Cross-reactivity among alphaviruses provides insight into viral emergence and novel defense strategies

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ACADEMIC ABSTRACT

Alphaviruses are a group of medically relevant arthropod-borne viruses (arboviruses) belonging to the *Togaviridae* family that are maintained by mosquito vectors. These zoonotic viruses are clustered into two groups: New World and Old World, depending on their geographical origin/distribution and clinical manifestations. Both of these groups cause disease symptoms of an acute febrile illness; however, each group has a distinct, hallmark disease symptom; New World alphaviruses, such as Eastern, Western, and Venezuelan equine encephalitis viruses (EEEV, WEEV, and VEEV, respectively), present with severe encephalitis while Old World alphaviruses, such as Sindbis, chikungunya, and Mayaro viruses (SINV, CHIKV, and MAYV, respectively) present with an incapacitating polyarthralgia that can persist for years following initial infection. To date, the most effective means of controlling these arboviral infections is through mosquito control programs. However, these programs have crucial limitations in their effectiveness; therefore, novel approaches are necessary to control the spread of these crippling pathogens and lessen their disease burden. Given the close phylogenetic and antigenic relationship between MAYV and CHIKV, we hypothesized that prior CHIKV immunity may affect the outcome of MAYV disease and/or limit its emergence in humans. Our work has shown that anti-CHIKV neutralizing antibodies can provide cross-protective immunity against MAYV disease. Alongside these studies, we have characterized the potency of a camelid-derived single-domain antibody (sdAb) that neutralizes a breadth of alphaviruses, including CHIKV and MAYV. With these data, we have designed and generated transgenic *Aedes aegypti* mosquitoes that express two anti-CHIKV sdAbs to target infection, dissemination, and transmission of MAYV and CHIKV within this deadly vector. These findings are particularly significant because they highlight the ability to co-target two

emerging alphaviruses that are crippling public health and obliterating quality of life around the globe within a single defense strategy.

PUBLIC ABSTRACT

Alphaviruses are arthropod-borne viruses (arboviruses) belonging to the *Togaviridae* family that infect millions of people annually via the bite of female mosquitoes. These viruses are major public health threats due to their ability to infect humans and animals and infections resulting in a range of debilitating diseases. Viruses within this genus are clustered into two groups: Old World and New World, based on geographical origin and distribution. While New World alphaviruses are known for inducing severe encephalitis (i.e., swelling in the brain), a hallmark symptom of the Old World alphaviruses is the development of incapacitating polyarthralgia (i.e., widespread joint pain) that can persist for years following initial infection. To date, the most effective means of combatting these viruses is through mosquito control programs. However, these programs have crucial limitations in their effectiveness; therefore, novel approaches are necessary to control the spread of these crippling pathogens. Given the close genetic relationship between chikungunya virus (CHIKV) and Mayaro virus (MAYV), our research has focused on harnessing cross-reactive immunity between these emerging alphaviruses. We discovered this cross-reactivity provides protective immunity to both viruses (i.e., CHIKV and MAYV) after exposure to only one (i.e., CHIKV) of the viruses. Next, we characterized the potency of a small, single-domain antibody (sdAb) to neutralize a breadth of alphaviruses, including CHIKV and MAYV. With these data, we have designed and generated transgenic *Aedes aegypti* mosquitoes that express this sdAb to target both CHIKV and MAYV within this deadly mosquito vector. These findings are particularly significant because they provide the foundation for a novel approach to controlling and preventing outbreaks of these emerging alphavirus pathogens that obliterate quality of life in public health settings around the globe.

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As a first-generation college freshman, I thought had my whole life figured out. That was until a professor stopped me in the hallway after my first semester and asked me to work in his research laboratory. I remember thinking, "Ew. I don't want to stare into a microscope for eight hours a day. I *know* I don't want to do research! " So, I started working for Dr. William H. Jackson researching HIV-1 and remained in his lab for the subsequent seven semesters of my undergraduate career. My preconceived notion that scientific research consisted of solely staring into a microscope couldn't have been further from the truth. Also, let's all laugh together at that whole eight-hour day thing. That being said, I would like to extend my immense gratitude to Dr. Jackson for seeing whatever it was he saw in that sassy 19-year-old seated in the back of his Genetics class. Had he never approached me, I know without a doubt I would not be where I am today.

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CHAPTER ONE: LITERATURE REVIEW

1.1 The mosquito

A tiny insect that has collapsed empires, reshaped civilizations, crippled economies, and obliterated quality of life around the globe: the mosquito. Mosquitoes belong to the order Diptera and are classified as true flies in the family Culicidae. Scientists believe the mosquito first originated in Africa and has since spread to every continent, except Antarctica. Evolutionary divergence events between *Anopheles* spp. and *Aedes* spp. mosquitoes date back to the early Jurassic period of the Mesozoic era, approximately 200 million years ago, and have since evolved to include over 3500 individual species (1). In the modern world, many of these changes in geographical distributions and production of evolved species are largely attributed to human trade and movement throughout the last several thousand years. Mosquitoes are a paramount example of the close ecological relationships between arthropod species and humans due to these insects relying on blood for their survival (2) and their adaptations to artificial breeding sites created in human settlements. This is often referred to as domestication or commensalism, especially in regard to mosquitoes, because these insects will evolve a preference for a stable source of blood—in this case, human blood (i.e., anthropophagy) (2). As humans have encroached on mosquito habitats for centuries, these insects have adapted to feeding on them for their primary supply of blood, which is necessary for egg laying and the production of offspring (i.e., anautogenous mosquitoes). This adaptation to anthropophagy has dire consequences for global public health as many of these anautogenous mosquitoes transmit potentially deadly pathogens while taking in a bloodmeal. Historians and scientists agree that this insect is responsible for killing over half of the world's cumulative human population; estimating 52 billion humans have succumbed to diseases caused by the pathogens vectored by mosquitoes throughout history (3).

1.2 Mosquitoes as vectors of disease

Since the 17th century, vector-borne diseases have caused more disease and deaths in humans than all other causes combined (4). In particular, the female mosquito is anticipated to be responsible for more deaths than every war ever fought (3). Arguably, the most notable example of a vector-borne disease that has resulted in an innumerable portion of human deaths is malaria: a protozoan parasitic disease that reached a peak annual human mortality rate of 1.8 million in 2004 (5). This pathogen, vectored by anopheline mosquitoes, has been greatly thwarted by control measures and developments in the field of vector-borne disease over the last few decades in economically developed countries; however, malaria remains a fundamental challenge in developing nations. Although the importance of vector-borne diseases has been identified for centuries, they still wreak havoc on global public health. In fact, the World Health Organization (WHO) estimates that vector-borne diseases are currently responsible for 700,000 deaths annually and account for 17% of the global burden of infectious diseases. Alongside the affliction of *Anopheles* spp. mosquitoes, *Aedes* spp. mosquitoes account for an abundance of the vector-borne illnesses that are devastating human populations. The majority of these vector-borne illnesses are viral pathogens known as arthropod-borne viruses, or arboviruses.

1.3 Aedes spp. mosquitoes and arboviruses

Arthropod-borne viruses (arboviruses) are a diverse group of viral pathogens that include hundreds of known viruses. Of the several hundred known arboviruses, approximately 100 of these are currently known to have pathogenic effects in humans (6). Interestingly, the majority of the most severe human pathogenic arboviruses (e.g., viruses causing yellow fever, dengue, Zika, and chikungunya) are vectored by *Aedes* spp. mosquitoes, particularly *Aedes aegypti*.

> "Recognizing its importance, *Aedes aegypti* should be studied as a long-term national, regional, and world problem rather than as a temporary local threat to the communities suffering at any given moment from yellow fever, dengue or other *aegypti*-borne disease. No one can foresee the extent of the future threat of *Aedes aegypti* to mankind as a vector of known virus diseases, and none can foretell what other virus diseases may yet affect regions where *Ae. aegypti* is permitted to remain." — Fred Lowe Soper (1966)

The incrimination of *Ae. aegypti* mosquitoes as deadly vectors of disease first began in the late 19th century when Cuban epidemiologist Dr. Carlos Finlay accurately hypothesized that the malignant effects of yellow fever—a disease that had been devastating Cuba for over 150 years—must be spread through a vector (7). Finlay hypothesized that yellow fever had to be carried from the blood vessels of an infected person to those of an uninfected person; declaring these characteristics of transmission to be, "All of which conditions the mosquito satisfied most admirably through its bite." (8) This mosquito-vector theory continued to be tested for nearly two decades, withstanding scrutiny and ridicule from physicians and colleagues, until Major Walter Reed and his team discovered that Finlay was correct. Not only was yellow fever vectored by mosquitoes, it was vectored by *Ae. aegypti*; a mosquito species that would become infamous in the study of vector-borne diseases.

To date, these widely distributed mosquitoes have been identified as vectors for various debilitating viral pathogens, including the arboviruses causing the most human suffering: yellow fever virus (YFV), dengue virus (DENV), Zika virus (ZIKV), and chikungunya virus (CHIKV) (9). Although a century of research has been focused on combatting these vectors of disease through control and prevention programs, the primary method of thwarting the spread of most arboviral diseases is

limiting the interaction among mosquitoes and their human hosts. While science has been successful in partially hindering the burden of these diseases through the latter mentioned vector control programs and through the development of arboviral vaccines, such as the YFV vaccine and Japanese encephalitis virus (JEV) vaccine, these diseases continue to have a stronghold on global economies and public health. Though the YFV vaccine is extremely efficacious, the global burden of yellow fever is believed to be drastically underrated (10, 11). In addition, dengue virus (DENV), a mosquito-borne flavivirus belonging to the *Flaviviridae* virus family, is primarily transmitted through the bite of *Aedes aegypti* mosquitoes. Infection with any of the four DENV serotypes (DENV 1-4) typically causes a febrile illness, commonly known as dengue fever, but can also cause severe forms of disease termed dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). These severe forms, which can result in a morality rate as high as 20%, present symptoms of vascular leakage, multiorgan malfunction, internal hemorrhaging and are most often observed in secondary heterologous infections (12, 13). Currently, DENV is endemic in 125 countries and results in over 390 million infections annually, with half the world's population at risk for being infected (14). Alongside these historic arboviral pathogens, newly emerging arboviruses are also vectored by *Ae. aegypti*. For example, Zika virus (ZIKV) is an arboviral pathogen that emerged in the Americas in 2015 (15). ZIKV had been known to cause disease in humans since the 1950s; however, it became particularly concerning throughout its rapid emergence in the Western Hemisphere within the last decade (16) when a more severe pathogenesis associated with ZIKV became apparent: congenital Zika syndrome (CZS) (15, 17, 18). CZS is characterized by seizures, ocular and auditory defects, cognitive impairment, and, most notably, microcephaly in the developing fetus (19). Since its emergence in the Western Hemisphere, ZIKV has been declared a public health emergency of international concern and has been reported in over 86 countries and

territories by the WHO. Recently, the Global Burden of Disease (GBD) estimates, by the WHO and the Institute for Health Metrics and Evaluation (IHME), released assessments for each of the diseases mentioned above (i.e., yellow fever, dengue, and Zika); however, there are other arboviral diseases of concern which include alphaviruses.

1.4 Alphaviruses

Alphaviruses are a group of medically relevant arboviruses belonging to the *Togaviridae* family that are maintained by mosquito vectors. These zoonotic viruses are enveloped, single-stranded, positive-sense RNA viruses that can be clustered into two groups: New World and Old World, depending on their geographical origin/distribution and clinical manifestations. Both of these groups cause disease symptoms of an acute febrile illness; however, each group has a distinct, hallmark symptom. For example, New World alphaviruses, such as Eastern, Western, and Venezuelan equine encephalitis viruses (EEEV, WEEV, and VEEV, respectively), present with severe encephalitis while Old World alphaviruses, such as Sindbis and chikungunya viruses (SINV and CHIKV, respectively) present with an incapacitating polyarthralgia that can persist for years following initial infection (20, 21). This dissertation will explicitly focus on the Old World alphaviruses.

