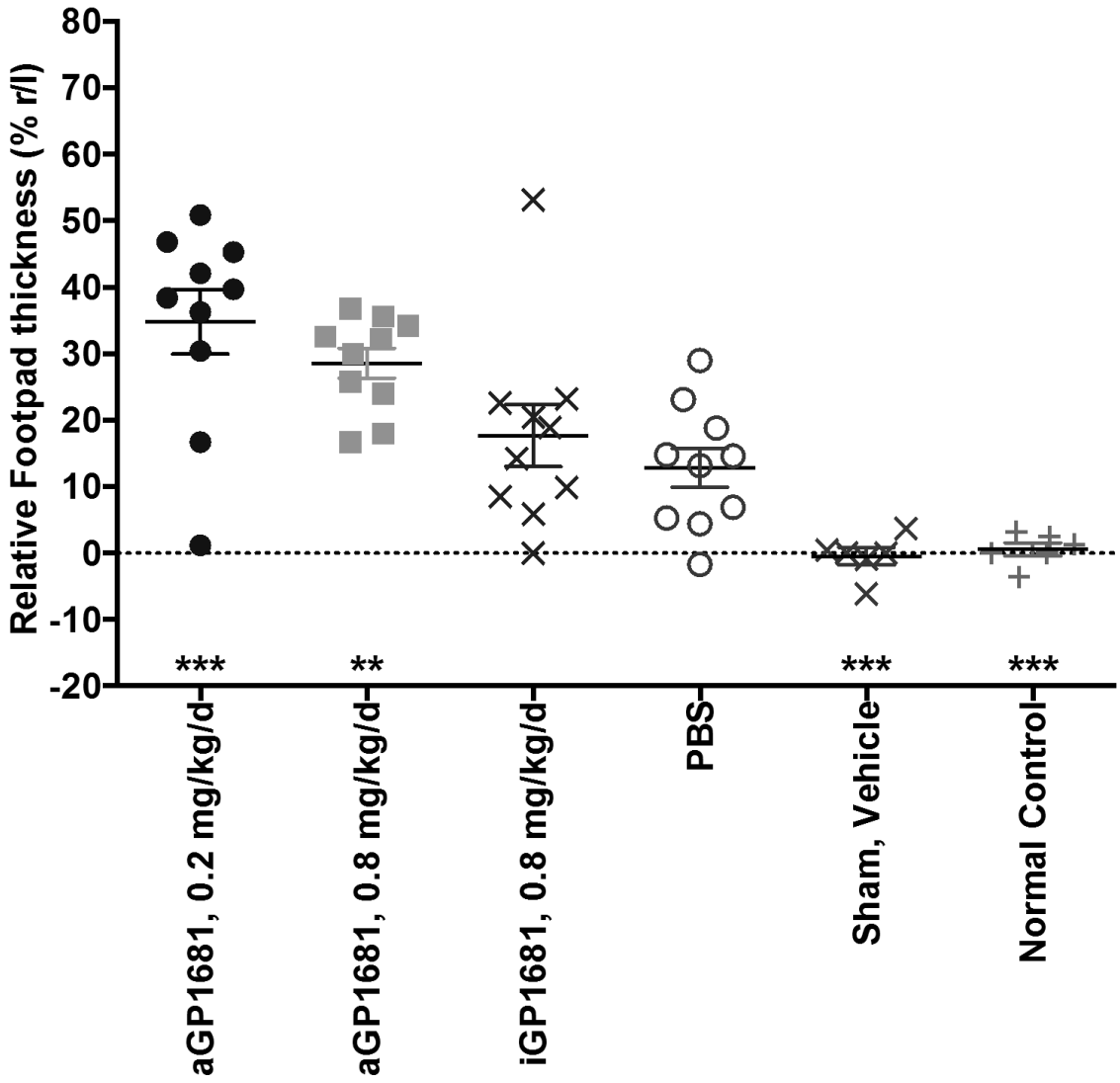

Fig. 4-6. The effect of two doses (0.2 and 0.8 mg/kg/d) of active GP1681 (aGP1681), inactive GP1681 (iGP1681), or vehicle control on percent increase in footpad thickness at the site of virus inoculation as compared with the contralateral footpad in CHIKV-infected mice. Mice were treated intraperitoneally twice daily for 10 days starting at 4 hours post infection (pi). Footpad thickness was increased at both concentrations of GP1681. (***P<0.001, **P<0.01, as compared with PBS control).