1.5 Old World alphaviruses

As mentioned, Old World alphaviruses are arthritogenic viruses that cause a chronic, debilitating polyarthralgia accompanied by myalgia, cutaneous rash, and fever. Of these Old World alphaviruses, a particular focus is placed on certain members of the Semliki Forest serocomplex: Ross River virus (RRV), o'nyong nyong virus (ONNV), chikungunya virus (CHIKV), and Mayaro virus (MAYV). These enzootic viruses are maintained in a sylvatic transmission cycle, circulating between non-human primates (NHPs) and arboreal mosquito species. Although these viruses have been historically maintained through their sylvatic cycle(s), spillover events have occurred,

particularly with CHIKV and MAYV, and stemmed urban outbreaks (22). It is assumed that that the majority of urban outbreaks that occur in the modern world are a result of viral emergence into an urban transmission cycle with virus maintained between humans and anthropophilic mosquito species, such as *Ae. aegypti* (22). Specifically, CHIKV spillover has led to its extensive emergence and is now among the most widely geographically distributed arboviruses in nature (23).

1.6 Chikungunya virus emergence and global burden

CHIKV was first described in Africa in 1952 and since has been classified into three distinct genotypes: Eastern/Central/Southern African (ECSA), Asian, and West African. CHIKV's first documented emergence event in the late 1950s occurred alongside outbreaks in India and Southeast Asia and were traced to an evolved ECSA lineage (22). This Asian lineage of CHIKV became endemic in Southeast Asia where it continued to persist in an urban transmission cycle between humans and *Ae. aegypti*. Next, CHIKV re-emergence was documented in 2004 in Coastal Kenya and the neighboring islands of the Indian Ocean (24), presumably via human travel (22) and resulted in the Indian Ocean lineage (IOL), which resides within the ECSA genotype. The IOL became of particular interest because, like the Asian genotype, it was determined to be a descendent of the ECSA lineage (25); however, the CHIKV IOL has adaptive mutations in the E1 and E2 envelope protein genes (26-28). These adaptative mutations played a critical role in CHIKV emergence as they allowed both *Ae. aegypti* and *Ae. albopictus* to more efficiently act as a vector for the virus. The development of CHIKV being vectored by *Ae. albopictus* was crucial because these mosquitoes can survive in a much broader geographical range due to their resistance to colder climates (29). This is suspected to have led to the autochthonous transmission in Italy (2007) (30) and France (2010) (31) after CHIKV was imported to these areas via infected travelers from India. Although the IOL continued to circulate throughout Asia, the Americas first experienced CHIKV emergence in

2013 with the virus being detected on the Caribbean Island of Saint Martin (32).This emergence was not exceptionally surprising given that the Americas had the presence of both *Ae. aegypti* and *Ae. albopictus*; however, studies revealed that this particular strain of CHIKV was actually that of the Asian genotype, not the ECSA; suggesting *Ae, aegypti* was the primary vector responsible for this emergence. Since CHIKV's emergence in the Americas, it has caused millions of human infections and has been reported in over 100 countries (33). To this end, CHIKV remains a growing concern for global public health. Alongside CHIKV, scientists suggest fellow Semliki Forest serocomplex member, Mayaro virus (MAYV), is a developing threat due to its shared characteristics with CHIKV and the potential for this virus to imitate the evolutionary and epidemiological patterns discussed thus far.

1.7 Mayaro virus emergence and concern

MAYV and CHIKV share disease symptoms and similar sylvatic transmission cycles, suggesting an understanding of CHIKV spread throughout the Americas may provide some opportunity to forecast the future emergence of MAYV. Since its isolation from Trinidadian forest workers in 1954 (34), MAYV has become of increasing concern for the tropical regions of the New World, predominantly in South America and the Caribbean. Although MAYV is typically known to cycle between canopy-dwelling mosquito species, such as *Haemagogus* spp., and non-human primates, De Thoisy et al. reported high seroprevalence rates for a large number of free-ranging mammals from French Guiana (35). However, no studies have been undertaken to identify the role of each in the amplification or maintenance of MAYV in nature. Alongside MAYV's detection in this wide range of vertebrate species, MAYV has also been detected in several urban-adapted mosquito species including those within the *Culex* and *Aedes* genera (36, 37), both of which are ubiquitous throughout the Americas. Experimental studies assessing the vector competence of these species, as well as several *Anopheles*

spp. mosquitoes, have shown some of these mosquitoes are competent laboratory vectors for MAYV (38), especially *Ae. albopictus* and *Ae. aegypti* (39). While MAYV is typically associated with small sporadic outbreaks, it has been shown to cause larger outbreaks in urban settings, chiefly throughout Brazil and Venezuela (40, 41).Genetic characterization of MAYV isolates from 1954 through 2010 suggest the existence of two genotypes: genotype D (widely-dispersed) and genotype L (limited). Both genotypes primarily vary in their geographic distribution, specifically genotype D being predominant throughout the Pan-Amazon region and genotype L mainly causing outbreaks in Brazil (42). Notably, there have been recent cases of these genotypes, specifically genotypes D and L, causing outbreaks in geographical areas that were not previously identified (43), suggesting increased prevalence of the virus in new areas. This has raised concern for MAYV's potential impact on global public health should the virus continue to increase its geographical range. To date, the presence of MAYV has been detected serologically, or the virus has been isolated, in countries ranging as far south as Brazil to as far north as Mexico. Due to similar clinical manifestations of MAYV with dengue fever and chikungunya disease, misdiagnosis and subsequent, inaccurate reporting likely impacts the true estimation of MAYV's incidence in these co-endemic regions (44). Along with these challenges, the knowledge regarding MAYV's host range is clearly lacking and leaves a gap in the ability to predict its spread and conduct appropriate surveillance. To this end, the best defense against these alphaviruses remains to be focused on combating the mosquito vector(s).

1.8 Current arbovirus defenses and vector control programs

Since their discovery, defense strategies and vector control programs have been at the forefront of combating arboviruses. These defense strategies and vector control programs have focused on both targeting the pathogen itself and, more predominately, their mosquito vectors. Unfortunately, pathogen-based prevention, or targeting the pathogen directly via therapeutics and/or prophylactics,

has not been established for the bulk of arboviruses, with the exception of the aforementioned successful full licensing of the YFV and JEV vaccines. It should be noted that a vaccine for DENV has been developed (e.g., Dengvaxia); however, its approval is conditional on prior DENV infection due to an increased risk of severe DENV disease in seronegative individuals (45). Due to the lack of approved vaccines, coupled with the efficacy of vector-based programs having seriously waned over time with environmental restrictions on chemical insecticides and the development of insecticide resistance in many of these arthropod vectors, vector-borne diseases are still at large. In combination with these difficulties, urbanization and climate change are factors that have contributed to the emergence and transmission of arboviral diseases. Controlling the interaction among mosquitoes and humans is the primary method of preventing the spread of these diseases. This is mostly conducted through two methods: (1) preventing the bite of female mosquitoes and (2) insecticide usage. The first method, or prevention of female mosquito bites, is typically accomplished by educating the public on the use of personal protective equipment (e.g., long- sleeved clothing), the use of insect repellents (e.g., DEET), utilizing screens and/or netting, and ridding properties of standing water, which mosquitoes use for egg laying (e.g., old tires, buckets, pools, etc.). While these tools have proven to be beneficial by hindering some of the interactions between mosquitoes and humans, some mosquito species, particularly *Ae. aegypti*, have evolved to live in close proximity to humans and are extremely anthropophilic (46). Therefore, the second method of insecticide usage is used to reduce populations of mosquitoes in, and around, urban settings. Decreasing these mosquito populations greatly diminishes the risk of interactions between mosquitoes and humans and, in turn, limits transmission of vector-borne diseases. However, the environmental impacts of these toxic chemicals have led to great concern and has even resulted in the restricted, or completely prohibited, use of some of the most aggressive insecticides (e.g., DDT).

Since this time, future development has focused on the production of insecticides that require fewer applications, have increased specificity, and do not bioaccumulate (47). These environmental concerns, the limited number of approved insecticides, the potency of available insecticides, and, most importantly, the development of insecticide resistance have all played a major role in the emergence and/or re-emergence of many arboviral diseases.

1.9 Insecticide resistance

The use of insecticides to battle our deadliest enemy, alongside its pest relatives, has been in practice for millennia and has proven to be successful in limiting the spread of diseases (47); however, within the last century insecticide resistance has become increasingly worrisome. To elucidate, several studies have reported on the developed resistance of *Ae. aegypti* to a number of insecticides used for mosquito population control (48). The mechanisms proposed to contribute to this insecticide resistance, particularly in mosquitoes, can be grouped into two categories: metabolic detoxification and target-site mutations. Metabolic detoxification is the process of increased biodegradation of the insecticide through the expression and overproduction of detoxification enzymes (e.g., insect P450s) (49). On the other hand, target-site mutations are the result of well-defined mutations in the target site of insecticides, resulting in the inefficacy of the insecticide. For example, mutations in the target site (i.e., voltage-gated sodium channels) of pyrethroid insecticides, the most widely used class of insecticides for vector control, have been identified in *Ae. aegypti* mosquitoes (50, 51), rendering the insecticides ineffective in these mutation-bearing populations. Both metabolic detoxification and target-site alterations, either alone or in combination (52), have greatly contributed to the spread of vector-borne diseases and added to the importance of developing novel strategies to control mosquito populations and/or impede disease transmission.

1.10 Novel strategies for vector control

In order to control mosquito populations and/or impede disease transmission, novel strategies have been explored over the last few decades. Perhaps the most promising among these strategies is the process of genetic modification of these mosquito vectors. While the idea of genetically modifying mosquitoes is not necessarily new, recent advances in this field have resulted in the theoretical release of genetically modified mosquitoes being a reality. The genetically modified mosquitoes being released today were developed on the basis of two concepts: (1) population replacement and (2) population suppression.

Population replacement: Bacterium-mediated disease control

Population replacement is the strategy in which natural mosquito populations that are able to transmit vector-borne diseases are replaced with modified mosquitoes that are resistant to infection with, or unable to transmit, these diseases. For example, in recent years researchers from the World Mosquito Program (WMP) have developed and released *Ae. aegypti* mosquitoes that carry a strain of *Wolbachia*; a bacterium that is known to naturally infect approximately 60% of insects but not *Ae. aegypti* mosquitoes (53). These transinfected *Ae. aegypti* mosquitoes were originally developed to combat DENV (54) but have since been shown to be resistant against infection with CHIKV (55) and ZIKV (56). According to scientists from the WMP, there are current field studies and release projects in more than twelve countries spanning throughout Asia, Latin America, and Oceania. Although this research has been ongoing for decades, a recent study published in April of 2020 reports on the establishment of these *Wolbachia*-carrying mosquitoes and the significant reduction of local DENV transmission in northern Queensland, Australia (57). Further, an even more recent press release (dated in August 2020) described a 27-month trial in Indonesia that suggests a 77% reduction in DENV incidence (58). Alongside these promising results, a study published earlier this year reported on the *Wolbachia*-induced arboviral resistance of *Ae. albopictus* mosquitoes (59), an

important implication given the vectorial capacity of this species and its co-localization with the genetically modified *Ae. aegypti* mosquitoes.

Population suppression: A modern adaptation of Sterile Insect Technique

Population suppression is the strategy in which natural mosquito populations are reduced. One of the first methods of population suppression dates to 1955 and is known as sterile insect technique (SIT) (60). Specifically, in regard to mosquitoes, this process involved the release of gamma-irradiated or chemically-sterilized male mosquitoes into natural mosquito populations in order for them to mate with wild females in these populations and, thus, produce few viable offspring (61). In a modern adaptation of this technique, the UK-based biotechnology company, Oxitec, has developed male mosquitoes that carry a self-limiting gene that is lethal to offspring produced by this male (i.e., Release of Insects with Dominant Lethal [genes]; RIDL) (62). The goal of releasing these mosquitoes is to promote mating with wild female populations, produce non-viable offspring, and, consequently, decrease the native mosquito population. The mechanism behind this lethality is dependent on the requirement of the tetracycline antibiotic for suppression of the lethal gene (i.e., because tetracycline is not available to larvae in the wild, the lethal gene is not suppressed, and the offspring die) (62). The company has conducted field trials in the Cayman Islands, Malaysia, and Brazil (63) and have reported up to 95% population reduction (64, 65). While these studies have been ongoing for years, the United States' Environmental Protection Agency (EPA), and local governmental agencies, have recently approved Oxitec's proposal of a pilot study to release these genetically modified mosquitoes in the Florida Keys. This is the first approval of release of genetically modified mosquitoes in the United States and sets an important precedent for future defense strategies against arboviral threats.

1.11 Future defense of CHIKV and MAYV

As CHIKV and MAYV have both become increasingly prevalent throughout the Americas within the last decade, a heavy focus on combatting these alphaviruses has ensued. In conjunction with sporadic outbreaks throughout the sub-tropical regions of the Americas, increased global travel to CHIKV- and MAYV-endemic areas has resulted in imported cases of both these viruses throughout the United States and Europe, as well as imported cases that facilitated local CHIKV transmission in the United States (66). While several CHIKV vaccine candidates are in development, to date, there are no licensed vaccines or antivirals specific to the prevention and/or treatment of CHIKV or MAYV. Fortunately, previous studies have been conducted to understand cross-protection among alphaviruses and, specifically, viruses within the Semliki Forest serocomplex (67-73). For example, the live-attenuated CHIKV/IRES vaccine candidate was shown to elicit a strong cross-neutralizing antibody response to ONNV and provide complete protection from ONNV disease in murine models (70); however, this protection did not extend to RRV. Furthermore, Fox *et al.* screened a panel of anti-CHIKV murine monoclonal antibodies (mAbs) for cross-neutralizing potential against other alphaviruses (e.g., ONNV, MAYV, Semliki Forest virus, and RRV), and found a single anti-CHIKV murine mAb completely protects mice from disease caused by MAYV (67). These data suggest that combating CHIKV and MAYV may be possible in a single defense measure.

1.12 In summary

The purpose of this dissertation was to further investigate the cross-reactivity among CHIKV and MAYV, to characterize the antibody-mediated cross-neutralization of alphaviruses, and to develop a single defense strategy to combat both emerging arboviruses. Our work has shown that neutralizing antibodies against CHIKV can reduce MAYV disease. Alongside these studies, we have characterized the potency of camelid-derived single-domain antibodies (sdAbs) to neutralize a breadth of medically-relevant alphaviruses, including CHIKV and MAYV. We then generated

transgenic *Aedes aegypti* mosquitoes that express these two anti-CHIKV sdAbs to reduce infection, dissemination, and transmission of MAYV and CHIKV within this deadly vector. This work moves the field forward because it's the first development of a single defense strategy to actively, and specifically, co-target two emerging alphaviruses that obliterate quality of life around the globe.

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CHAPTER TWO: EVALUATING CROSS-REACTIVITY AMONG CHIKV AND MAYV

Effects of chikungunya virus immunity on Mayaro virus disease and epidemic potential

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2.1 Abstract

Mayaro virus (MAYV) causes an acute febrile illness similar to that produced by chikungunya virus (CHIKV), an evolutionary relative in the Semliki Forest virus complex of alphaviruses. MAYV emergence is typically sporadic, but recent isolations and outbreaks indicate that the virus remains a public health concern. Given the close phylogenetic and antigenic relationship between CHIKV and

MAYV, and widespread distribution of CHIKV, we hypothesized that prior CHIKV immunity may affect MAYV pathogenesis and/or influence its emergence potential. We pre-exposed immunocompetent C57BL/6 and immunocompromised A129 or IFNAR mice to wild-type CHIKV, two CHIKV vaccines, or a live-attenuated MAYV vaccine, and challenged with MAYV. We observed strong cross-protection against MAYV for mice pre-exposed to wild-type CHIKV, and moderately but significantly reduced cross-protection from CHIKV-vaccinated animals. Immunity to other alphavirus or flavivirus controls provided no protection against MAYV disease or viremia. Mechanistic studies suggested that neutralizing antibodies alone can mediate this protection, with T-cells having no significant effect on diminishing disease. Finally, human sera obtained from naturally acquired CHIKV infection cross-neutralized MAYV at high titers *in vitro*. Altogether, our data suggest that CHIKV infection can confer cross-protective effects against MAYV, and the resultant reduction in viremia may limit the emergence potential of MAYV.

2.2 Introduction

Mayaro virus (MAYV) is an arthropod-borne virus and a member of the family *Togaviridae*, genus *Alphavirus*. Since its isolation from Trinidadian forest workers in 1954[1], MAYV has become of increasing concern for the neotropics[2-4]. MAYV is the etiological agent of Mayaro fever (MAYF), a disease often misdiagnosed due to its similar clinical presentation to dengue and chikungunya fevers, as well as many other tropical diseases. The co-circulation of these viruses in many Latin American countries further complicates accurate diagnoses[5-7]. Infection with MAYV typically results in an acute febrile illness, with flu-like signs and symptoms such as a cutaneous rash, headache, myalgia, and debilitating arthralgia that can persist for months or, in some cases, years following infection[8]. MAYV is a 65–70 nm enveloped virus with a ~11.5 kb single-stranded, positive-sense genomic RNA that is packaged within a nucleocapsid. The genome encodes five

structural proteins (C, E1-3, and 6K), including capsid and envelope proteins, and four non-structural proteins (nsP1-4), encoding the virus' replication machinery[9]. Phylogenetic studies have revealed three distinct MAYV lineages: genotype D (widely dispersed), genotype L (more limited distribution detected), and genotype N (newly identified)10. Genotypes D and L are considered major lineages whereas genotype N consists of one strain isolated in Peru in 2010[10]. Genotype L has been reported only in Brazil and Haiti, while genotype D contains isolates from Trinidad, Suriname, French Guiana, Peru, Bolivia, Venezuela, and Brazil[11]. Recently, MAYV exposure has been detected serologically or by virus detection in countries ranging as far south as Brazil and as far north as Mexico[12-22].

MAYV outbreaks are typically relatively small and occur primarily in Brazil and Peru[8,11,22-25]; however, MAYV has demonstrated its potential for large outbreaks such as the ~800 persons affected in Brazil in 1978[22]. Interestingly, a recent case in Haiti reported an eight-year-old boy presenting with fever, abdominal pain, who was co-infected with dengue virus (DENV) and MAYV[13]. The detection of MAYV is unprecedented in Haiti and surprising due to the absence of indigenous nonhuman primates (NHP; key amplification hosts in MAYV's proposed enzootic cycle) in Haiti. While this cycle has not been fully characterized, MAYV is believed to circulate between canopy-dwelling *Haemagogus* mosquitoes and NHPs, similar to sylvatic yellow fever virus in the neotropics[26]. However, in 2003, Thoisy *et al*. conducted a serological survey for MAYV in wild animals from French Guiana and found seropositive birds, rodents, NHPs, and other small mammals[18]. Furthermore, mosquitoes from the *Aedes* genus (*e.g., Ae. albopictus* and *Ae. aegypti*) can experimentally transmit MAYV[27-28], and MAYV has been isolated from *Ae. aegypti* and possibly *Culex quinquefasciatus* mosquitoes in the wild[29]. Recent studies show that *Culex quinquefasciatus* are incompetent MAYV vectors but highlight the potential importance of

Anopheline mosquitoes in transmission[30]. These data, coupled with recent outbreaks, suggest the existence of alternative enzootic vertebrate hosts, undescribed vectors, and/or the beginning of MAYV's adaptation to an urban or peridomestic epidemic transmission cycle.

Chikungunya virus (CHIKV) is a close alphavirus relative of MAYV and a fellow member of the Semliki Forest complex. CHIKV infection results in a similar disease presentation, characterized by persistent, debilitating arthralgia, and has a comparable sylvatic transmission cycle to that of MAYV, but in sub-Saharan Africa. However, it has a long history of emergence into a peridomestic, human-amplified cycle and of invading Asia, the Indian Ocean Basin[31], and recently the Americas in 2013[32]. CHIKV is now among the most widely distributed alphaviruses and circulates in all countries with evidence of MAYV transmission[33].

Given the similarities in disease presentation and potentially similar peridomestic transmission cycles, as well as the close phylogenetic and antigenic relationship between MAYV and CHIKV, we hypothesized that the recent CHIKV spread throughout the Americas and resultant herd immunity in humans, as well as the potential for CHIKV to establish enzootic transmission there, will affect transmission and spread of MAYV in the Americas. Previous studies have been conducted to understand cross-protective immunity among alphaviruses and, specifically, viruses within the Semliki Forest virus complex[34-40]. A live-attenuated CHIKV vaccine candidate (*e.g.*, CHIKV/ IRES) elicits a strong cross-neutralizing antibody response to o'nyong-nyong virus (ONNV), a close relative of CHIKV, and provides complete protection from ONNV but not Ross River virus disease in mouse models[34]. This study suggests that neutralizing antibodies are the sole mediator of the observed cross-protection. Furthermore, Fox *et al*. screened a panel of anti-CHIKV mouse monoclonal antibodies (mAbs) for cross-neutralizing potential against other alphaviruses (*i.e.*, ONNV, MAYV, Semliki Forest virus, and Ross River virus), and demonstrated that a single anti-

CHIKV mouse mAb completely protects mice from MAYV disease and mortality[36].

Given the phylogenetic and serological relationship between CHIKV and MAYV, we investigated the potential effects of CHIKV immunity on MAYV infection and disease using established immunocompetent and immunocompromised murine models[36,41-42]. Additionally, we studied the potential application of leading CHIKV vaccine candidates to control MAYV disease and potential emergence. Our results demonstrate that immunity to CHIKV can significantly reduce murine MAYV disease and that neutralizing antibodies alone can mediate this protection, with T-cells not playing a significant role in protection. Our data also show that naturally acquired CHIKV immunity in humans can neutralize MAYV *in vitro*. These data also suggest a certain threshold of CHIKV immunity is needed to protect against MAYV disease, such that the chimeric or live-attenuated CHIKV vaccines are unlikely to control a MAYV outbreak, while naturally acquired CHIKV immunity (wild-type CHIKV infection) may be adequate to provide protection and reduce viremias sufficiently to impede outbreaks.

2.3 Materials and Methods

Viruses and cell cultures

The wild-type and attenuated vaccine CHIKV strains used in this study were provided by the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) at the University of Texas Medical Branch (Galveston, Texas, USA). These included CHIKV/IRES (i.e., a live-attenuated CHIKV vaccine)[57], EILV/CHIKV (i.e., a chimeric host-restricted CHIKV vaccine)[58], CHIKV-99659 (i.e., an Asian-American, wild-type CHIKV isolated from the British Virgin Islands in 2014)[59] and CHIKV 181/25 (i.e., vaccine strain)[60]. MAYV strains included MAYV/IRES (i.e., a live-attenuated MAYV vaccine)[41] and 12A (i.e., a genotype D wild-type MAYV isolate from a 2010 outbreak in Venezuela)[10]. Additionally, Zika virus (ZIKV) strain FSS13025 (i.e., a Cambodian

ZIKV strain)[61] and Venezuelan equine encephalitis virus (VEEV) strain TC-83 (i.e., a live-attenuated VEEV vaccine)[62-63] were used for the study. Vero 76 (African green monkey kidney), were obtained from the American Type Cell Collection (Bethesda, MD) and maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 5% fetal bovine serum (FBS), and Penicillin and Streptomycin (Pen/Strep) (100 U/ ml) in a 37°C, 5% CO_2 incubator. C7/10 (*Aedes Albopictus*) cells were obtained from the WRCEVA and maintained in DMEM supplemented with 10% FBS, 1% minimal essential medium non-essential amino acids, 1% tryptose phosphate broth and Pen/Strep (100 U/ml) in a 29°C, 5% CO_2 incubator. C7/10 cells were used to prop- agate EILV/CHIKV (i.e., it does not replicate in vertebrate cells) for vaccination and Vero cells were used to propagate all remaining viruses and vaccine strains, and used during plaque assays and $PRNT_{80}$ tests.