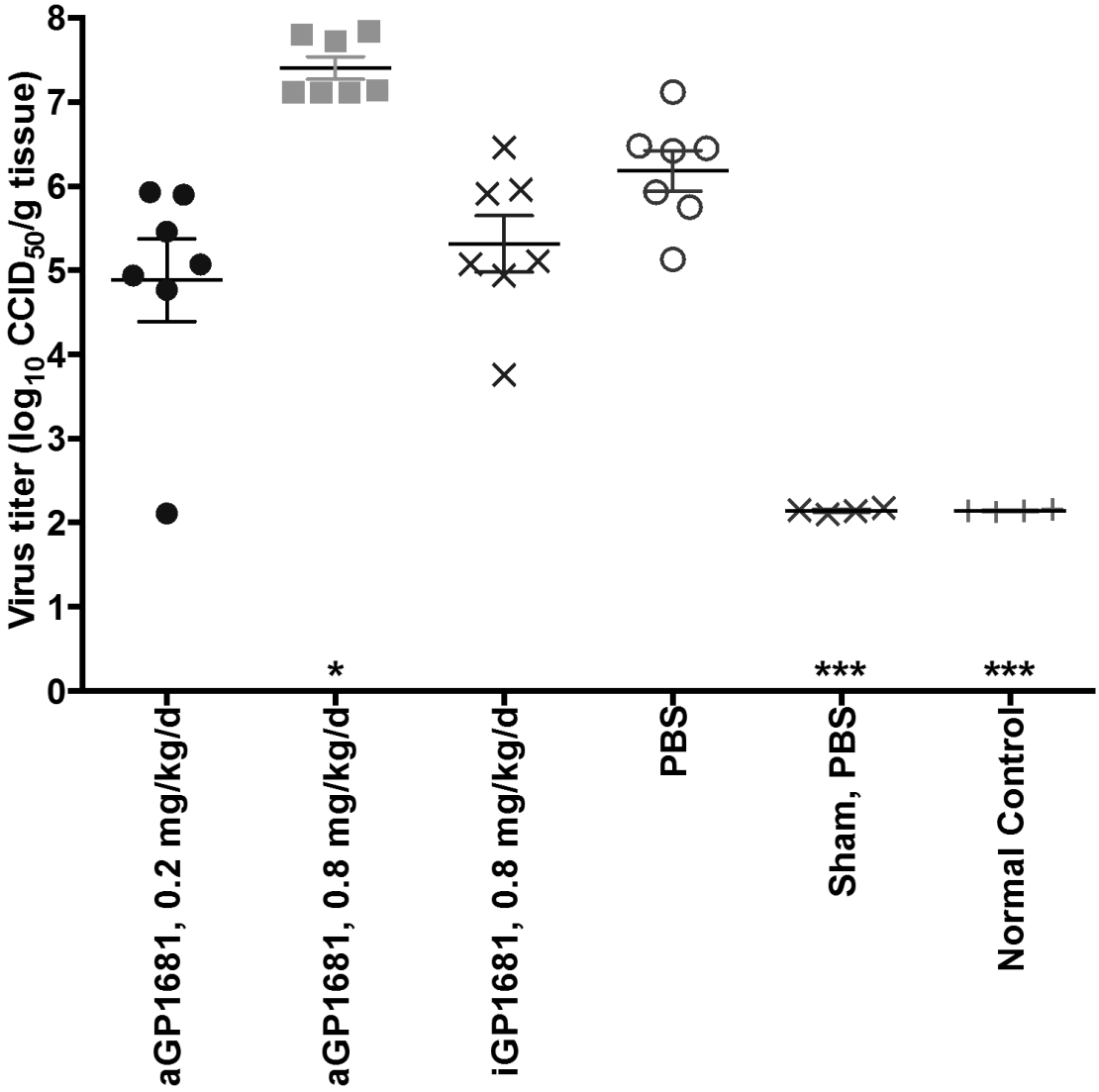

Fig. 4-7. The effect of GP1681 on virus titer of the hind limb at the site of virus inoculation in CHIKV-infected mice when treated twice daily for 10 days starting 4 hrs post infection (pi). Viral titer was significantly increased at 0.8 mg/kg/d. (***P<0.01, *P<0.05, as compared to control).

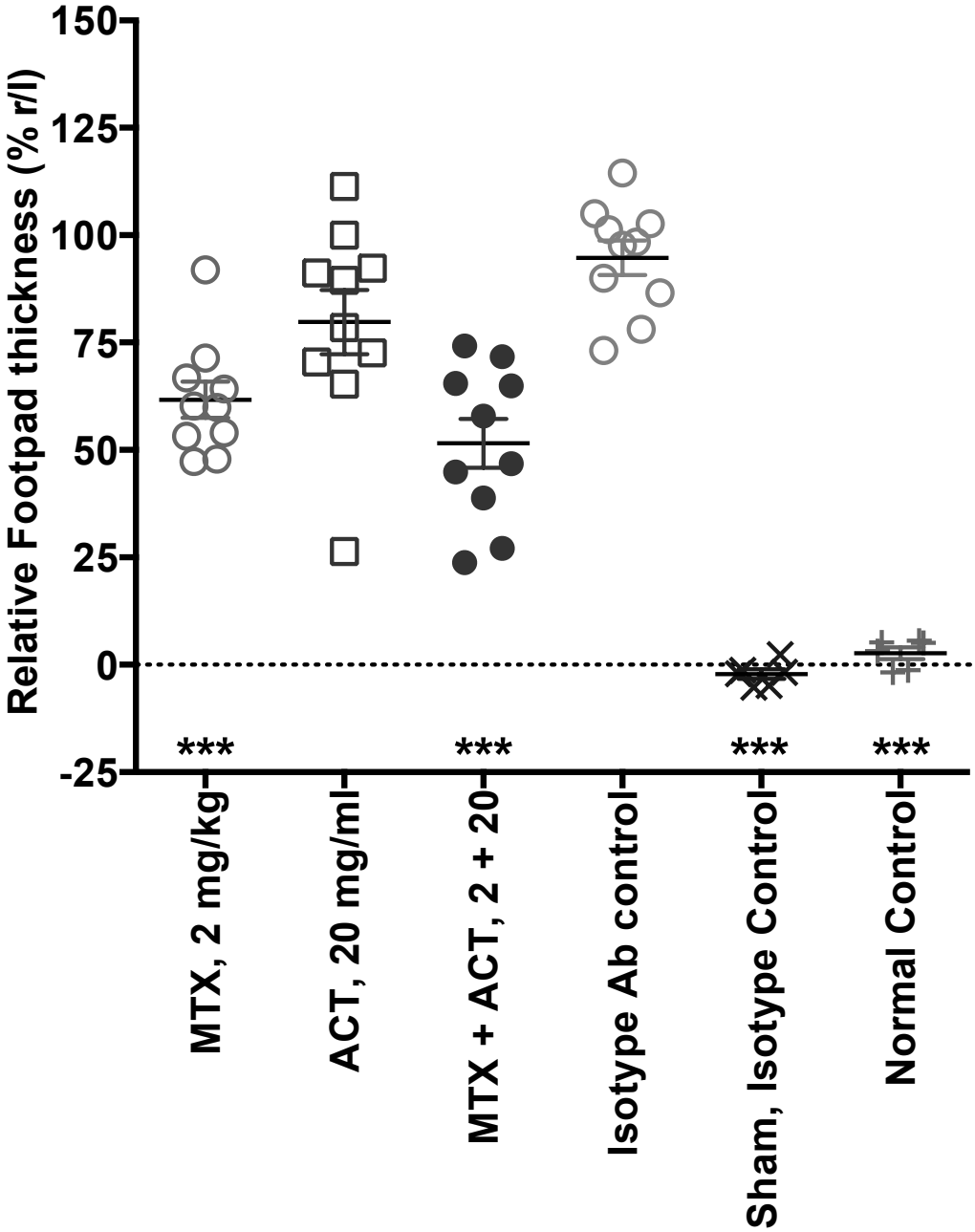

Fig. 4-8. The effect of methotrexate (MTX) at 2 mg/kg and, Actemra (ACT) at 20 mg/ml or a combination of both on footpad thickness at the site of virus inoculation as compared with the contralateral footpad in CHIKV-infected mice. MTX alone and in combination with ACT significantly decreased footpad swelling. (***P<0.001, as compared with normal control).