Cross protection in an immunocompetent mouse model

Four-week-old C57B6/J mice (Jackson Laboratory, Bar Harbor, ME, n = 8) were vaccinated or infected subcutaneously with 104 plaque-forming units (PFU) of CHIKV/IRES, CHIKV-99659, MAYV/IRES, TC-83, 105 PFU of ZIKV, 108 PFU of EILV/CHIKV, or sham-vaccinated with PBS. A PBS group (n = 8) was also employed as uninfected controls. Virus and vaccine doses were administered based on their initial reports to achieve sufficient preexisting immunity for homologous protection. Twenty-eight days post-vaccination, mice were bled to evaluate humoral immune responses via neutralization assays. Thirty-one days post-vaccination mice were challenged intradermally in the hind footpad with 105 PFU of MAYV. This dose was selected based on previous studies to generate sufficient MAYV disease signal to allow for statistical comparison in both murine models employed[41-42]. Mice were bled on alternating days (i.e., days one and three or

days two and four) for viremia measures. Viremia was determined by plaque assay using Vero cells with standard protocols and eighty percent plaque reduction neutralization tests ($PRNT_{80}$) were also performed on Vero cells using CHIKV-99659 as the control CHIKV and 12A as the control MAYV[64]. For $PRNT_{80}$, serum was heat inactivated at 56°C for 30 min and serially diluted in DMEM containing 1% FBS. All dilutions were then further diluted an additional 2-fold by the addition of ~100 PFU of MAYV or CHIKV, mixed, and incubated at 37°C for 1 hr, then 100 PFU was transferred to 90% confluent monolayers of Vero cells in 12-well plates and incubated at 37°C for 1 hr. PRNTs were done in duplicate. Overlay containing 0.4% agarose in DMEM with 2% FBS and Penicillin and Streptomycin (Pen/Strep) (100 U/ml) was added, and plates were incubated for 2 days at 37 °C. Cells were then fixed in 10% formaldehyde, and plaques visualized following crystal violet staining. Weight changes and footpad swelling were measured daily for 14 days post-challenge.

Cross protection in an immunocompromised mouse model

A129 (i.e., Interferon alpha/beta receptor null) mice were bred locally at UTMB. At four weeks of age, mice (n = 5 or 6) were vaccinated with 104 PFU of CHIKV/IRES, MAYV/IRES, 108 PFU of EILV/CHIKV, or sham-vaccinated with PBS. A PBS group (n = 5) was also employed as uninfected controls. Twenty-eight days post-vaccination, mice were bled for neutralization assays, and then challenged as described above 31 days post-vaccination with a lethal dose (105 PFU) of MAYV. Mice were bled for viremia as described above, and weights and footpad swelling were measured until mortality.

Passive transfers of immune sera to an immunocompromised mouse model

At four weeks of age, C57/B6J mice (n = 4) were vaccinated or infected with 104 PFU of CHIKV/IRES, CHIKV-99659, MAYV/IRES, 105 PFU of ZIKV, 108 PFU of EILV/CHIKV or

sham-vaccinated with PBS. A PBS group (n = 5) was also employed as uninfected controls. Six weeks post-vaccination, mice were terminally bled to measure neutralizing antibody titers and to collect immune sera for passive transfers. Sera from each group were pooled and $PRNT_{80}$ were completed using respective antigens. 100 µl of pooled sera were administered intraperitoneally to A129 mice (n = 5 or 6) 36 hours prior to challenge with a lethal dose (105 PFU) of MAYV. Mice were bled daily for four days for viremia measurements. Weight changes and footpad swelling were measured for 14 days post-challenge or until mortality.

T-cell stimulation assays

C57BL/6J mice were vaccinated or infected with CHIKV/IRES, EILV/CHIKV, CHIKV-99659, ZIKV, MAYV/IRES, or sham-vaccinated with PBS using doses described above. Six weeks post-vaccination, mice were sacrificed and spleens were collected for splenocyte isolation. Splenocytes were stimulated for 24 hours with MAYV 12A (MOI = 1) in a Golgi-plug (BD Biosciences, San Jose, CA) containing medium. Cells were then harvested, stained with antibodies for CD3, CD4, or CD8, fixed, and permeabilized with BD Cytofix/Cytoperm (BD Biosciences, San Jose, CA) before adding PE-conjugated anti-IFN-γ, or control PE-conjugated rat IgG1 (eBiosciences, San Diego, CA). Cells were then washed and analyzed using a C6 Flow Cytometer (Accuri cytometers, Ann Arbor, MI).

T-cell depletion studies

Five-week-old IFNAR knockout mice (n = 15) were vaccinated with 104 PFU of CHIKV/IRES, 108 PFU of EILV/CHIKV, or sham-vaccinated with PBS. Ten weeks later, anti-CD4 (clone GK1.5), anti-CD8 (clone 2.43), or rat IgG2b isotype control (clone LTF-2) antibodies (Bio X Cell, West Lebanon, NH) were administered intraperitoneally to mice (e.g., n = 5/antibody group) on days −3, −1, and 3 post-challenge. On the day of challenge, blood samples were taken from three mice per

each group and circulating T-cells were quantified by flow cytometry to determine knockdown efficiency. After confirming >95% knockdown in all groups, mice were challenged with a lethal dose (105 PFU) of MAYV. Mice were bled daily for four days for viremia measurements. Weight changes and footpad swelling were measured for six days post-challenge or until mortality.

Cross-neutralization studies of MAYV 12A in human sera samples

Twenty-four (24) serum samples from 20 individuals who had presented with clinical symptoms of CHIKV infection during the 2014–2015 CHIKV epidemic in Trinidad (n = 18) and in 2016 (n = 2) were screened for neutralizing activity against CHIKV vaccine strain 181/25 and MAYV strain 12A using PRNTs. Neutralization titers were calculated and expressed as the reciprocal of the initial serum dilution yielding greater than 80% reduction ($PRNT_{80}$) in the number of plaques as compared to control wells. Serum samples were tested for the presence of CHIKV specific antibodies using EuroImmun Anti-Chikungunya Virus ELISA IgM and IgG kits (EuroImmun AG, Germany), with the IgM ELISA used only for early convalescent (<180 dpo) samples. A subset of samples (TT10 – TT57) were also tested using AbCam Human Anti-Chikungunya Virus ELISA kits (AbCam, Cambridge, UK) for IgG. Samples were deemed IgG positive if a positive result was returned by either the EuroImmun ® or AbCam ® IgG kit.

Statistics

Data normalcy was tested using a combination of Q-Q plot and box-plot analyses. However, uneven group numbers, especially in the context of lethal models, precluded ranks-based analyses and parametric tests were universally employed. One-way and repeated measures ANOVAs, as well as Kaplan-Meier survival curves were performed using SPSS statistics software (IBM Corporation, Armonk, NY).

2.4 Results

Wild-type CHIKV protects against MAYV disease in immunocompetent mice. To investigate the cross-reactive immune potential between CHIKV and MAYV, we first evaluated neutralizing antibody titers in CHIKV-infected C57/B6J mice (Fig.1a). These mice were vaccinated or inoculated with CHIKV strain 99659, vaccine candidates CHIKV/IRES, EILV/CHIKV, or MAYV/IRES, Venezuelan equine encephalitis virus (VEEV; a distantly related alphavirus) vaccine strain TC-83, the unrelated flavivirus Zika (ZIKV) or were sham-vaccinated (N.B. see materials and methods for details on viruses used). Mice inoculated with CHIKV-99659 and all CHIKV-vaccinated groups developed moderate (>320) CHIKV-neutralizing antibody titers, as expected. Mice sham-vaccinated with PBS served as negative controls and developed no detectable neutralizing antibodies. Mice vaccinated with MAYV/IRES developed high (>640) MAYV-neutralizing antibody titers and mice vaccinated with TC-83 presented with typical VEEV-neutralizing antibody titers (≤320). Mice infected with ZIKV developed low (≤40) ZIKV-neutralizing antibody titers.

Prior to MAYV challenge, little-to-no cross-reactive neutralization activity was detected in the sera of the CHIKV-vaccinated groups, the PBS-vaccinated group, or in the TC-83- and ZIKV-infected groups. Mice infected with wild-type CHIKV-99659 presented the highest (≤160) cross-reactive (MAYV) neutralizing anti- body titers out of all non-MAYV vaccine/virus groups, with the CHIKV vaccines generating lower levels of cross-neutralization. Mice were then challenged with 105 PFU of MAYV. There were no significant changes in weight among any of the vaccine/virus groups. This is expected since weight loss offers a less sensitive disease signal in immunocompetent mice as seen previously[41-42] However, MAYV/IRES completely protected against foot- pad swelling and CHIKV-99659 also provided significant protection when compared to all other groups (Fig.1b). These data also correlated with the viremia studies; both MAYV/IRES and CHIKV-99659

vaccination/infection prevented detectable MAYV viremia throughout four days post-challenge (Fig.1c). There was no evidence for diminished MAYV disease or viremia in any of the CHIKV-vaccinated groups or other virus control groups.

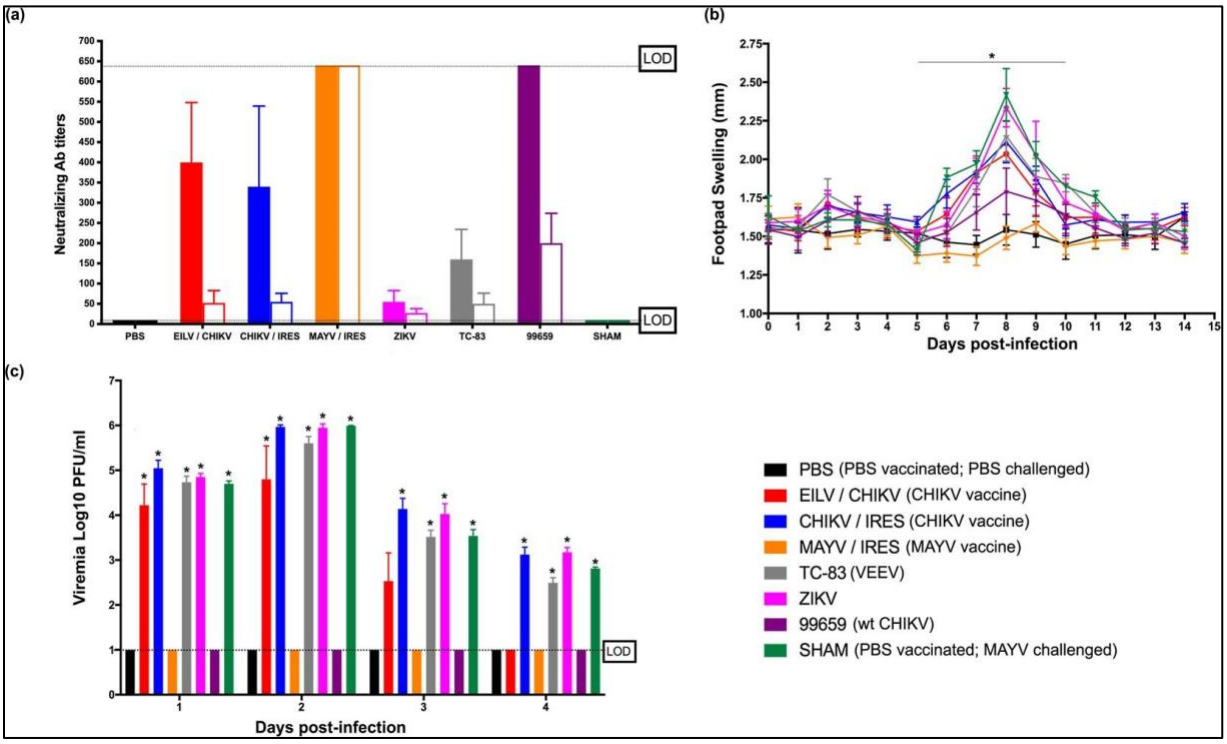

Figure 1. CHIKV immunity provides cross-protection against MAYV infection and disease in an immunocompetent mouse model. (**a**) Virus-specific neutralizing antibody titers (solid bars) and MAYV cross- neutralization antibody titers (empty bars) were determined prior to MAYV challenge. Dashed lines indicate the upper and lower limits of detection (1:640 and 1:20, respectively); (**b**) footpad swelling was determined daily throughout the study; (**c**) Viremia was measured daily for four days post-infection. All plotted values in (**b, c**) are mean ± S.D. Data in (**b**) were analyzed using a repeated measures ANOVA with a Bonferroni multiple comparison post hoc analysis, and data

in (**d**) were analyzed with a one-way ANOVA with a Bonferroni post hoc analysis. There were no significant differences in percent weight change among groups; however, footpad swelling was significantly different between the PBS (i.e., PBS-vaccinated and PBS-challenged) control and all other groups except CHIKV-99659 and MAYY/IRES. Additionally, viremia was significantly different between the PBS control and all other groups except CHIKV-99659 and MAYY/IRES on days one and two, and EILV/ CHIKV, CHIKV-99659 and MAYY/IRES on days three and four. Statistically significant values are denoted by $*p < 0.05$.