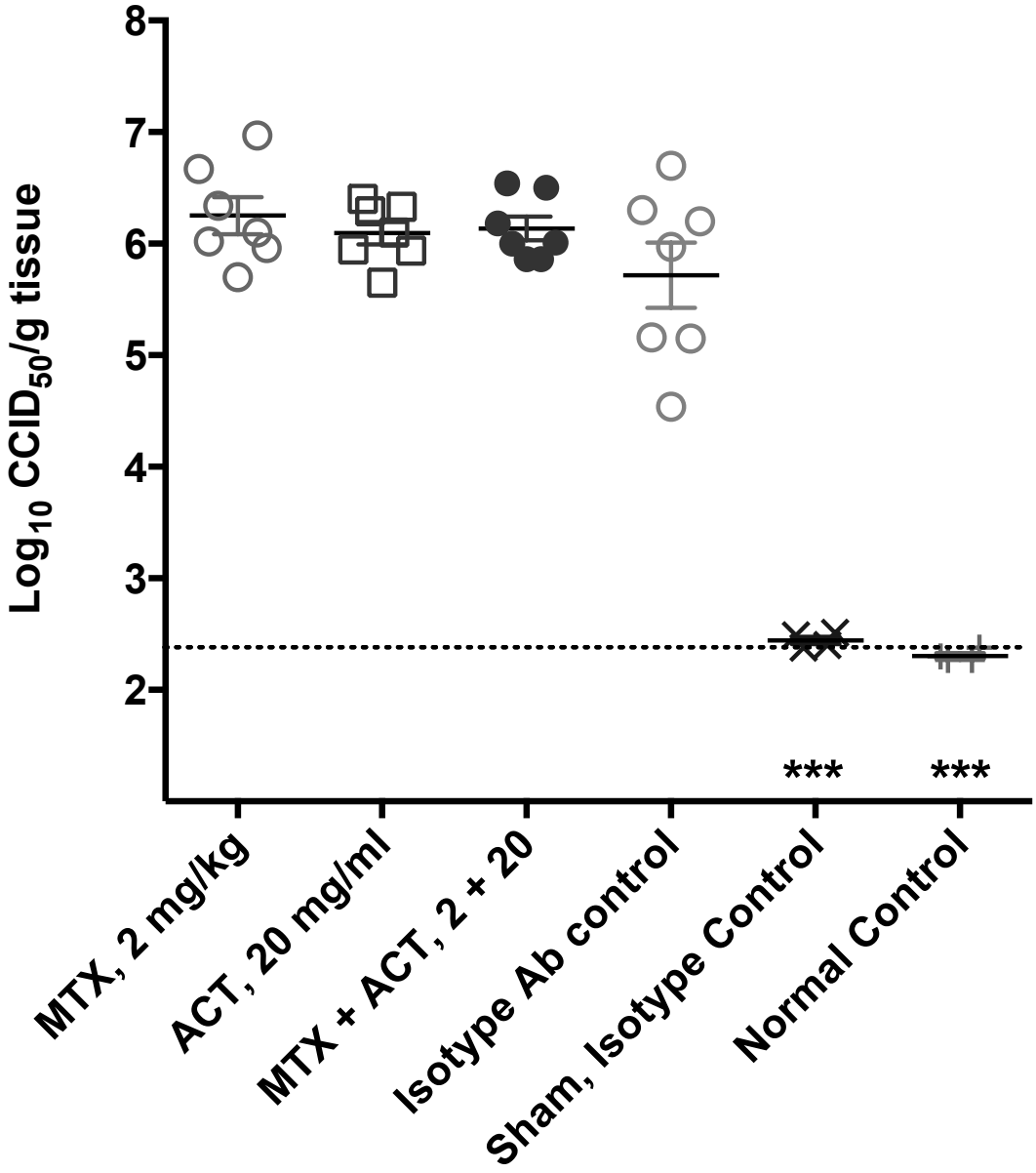

Fig. 4-9. The effect of methotrexate (MTX) at 2 mg/kg and, Actemra(ACT) at 20 mg/ml or a combination (MTX 2 mg/kg, ACT 20 mg/ml) on virus titer of hind limb in CHIKV-infected mice. No effect. (***P<0.001, as compared to control).

Table 4-1
Efficacy of Actemra (anti IL-6 mAb, ACT) and methotrexate (MTX) alone or in combination on disease of mice infected with CHIKV.

Animals: Female 13-17 g DBA/1J mice. *Duration of experiment*: 21 days
Virus: Chikungunya virus strain LR06 (LR2006-OPY1, 2C6) *Treatment route*: MTX- s.c., ACT- i.p.
Virus route: s.c. footpad inoculation

		Toxicity controls[a]		Infected, treated	
Treatment	Dose, schedule, route	Footpad swelling (%)[a]	Log_{10} right leg virus titer[b]	Footpad swelling (%)[a]	Log_{10} rt leg virus titer[b]
MTX	2 mg/kg/d, qd X 7 beg. -1 dpi	0.4 ± 3.0	2.3 ± 0.0	61.7 ± 13.2***	6.2 ± 0.4
ACT	20 mg/ml, single inj. +4 h	--	--	79.7 ± 23.5	6.1 ± 0.3
MTX + ACT	2 mg/kg + 20 mg/ml	--	--	51.5 ± 18.1***	6.1 ± 0.3
Isotype cont.	40 mg/ml, single inj. +4 h	-2.1 ± 2.8	2.4 ± 0.1	94.8 ± 12.7	5.7 ± 0.8
Normal control	NA	2.7 ± 3.4	2.3 ± 0.1	--	--

[a]Percent increase in footpad at the site of inoculation as compared with the contralateral foot on 6 dpi.
[b]Right hind limb was removed 6 dpi to determine virus titer.
***P <0.001, as compared with respective placebo treatment