CHIKV immunity protects against MAYV disease in immunocompromised mice.

We next analyzed cross-protective immunity in an immunocompromised, lethal mouse model. A129 mice vaccinated with CHIKV/IRES, MAYV/IRES, EILV/CHIKV, or sham-vaccinated were analyzed for virus-specific neutralizing antibodies. Other virus controls could not be used because of the susceptibility of this model to disease and death following administration of some viruses and vaccines. A129 mice developed high CHIKV neutralization titers (>640) after administration of all CHIKV vaccines, and high MAYV neutralization titers (>640) in the MAYV/ IRES group. Interestingly, in A129 mice, we observed similar cross-reactive neutralizing antibody titers (≥80) in the CHIKV/IRES group compared to those of CHIKV-99659 in the immunocompetent mouse model study described above. The EILV/CHIKV group developed less cross-reactive immunity (≤80). Figure 2a describes the virus-specific and MAYV-cross-reactive neutralizing antibody titers for these experimental groups. Following challenge with a lethal dose of MAYV, EILV/CHIKV- and sham-vaccinated groups displayed significant weight loss and footpad swelling (Fig. 2b-c), with mortality resulting five days post-challenge (Fig. 2d). Footpad swelling observations suggested that

MAYV/IRES completely protected against disease, as expected, and CHIKV/IRES displayed similar protection with a small spike in footpad swelling on day two post-challenge. Both CHIKV/IRES and MAYV/IRES vaccination protected against detectible MAYV viremia and fatal outcomes. EILV/CHIKV offered no protection from disease (i.e., footpad swelling), viremia, or death.

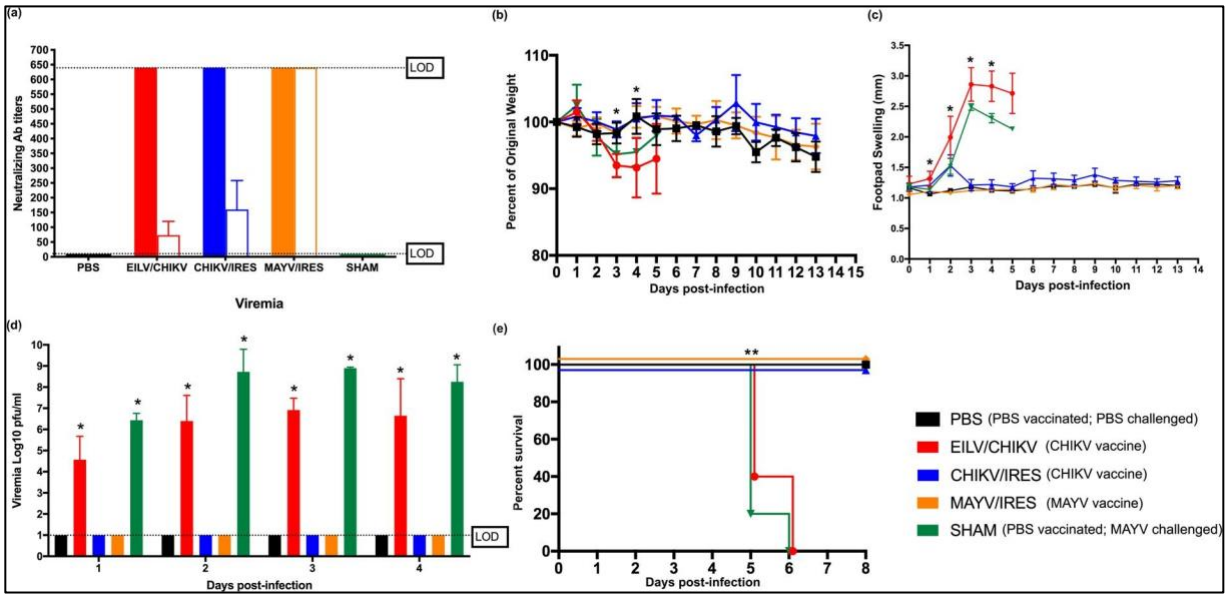

Figure 2. CHIKV immunity provides cross-protection against MAYV infection and disease in an immunocompromised mouse model. (a) Virus-specific neutralizing antibody titers (solid bars) and MAYV cross-neutralizing antibody titers (empty bars) were determined prior to MAYV challenge. Dashed line indicates the upper and lower limits of detection (1:640 and 1:20, respectively). **(b)** Weight change, expressed as percent of original, and **(c)** footpad swelling was determined daily throughout the study. (d) Viremia was measured daily for four days post-infection and (e) survival was recorded. All plotted values in (b–d) are mean ± S.D. Data in **(b–d)** were analyzed using a one-way ANOVA with a Bonferroni post hoc analysis, and survival curves (e) were analyzed by Kaplan-Meier survival analysis. Footpad swelling

was significantly different between the PBS control (i.e., PBS-vaccinated and PBS-challenged) and EILV/CHIKV groups on days one and two, and between the PBS control and EILV/CHIKV and sham-vaccinated groups on day three, and PBS and sham on day four. Weight change was statistically significant between the PBS control and EILV/CHIKV on days three and four. Viremia and survival were statistically significant for EILV/CHIKV and sham throughout four days post-infection. Statistically significant values are denoted by *****$p < 0.05$.

Passive transfer of CHIKV immune serum is not protective against MAYV challenge in immuno- compromised mice. To further explore whether cross-protective immunity is T-cell- or antibody-mediated, C57/B6J mice were vaccinated or infected with the viruses/vaccines described above in the C57/B6J cross-protection study. Next, 100 µl of immune sera (i.e., collected 6 weeks post-infection) was administered to A129 mice intraperitoneally prior to challenge with a lethal dose (105 PFU) of MAYV. Figure 3a describes the neutralizing antibody titers for the immune sera transferred. No cross-protection was observed among any of the groups except the MAYV/IRES positive control group. Changes in weight were consistent among all groups (Fig. 3b); however, footpad swelling (Fig. 3c) and viremias (Fig. 3d) were elevated in all heterologous CHIKV vaccine/virus groups, and all experimental groups succumbed to disease six days post-MAYV-infection (Fig. 3e). The sham-challenged and MAYV/IRES-vaccinated, challenged control groups survived the entire study and showed no signs of illness.

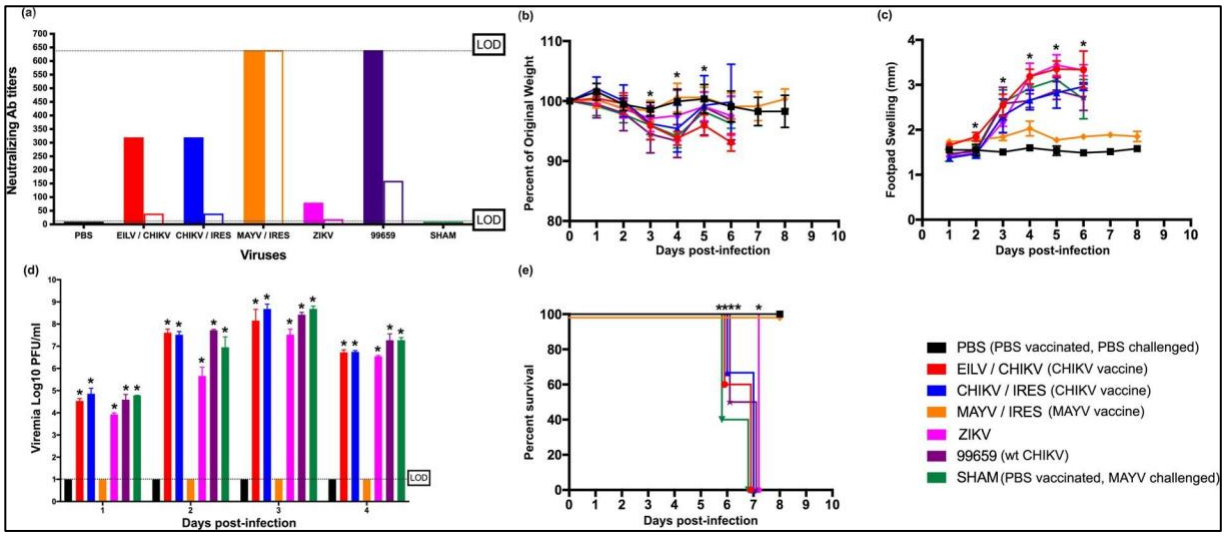

Figure 3. Passive transfer of CHIKV immune sera does not provide cross-protection against MAYV infection and disease in an immunocompromised mouse model. (a) Virus-specific neutralizing antibody titers (solid bars) and MAYV cross-neutralizing antibody titers (empty bars) were determined prior to MAYV challenge. Dashed line indicates the upper and lower limits of detection (1:640 and 1:20, respectively). **(b)** Weight change, expressed as percent of original, and **(c)** footpad swelling was determined daily throughout the study. **(d)** Viremia was measured daily for four days post-infection and **(e)** survival was recorded. All plotted values in (b– d) are mean ± S.D. Data in (b-d) were analyzed using a one-way ANOVA with a Bonferroni post hoc analysis, and survival curves (e) were analyzed by Kaplan-Meier survival analysis. When compared to the PBS control (i.e., PBS-vaccinated and PBS-challenged), statistically significant footpad swelling was observed in mice from EILV/CHIKV on days 2–6, CHIKV/IRES on days 1 and 3–6, and ZIKV, CHIKV-99659 and sham on days 3–6. Weight change was statistically significant for EILV/CHIKV on days three and five, and CHIKV-99659 on days three and four, and

sham on day four. Viremia (days 1–4) and survival was statistically significant for all groups except MAYV/IRES. Statistically significant values are denoted by ***** $p<0.05$.

T-cells from CHIKV-infected mice are reactive to MAYV 12A antigen. To investigate the cellular response to MAYV 12A antigen (i.e., the virus strain used in challenges throughout the study) following exposure to PBS, ZIKV, EILV/CHIKV, CHIKV/IRES, CHIKV-99659, or MAYV/IRES, we measured *ex vivo* intracellular IFN-γ production among murine CD4+ and CD8+ T-cells following stimulation with MAYV 12A. All groups produced significantly ($p \leq 0.05$) higher numbers of MAYV-specific IFN-γ+- CD4+ and CD8+ T-cells when com- pared to the PBS control group (Fig. 4). As expected, the MAYV/IRES group produced the highest numbers of IFN-γ+ T-cells; however, the CHIKV-99659 group also produced large quantities of IFN-γ+ T-cells.

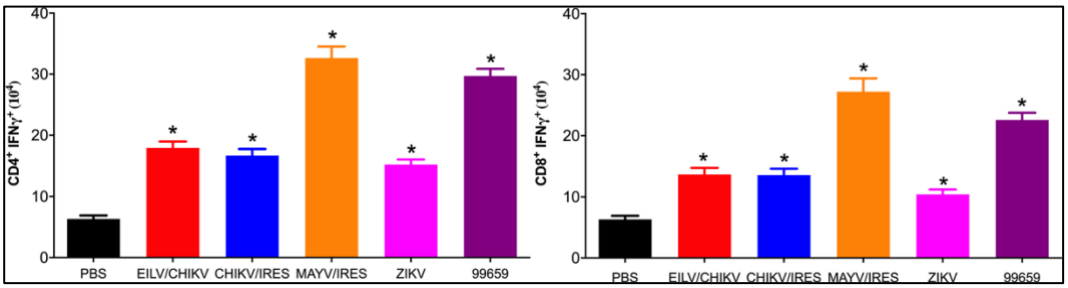

Figure 4. CHIKV immune T-cells are reactive upon stimulation with MAYV 12A. C57/B6J mice were vaccinated or challenged with CHIKV/IRES, EILV/CHIKV, CHIKV-99659, ZIKV, MAYV/IRES or sham- vaccinated with PBS. Six weeks post-vaccination, mice were sacrificed, splenocytes were isolated and stimulated for 24 hours with MAYV, stained with antibodies for CD3, CD4, CD8, IFN-γ and analyzed using flow cytometry. Number of (**a**) CD4+ IFN-γ+ and (**b**) CD8+ IFN-γ+ T-cells are plotted as mean ± S.D. Data were analyzed using a one-way ANOVA with a

Bonferroni post hoc analysis. Statistically significant values are denoted by *****$p < 0.05$.

T-cell depletion studies suggest cross-protection is antibody-mediated. IFNα/βR−/− mice were vaccinated with 104 PFU of CHIKV/IRES, 108 PFU of EILV/CHIKV or sham-vaccinated with PBS. Ten weeks post vaccination, CD4+ and CD8+ T-cells were independently depleted with antibodies (>98% depletion efficiency compared to isotype controls; data not shown) and mice were then challenged with a lethal dose (105 PFU) of MAYV. Since previous studies showed that depletion of both CD4+ and CD8+ T-cells in MAYV/IRES vaccinated A129 mice did not have any effect post MAYV challenge (data not shown), and given the pathogenicity of this vaccine in 6-week old IFNα/βR−/− mice, this group was excluded from this study. As seen previously, EILV/CHIKV vaccination did not protect against MAYV-induced disease, but the CHIKV/IRES group was protected from mortality (Fig. 5). The viremia studies also suggested a reduction in the CHIKV/IRES-vaccinated but not EILV/CHIKV-vaccinated mice (Fig. 5g-i). No statistically significant trend or pattern was detected when CD4+ or CD8+ depleted groups were compared to the isotype controls. These results suggest little or no role for T cells in the protection against MAYV challenge.

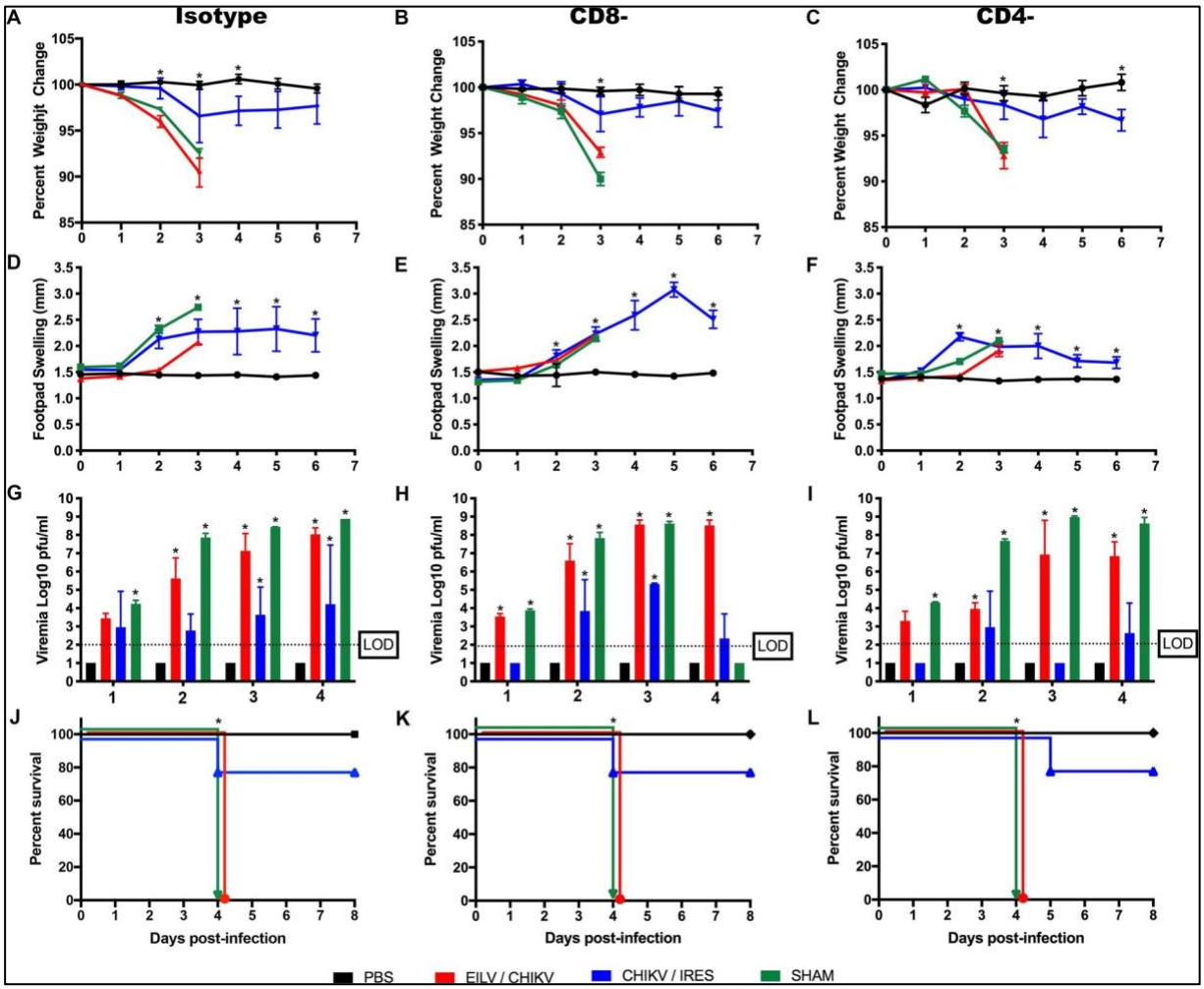

Figure 5. T-cell depletion studies reveal no significant role for CD4+ and CD8+ T-cells in diminishing MAYV disease. (**a**–**c)** Weight change, expressed as percent of original and (**d**–**f**) footpad swelling was determined daily throughout the study. (**g**–**i**) Viremia was measured daily for four days post-infection, (**j**–**l**) and survival was recorded. All plotted values in (**a**–**i)** are mean ± S.E.M. (**a**–**c**) were analyzed using a repeated measures ANOVA with a Bonferroni multiple comparison post hoc analysis, and data in (**d**–**i**) were analyzed using a one-way ANOVA with a Bonferroni post hoc analysis, and survival curves (**j**–**l**) were analyzed by Kaplan-Meier survival analysis. Statistically significant values are denoted by *****$p < 0.05$.

CHIKV-immune human sera cross-neutralizes MAYV *in-vitro*

To investigate the extent to which naturally acquired CHIKV immunity in humans can cross protect against MAYV infection, 24 serum samples from 20 individuals who presented with suspected CHIKV infection in Trinidad during the 2014/15 CHIKV outbreak (18 individuals) and in 2016 (2 individuals) were screened by ELISA for CHIKV-specific antibodies. These samples were then analyzed for the ability to neutralize CHIKV (i.e., vaccine strain 181/25) and MAYV 12A infection on Vero cells by plaque reduction neutralization tests (PRNT). As indicated in Table 1, both early (*i.e.*, <180 days post-onset of illness [dpo]) and late (≥180 dpo) convalescent samples were available for four individuals (TT93, TT103, TT112 and TT114). All other individuals were represented by serum from a single time point, of which four were during early convalescence (TT35, TT47, TT107, and TT122). CHIKV-specific IgG antibodies were detected in 22 out of the 24 samples, and CHIKV-specific IgM antibodies in one out of ten samples tested (i.e., TT47). CHIKV-neutralizing antibodies were detected in all of the CHIKV IgG-positive samples with $PRNT_{80}$ titers ranging from 40 to 40,960. All but two of the CHIKV IgG-positive sera (i.e., TT103 and TT112) also neutralized MAYV with titers ranging from 40 to 2,560. Two samples collected during the CHIKV outbreak (TT10 and TT38) had eight- and two-fold higher PRNT80 titers for MAYV 12A than for CHIKV 181/25 respectively, suggesting possible MAYV or another alphavirus infection.

Table 1. Serological status and $PRNT_{80}$ titers of human sera from individuals with suspected CHIKV infections. *Indicates early convalescent samples (i.e., serum samples collected less than 180 dpo). NT indicates "not tested", " – "indicates "not applicable". †Results of IgG ELISAs were discordant (positive by AbCam and negative by EuroImmun Anti-Chikungunya Virus ELISA). §Presented with suspected

CHIKV in 2016; all others presented during 2014/15 CHIKV epidemic period.

#Indicates a possible MAYV or another alphavirus infection.

Sample ID	Days post onset of illness (dpo)	CHIKV serostatus	$PRNT_{80}$ titer		
		IgG	IgM	CHIKV	MAYV
TT10	312	Positive	—	**40**	**320#**
TT13	336	Negative	—	<20	<20
TT35	135*	Positive	—	**2560**	**2560**
TT38	210	Positive	—	**320**	**640**
TT39	210	Positive	—	**2560**	**640**
TT47	130*	Positive	Positive	**5120**	**640**
TT48	330	Positive	—	**10240**	**640**
TT49	300	Positive	—	**10240**	**640**
TT52	360	Positive	—	**10240**	**640**
TT54	330	Positive†	—	**2560**	**640**
TT57	360	Positive	—	**40960**	**640**
TT65	363	Positive	—	**640**	**640**
TT68	389	Positive	—	**640**	**160**
TT92	360	Positive	—	**640**	**320**
TT93	30*	Negative	Negative	**40**	<20
	367	Negative	—	<20	<20
TT103§	30*	Positive	Negative	**640**	<20
	360	Positive	—	**320**	**40**
TT107§	30*	Positive	Negative	**640**	**40**
TT112	20*	Positive	NT	**80**	<20
	427	Positive	—	**640**	**80**
TT114	10*	Positive	Negative	**640**	**160**
	415	Positive	—	**640**	**160**
TT122	23*	Positive	Negative	**640**	**40**

2.5 Discussion

MAYV is an important re-emerging alphavirus in the Americas. Despite its history of small sporadic outbreaks, MAYV has the potential to cause large devastating outbreaks through potential peridomestic, human-amplified transmission. Therefore, identifying and understanding the factors that can influence the epidemic potential of MAYV is important to forecasting its spread and disease burden. MAYV and CHIKV share many characteristics and are phylogenetically and serologically related members of the Semliki Forest complex. There is no published information available on

whether or not MAYV circulation or immunity is affecting the epidemic dynamics of CHIKV in the Americas, but CHIKV is now probably endemic in many parts of Latin America and its reciprocal effects on MAYV emergence warrants investigation.