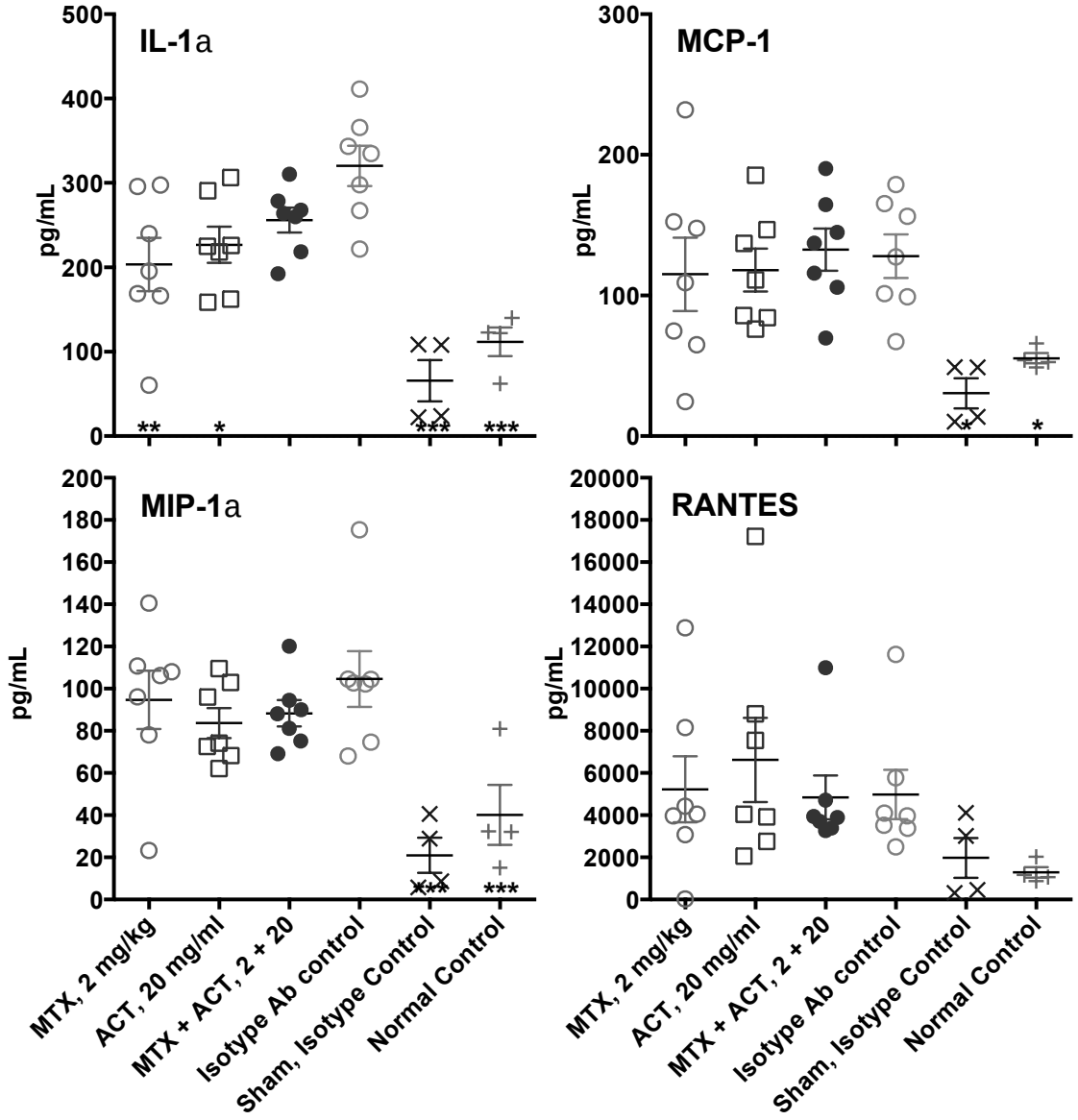

Fig. 4-10. The effect of monotherapy or combination therapy with MTX and ACT on cytokine profile of the hind leg at the site of virus inoculation in CHIKV infected mice on 6 days post infection (dpi). (***P<0.001, **P<0.01, *P<0.05, as compared with placebo treatment)

4.5 Discussion

Treatment with Methotrexate was effective in significantly reducing footpad swelling as compared with placebo treatment. Virus titers, however, were increased in a dose dependent manner, which is consistent with an indirect mechanism for disease amelioration as well as the immunosuppressive state induced by methotrexate (Ichikawa et al., 2013). The effectiveness of methotrexate treatment coincides with studies conducted on humans who experience rheumatic manifestations and were treated with methotrexate. In one particular study 54 of 72 CHIKV-infected patients who experienced rheumatic manifestations responded well to methotrexate treatment (Javelle et al., 2015). During this time viral levels are not an issue as compared with initial infection. In contrast to what we see in CHIK, when mice are infected with Ross River Virus (RRV) and treated with methotrexate their symptoms are exacerbated (Taylor et al., 2013), possibly due to the suppression of the immune response and increase in viral titers is more detrimental during RRV infection. Virus titer increases were consistent in both virus models after methotrexate treatment.

Treatment with ACT at 20 mg/ml, in combination with MTX at 2 mg/ml, significantly improved footpad swelling, spleen weights, and

cytokine levels in hind leg and spleen. There have been few studies conducted looking at the use of ACT during viral infection, as the drug is most often used to combat autoimmune disease inflammatory symptoms, but there has been some study of the effect of ACT in the treatment of chronic hepatitis C virus (HCV) infection (Felis-Giemza et al., 2015). Virus load was not increased when HCV patients suffering from RA were treated with ACT (Nagashima et al., 2012), possibly indicating the involvement of IL-6 in disease manifestation. It remains to be seen if ACT is efficacious in ameliorating disease in people infected with CHIKV, and further study is warranted.