Our studies in immunocompetent C57/B6J mice show that immunity derived from wild-type CHIKV (CHIKV-99659) infection diminished MAYV disease and completely prevented MAYV viremia. However, immunity induced by two highly immunogenic and efficacious CHIKV vaccines, CHIKV/IRES or EILV/CHIKV, offered no protection from MAYV disease or viremia. The differential protective effects observed here may be attributed to the significant disparity in CHIKV-specific and MAYV cross-reactive neutralizing antibody titers. The sera from CHIKV-99659-infected mice contained CHIKV-neutralizing antibody titers >640, at least dou- ble those of the vaccinated groups. As expected, the degree of CHIKV immunity significantly correlates with MAYV cross-reactive neutralizing antibody titers, such that higher CHIKV-specific neutralizing antibody titers are correlated with higher cross-reactive neutralizing antibody titers. Our data also suggest a cross-neutralizing antibody threshold needed for protection from MAYV infection. The complete absence of any protective effects by the TC-83 groups, which are either more distantly related or unrelated viruses, respectively, confirms that the observed CHIKV cross-protection is specific and likely due to antigenic overlap between CHIKV and MAYV. Immunity to CHIKV-99659 infection also completely prevented the development of MAYV viremia, which has important implications for MAYV's emergence potential since the absence of or even reduction of MAYV viremia in CHIKV-immune persons could prevent transmission. This suggests that CHIKV herd immunity, which is high in many parts of Asia[43-45] and Latin America[46-48] may reduce MAYV's epidemic potential.

Our cross-protection study performed in 8-wk old A129 mice, a more sensitive model for MAYV

disease, also demonstrated limited cross-protection from CHIKV vaccines. Of the vertebrate replication-competent viruses, only the attenuated CHIKV/IRES and MAYV/IRES vaccine strains could be employed because of the high susceptibility of this model to fatal disease with more virulent virus strains. The vertebrate replication-incompetent EILV/CHIKV-derived immunity provided no protection against MAYV viremia or mortality, but in fact exacerbated weight loss and footpad swelling in this model. Although sub-neutralizing levels of cross-reactive antibodies against MAYV could result in antibody dependent enhancement (ADE) of disease as experimentally demonstrated with CHIKV[49] and Ross River virus[50-52], there is no evidence of this phenomenon for alphavirus infection of humans. Further experimental and epidemiological research is required to explore the possibility of ADE among antigenically related alphavirus infections.

In contrast, CHIKV/IRES-derived immunity protected against MAYV viremia and mortality, and greatly reduced footpad swelling on day two post-challenge. Remarkably, evaluation of CHIKV-specific and MAYV-cross-reactive neutralizing antibody titers from the CHIKV/IRES group closely reflected those induced by CHIKV-99659 infection of C57/B6J mice. The enhanced susceptibility of this immunocompromised model to viral replication in the absence of an interferon type I response presumably allows a live-attenuated vaccine such as CHIKV/IRES to illicit immunity similar to that following a wild-type CHIKV infection in immunocompetent mice, and hence a similar degree of cross-protection was observed. Our study suggests that a minimum CHIKV immunity threshold must be achieved to provide effective cross-protection against MAYV disease. Our data suggests that cross-protection may be conferred when a cross-neutralization threshold of ≥80 is achieved *in vivo*. Given the evidence that CHIKV immunity can reduce MAYV disease and prevent MAYV viremia, thereby hindering its emergence potential, we sought to determine the influence of humoral and cell-mediated immunity on the observed cross-protection.

There were high numbers of MAYV antigen-cross reactive T-cells in CHIKV/ IRES vaccinated and wild-type CHIKV-infected mice suggesting shared T-cell epitopes among the viruses. With the exception of MAYV/IRES, passive transfer of immune sera from EILV/CHIKV-, CHIKV/IRES-, CHIKV- 99659-, TC-83-, and ZIKV-infected mice provided no protection from MAYV disease in A129 mice. Given IACUC protocol limitations, circulating neutralizing antibody titers were not estimated after passive transfer, however, previous studies indicate that transferring a neutralizing antibody titer of 320 or 640, typically results in a circulating titer of 20–40 or 40–80. This result can therefore be explained by the considerable dilution of the neutralizing antibodies circulating within the mouse (≈10-fold dilution), such that the minimum antibody threshold needed for protection was not obtained. To ensure that the previously observed minimum neutralizing antibody threshold was maintained, T-cell depletion studies were performed in IFNα/βR−/−mice. As expected, these studies generated similar results with the initial cross-protection studies done in the 8-week A129 mice with an intact T-cell response, although this cellular response was possibly primed to a lesser extent given the longer period (4 weeks versus 10 weeks) between CHIKV exposure and MAYV challenge. Consistent with our previous studies, EILV/CHIKV vaccination failed to protect against footpad swelling, weight loss, or viremia, while, CHIKV/IRES-vaccinated mice depleted of T-cells survived challenge and exhibited reduced viremia. The absence of any significant trend in the knockout models indicated that CD4+ and/or CD8+ T-cells have little or no effect on the immune protection observed. These data suggest that neutralizing antibodies against CHIKV are sufficient to reduce MAYV disease and prevent viremia. Although many studies demonstrate that humoral immunity alone is adequate to provide homologous and heterologous protection from alphaviral disease[34-36, 53, 54], previous work provides strong evidence of the role of T-cells in homologous and heterologous protection among members of the Semliki Forest complex[34,38,40,55].

While our mechanistic studies suggest the humoral immune response alone can provide protection and T-cells play little or no role in diminishing disease, further studies such as adoptive transfers may be warranted to determine the exact degree, if any, of cross-protection afforded specifically by the cellular immune response.

Finally, our results indicate that naturally acquired CHIKV-specific human antibodies from Trinidad can strongly cross-neutralize MAYV infection *in vitro*. Although our sample size was small, a recent report based on sera from 35 individuals also supports our findings[56]. Our neutralization titers were generally higher (at least four-fold) against CHIKV than MAYV, delineating CHIKV infections in these individuals. Of note, there were two individuals whose neutralization titers were eight- and two-fold higher against MAYV than CHIKV indicating possible MAYV or another alphaviral infection respectively. No MAYV outbreaks have been documented in Trinidad, but the virus was originally isolated in forested regions within Trinidad[1] and presumably remains in circulation. Typical MAYV cross-neutralization titers in humans were above the apparent $PRNT_{80}$ threshold of 80 for cross-protection in mice (Table 1), suggesting that cross-protection may be conferred in human samples. However, further studies aimed at elucidating the cross-protection PRNT titer threshold of human serum are warranted to test this hypothesis. There is currently insufficient epidemiologic data available throughout the Americas to determine with any certainty whether MAYV emergence has been limited by preexisting CHIKV immunity since 2013. This is further compounded by the absence of adequate diagnostics for MAYV or inclusion of MAYV as a differential diagnostic in some countries. Improved epidemiological studies with rigorous and thorough diagnostic testing are necessary to provide sufficient information to further test this hypothesis.

In conclusion, our results indicate a significant cross-reactive immunological response between

MAYV and CHIKV, which could reduce the risk of MAYV emergence in neotropical regions with high levels of natural CHIKV herd immunity due to epidemic transmission since 2013. However, cross-reactivity based on a CHIKV vaccine may not be strong enough to limit the emergence of MAYV. MAYV-specific vaccines should therefore continue to be developed.

Ethical approval and informed consent

All experimental protocols were approved by the Virginia Tech Institutional Biosafety Committee. All animal study protocols and experiments were approved by Virginia Tech's Institutional Animal Care and Use Committee (IACUC). All animal experiments were performed in compliance with the guidelines of the Virginia Tech's IACUC. The study protocols regarding archived human sera samples were approved by the Ethics Committee of the University of the West Indies, St. Augustine campus, and methods use were in accordance with the relevant guidelines and regulations. Written informed consent was obtained from all participants. Written informed consent was obtained from all participants.

Data availability

All reagents, data and associated protocols are available to readers upon request.

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Author contributions

E.M.W., S.R.A., S.L.H. and A.J.A., C.E.C., H.L., K.P. performed the experiments. R.M.L., S.C.W. and A.J.A. conceived the project. A.T.R., T.W., G.S., C.V.C., S.L.R. provided samples and resources. All authors contributed to writing and editing the manuscript.

Competing interests

S.C.W. is a holder of patents related to alphavirus vaccine development. All other authors declare there are no competing interests related to this work.

CHAPTER THREE: SDAB CROSS-NEUTRALIZATION OF MEDICALLY RELEVANT ALPHAVIRUSES

Stabilization of a Broadly Neutralizing Anti-Chikungunya Virus Single Domain Antibody

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Key words: Single-domain antibody, sdAb, alphavirus, chikungunya virus, virus neutralization, broadly neutralizing antibody

3.1 Abstract

A single domain antibody (clone CC3) previously found to neutralize a vaccine strain of the chikungunya virus ($PRNT_{50}$ = 2. 5 ng/mL) was found to be broadly neutralizing. Clone CC3 is not only able to neutralize a wild-type (WT) strain of chikungunya virus (CHIKV), but also neutralizes WT strains of Mayaro virus (MAYV) and Ross River virus (RRV); both arthralgic, Old World

alphaviruses. Interestingly, CC3 also demonstrated a degree of neutralizing activity against the New World alphavirus, Venezuelan equine encephalitis virus (VEEV); albeit both the vaccine strain, TC-83, and the parental, WT Trinidad donkey strain had $PRNT_{50}$ values ~1,000-fold higher than that of CHIKV. However, no neutralization activity was observed with Western equine encephalitis virus (WEEV). Ten CC3 variants designed to possess a range of isoelectric points, both higher and lower, were constructed. This approach successfully identified several lower pI mutants which possessed improved thermal stabilities by as much as 10ºC over the original CC3 (T_m= 62°C), and excellent refolding abilities while maintaining their capacity to bind and neutralize CHIKV.

Keywords: chikungunya virus, old world, new world, alphavirus, neutralization, melting temperature, single domain antibody

3.2 Introduction

Chikungunya fever is a reemerging infectious disease caused by the chikungunya virus (CHIKV), a mosquito-borne alphavirus. Old World alphaviruses typically result in persistent, or recurring, arthralgia after acute infection (1, 2), while the more virulent New World alphaviruses can cause lethal encephalitis (3). In late 2013, CHIKV emerged in the Americas where it has caused millions of human infections (4). Neutralizing antibodies have shown promise as both prophylactic and therapeutic agents against CHIKV (5). To date, both polyvalent immunoglobulin (Ig) and monoclonal antibodies (mAbs) have been studied (6, 7). Some mAbs have been reported to be broadly neutralizing, being effective against CHIKV and several other arthralgic Old World alphaviruses (8).

Recombinantly expressed antibody binding domains, such as single domain antibodies (sdAb), offer an alternative format for antiviral therapeutics (9). Comprised of the variable domain of unconventional heavy-chain only antibodies found in camelids, sdAb function as small and robust

recognition elements with affinities comparable to those of conventional IgG (10, 11). Advantages of sdAb over conventional immunotherapeutics include their ability to access cryptic epitopes, low molecular weight, and ease of production in *E. coli* (12, 13). Several sdAb have been tailored to a variety of specific applications such as protease resistance (14), ability to function in the presence of denaturants (15, 16), and have the ability to maintain their antibody-antigen complexes even at elevated temperatures (17, 18). Fast clearance, although a potential drawback, can be overcome through strategies such as PEGylation or genetic fusion with an anti- albumin sdAb or Fc-domains (19–21).

Previously, we described five sdAb able to bind CHIKV virus-like particles (VLPs), or recombinant CHIKV envelope protein. Two of the clones (CC3 and CA6) were evaluated for their ability to neutralize CHIKV; whereas both clones showed neutralization, CC3 was ∼80 times more effective (22). In this study, we demonstrate that CC3 can also neutralize other Old World as well as New World alphaviruses. In addition, we constructed and characterized a series of CC3 isoelectric point (pI) variants and identified mutants with improved stability and increased ability to refold after heat denaturation that retain their neutralization capability.