GP1681 is an isomer of beraprost, it is thought that beraprost works in a similar manner to prostaglandin I2 (prostacyclin) (PGl2) by suppressing TNF-α, IL-1β, and IL-6 production in lungs, human monocytes, and endothelial cells (Vicil and Erdogan, 2015). PGI_2 is an important mediator of edema and pain that accompany acute inflammation, interestingly PGI_2 is the most abundant prostanoid found in synovial fluid in human arthritic knee joints (Ricciotti and FitzGerald, 2011). However, TNF-α, IL-1β and IL-6 are key players in CHIKV infection with IL-6 increasing during the acute phase of infection and TNF-α, and IL-1β increasing during disease

progression when patients began to recover from acute illness (Kelvin et al., 2011). We can assume that when all 3 of these key player cytokines are suppressed, disease symptoms are exacerbated.

These studies provide a proof of principle that treatment of CHIK with immune modulators may ameliorate or exacerbate disease, shows the complexities of the immune system and that variability exists between the responses of different alphaviruses to immune modulatory therapy.

Acknowledgements

We thank Kelsey Tolbert, Brittney Downs, Brett Hurst and Jorgen Madsen for expert technical assistance. This work was supported by funding from the Virology Branch, DMID, NIAID, NIH (HHSN272201000039I/HHSN27200004/A21).

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5. SUMMARY

CHIK is a complex viral disease that is becoming a major health concern to people in the USA and throughout the world. The work being done with CHIKV is greatly expanding our knowledge of this virus but as of yet has not produced an FDA-approved vaccine or treatment. To facilitate the discovery of novel antiviral preventative and therapeutic options, we have developed a model that can be used in CHIK research, including testing antivirals, vaccines and other intervention strategies. With relevant features of footpad swelling and inflammatory cytokine involvement, preliminary research can be conducted that can direct further clinical development in higher order models and in human clinical trials.

The DBA1/j mouse model was selected due to the aforementioned similarities with human disease, as compared with immune compromised models, such as AG129 mice and suckling mouse models that often include lethality and neurologic symptoms that are generally atypical disease manifestations in human patients. We have not identified any features of chronic infection, which would be very important to further model clinically relevant human disease (Poo et al., 2014), although indication of chronic infection has been observed in other mouse models (Seymour et al., 2015).

Our results indicate that prophylactic treatment with mDEF201, even several weeks in advance, may be a viable option during a CHIK epidemic, especially in the absence of a vaccine. Future studies should focus on the therapeutic window of pre-treatment with mDEF201 to discuss the potential of treating at-risk naïve populations, then studies need to be elevated in model level possibly using the hamster model which treats with a human DEF201, to test for efficacy.

Studies conducted with GP1681 demonstrate the importance and complexities involved with the CHIKV immunologic response and the involvement of various cytokines during different phases of CHIKV infection (Partidos et al., 2011). Levels of IL-6 are significantly increased in mouse models of CHIKV infection, which prompted the selection of ACT to determine if reducing IL-6 would positively impact infection. Treatment with 40 mg/ml was effective in improving disease, suggesting a role of IL-6 in pathogenesis. Similar results were seen with Bindarit, an MCP-1 inhibitor, which was protective in C57BL/6 mice infected with CHIKV (Rulli et al., 2011). This supports targeting of specific inflammatory mediators in order to ameliorate disease after CHIKV infection, although the timing of treatment is likely very critical.

Overall, we have provided evidence that treatment with immune modulators may be beneficial to patient outcome after CHIKV infection. Currently researchers are working on producing vaccines to CHIKV but rapid gene mutations make this difficult. Anti-viral testing is currently being done to target specific phases of viral infection, this research should be continued and special emphasis placed on CHIK because of the rapidity with which it is spreading.

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