3.3 Materials and methods

Materials

The CHIKV-specific sdAb CC3 was previously described (22). All enzymes used for cloning were from New England Biolabs (Ipswich, MA). CHIKV VLPs and recombinant envelope proteins were from the Native Antigen Company (Oxford, UK). The BSL2 CHIKV strain 181/25 was kindly provided through the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA, Galveston, TX). The RRV Rarotonga strain was obtained from the U.S. Centers of Disease Control and Prevention (CDC, Atlanta, GA). The WT MAYV strain TRVL 4675 (23), WT CHIKV strain

SL-15649 (24) (a gift from Dr. Mark Heise), VEEV TC-83 vaccine strain (25) (a gift from Dr. Scott Weaver), and WEEV Imperial 181 strain (26) (a gift from Dr. Aaron Brault) were rescued from infectious clones. Viral rescue was performed as described previously (23). Lassa VLPs were from Zalgene (Germantown, MD). Unless otherwise specified, common reagents were from Sigma-Millipore, VWR, or Thermo Fisher.

Construction and Production of sdAb Variants

Genes for sdAb variants were synthesized by Eurofins Genomics (Louisville, KY) with flanking NcoI and NotI sites. Mutants were designed based on the consensus sequence of the CA6 neutralizing sdAb as well as toxin binding sdAb (22, 27). The hop tail is based on the patent application by Neal Anthony Eric Hopkins (28), and was flanked by NotI and XhoI sites. All sdAb were expressed in *E. coli* and purified as previously described (27). The amino acid sequences of the produced sdAb with the hop tail is provided in the Supplementary Information. Variants with the hop tail are denoted with the clone name followed by "hop." Theoretical pI was determined using the online tool ExPASy (29).

Measuring Melting Temperatures and Binding Abilities

Thermal denaturation was monitored using circular dichroism (CD) and binding ability was assessed by MagPlex direct binding assays as previously described (22).

Neutralization

Neutralization studies were similar to those previously described (22). Minor differences in plaque reduction and neutralization testing (PRNT) protocols between the three laboratories are detailed in the Supplementary Information.

3.4 Results and Discussion

Neutralization of Alphaviruses

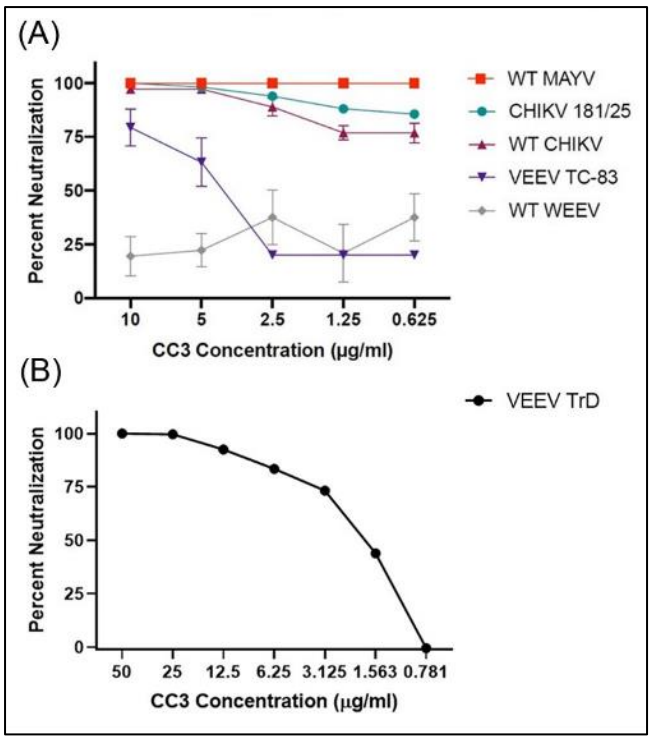
We had previously identified five CHIKV binding sdAb. The neutralization capacity of two of the five (clones CC3 and CA6) was determined by IBT Bioservices (Rockville, MD). Both neutralized CHIKV 181/25 (22); however, CC3 was much more effective than CA6. Further testing of all five clones at the Naval Research Laboratory (NRL) and Virginia Tech (VT) confirmed that CC3 demonstrated far superior neutralization than any of the other clones identified (Supplementary Table 1). The ability of CC3 to neutralize CHIKV 181/25 also was independently verified by the US Army Medical Research Institute for Infectious Diseases (USAMRIID). With a focus on CC3, we determined its ability to neutralize WT strains of CHIKV, MAYV, and RRV; all arthralgic, Old World alphaviruses. CC3 showed potent neutralization of each Old World alphavirus tested (PRNT50 values all <0.625μg/ml; Figure 1). In addition, we examined the ability of CC3 to neutralize two New World alphaviruses: VEEV and WEEV. Interestingly, while CC3 showed some ability to neutralize both the TC-83 vaccine strain of VEEV (PRNT50: 4.0μg/ml) as well as the parental WT Trinidad donkey strain (PRNT50: 1.9μg/ml), no neutralization was observed with WEEV (Figure 1). A toxin binding sdAb [ACVE (30)] was run as an isotype sdAb control, and as

expected no neutralization of any of the viruses was observed (Supplementary Figure 1).

Figure 1. Ability of clone CC3 to neutralize the indicated alphaviruses. Top panel shows neutralization of WT CHIKV, WT MAYV, WT WEEV, CHIKV 181/25, and VEEV TC-83. Bottom panel shows neutralization of WT Trinidad donkey (TrD) strain of VEEV.

Variants of CC3

Adding charges is a known path toward stabilizing antibody binding fragments that can result in increased melting temperatures or decreased aggregation, or both. We have improved melting temperatures and refolding ability of sdAb by adding negative charge (27, 31). Others have also found that negative charge within sdAb domains is correlated with thermal stability and refoldability (32). However, adding positive charges to a conventional antibody binding domain was also shown to improve stability (33), and in a study of artificial human sdAb, the charge of the scaffold determined if the addition of negative or positively charged amino acids prevented aggregation (34). Although typically negative charges are associated with the stability of sdAb, we aimed to explore a



range of charge variants. For this study, a series of ten CC3 variants were constructed. These variants have a range of pIs both higher and lower than the WT-CC3 through specific charge changes to framework residues known to accommodate such a change (Figure 2, Table 1, Supplementary Table 1).

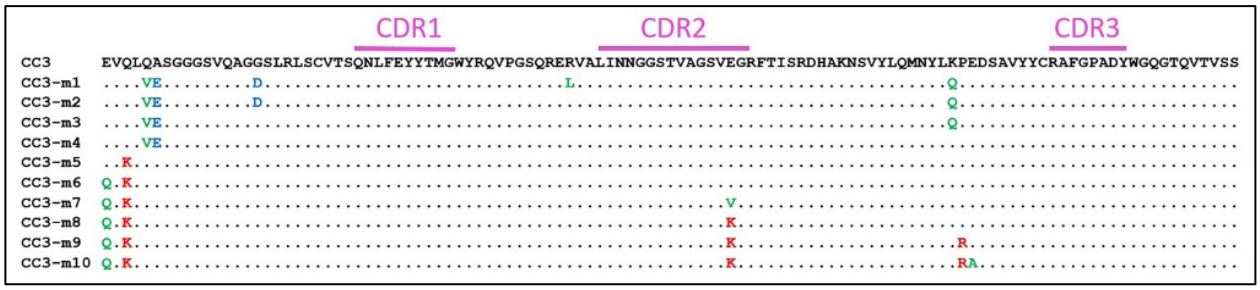


Figure 2. Amino acid sequence (single letter abbreviation) of CC3 and the variants. The CDR regions are indicated by a purple bar. Unchanged amino acids are indicated with a dot. Substitutions to negative amino acids are in blue, substitutions to uncharged amino acids are in green, and substitutions to positively charged amino acids are in red.

Table 1. **Theoretical pI, protein yield, melting temperature (Tm), and percent refolding of the CC3-hop variants.** The Tm and percent refolding for the mutants with a more negative pI than CC3-hop are shown in bold. A representative protein yield is show; although variation is seen between preparations, typically very similar yields are observed. The error on Tm is ± 1 °C.

Clone	Theoretical pI	Yield mg/L	T_m (°C)	% refold
CC3-hop	7.75	12.4	62	80
CC3-m1hop	6.12	28	**76**	**95**
CC3-m2hop	6.35	18	**69**	**91**
CC3-m3hop	6.63	9.9	**72**	**91**
CC3-m4hop	7.04	9.2	**70**	**81**

CC3-m5hop	8.40	20.5	58	46
CC3-m6hop	8.75	9.6	55	38
CC3-m7hop	9.0	11	55	19
CC3-m8hop	9.18	11	56	19
CC3-m9hop	9.35	5.8	54	5
CC3-m10hop	9.51	3.4	49	15

sdAb Production

All sdAb variants were purified by immobilized metal affinity chromatography followed by gel filtration; only monomeric sdAb was used for further characterization. Each of the sdAb variants was first produced with only a hexa-histidine tag for purification (Supplementary Table 2). However, it can be advantageous to express the sdAb with residues that can be used for covalently conjugating biotin, fluorophores, or polyethylene glycol (PEG). To this end, a second version of each clone was produced with a C-terminal cysteine before the histidine tag which could be used to specifically label the sdAb with molecules containing a maleimide group. Unfortunately, the addition of the terminal cysteine to these constructs resulted in poor yields and a large dimer peak in gel-filtration (Supplementary Figure 2). The monomeric protein yields were only ~0.4–1.5 mg/L. More robust production is needed for reagents that may potentially be examined for their therapeutic potential. In an effort to enhance production yields, the “hop tail,” a peptide tag that contains a short linker and two cysteine residues plus an amino acid sequence for substrate recognition by E. coli disulfide isomerase was added (26). Fusions of CC3 and the hop tail significantly enhanced yields, giving between 3.4 and 28 mg/L of monomeric protein (Table 1, Supplementary Figure 2). The two most positively charged mutants (CC3-m9hop and CC3-m10hop) produced the least, with yields of

3.4 and 5.8 mg/L and had pI values of 9.51 and 9.35, respectively. Others have also observed lower yields for more positively charged variants of a sdAb (34). However, overall, there was no clear correlation between pI and sdAb yield.

Binding Ability

The CC3 variants with the hop tail were assessed for their ability to bind to CHIKV VLPs to ensure maintenance of function (Supplementary Figures 3, 4). Every mutant was able to bind the immobilized VLP; however, the most negatively-charged variant (CC3-m1hop) appeared to bind poorly. This variant contains an arginine to leucine substitution in framework 2 at a position which is frequently a leucine in sdAb, however this position has been observed to make contacts with antigen which could explain the observed reduction in binding ability (35). Variants 8, 9, and 10, contain a glutamic acid to lysine substitution at the end of complementarity determining region 2 (CDR2). In a study of sdAb sequences and structures, this position is often a lysine and was observed to be involved in antigen binding in over 10% of the structures examined (35), however no reduction in binding was observed with the CC3 variants.

Positively charged antibody binding domains have previously been correlated with higher non-specific binding (36), therefore, binding to Lassa VLPs was examined to assess specificity of the CC3 variants (Supplementary Figure 3). The most positive variant, CC3-m10, showed a modest signal of ∼170 on the Lassa VLPs at the highest concentration vs. a signal of ∼10,000 on CHIKV VLPs. The other variants showed no binding to irrelevant target.

Melting Temperature and Refolding

Melting temperatures and refolding ability were assessed by CD and are shown in Table 1, Supplementary Table 2 for variants with and without the hop tail, respectively. Representative

melting and refolding curves are shown in Supplementary Figure 5. The melting temperatures of the variants were not substantially affected by the addition of the hop tail, although some clones showed somewhat poorer refolding with the tail. The two most positively charged mutants were much less stable, with lower melting temperatures, and greatly reduced refolding ability compared to the original CC3. All negatively charged mutants had increased melting temperatures and superior refolding ability compared to the original clone. This is consistent with our previous observation that introducing negative charge effectively stabilizes the sdAb, increasing their melting temperatures and enhancing their ability to refold after heat denaturation (31). We previously observed that changing the 5 and 6 positions in framework 1 of a sdAb to V and E, respectively, led to an increase in melting temperature of up to 7◦C (37). Separately, other researchers showed that the 5V mutation can be stabilizing (38). Because of our previous observations, we included the 5V substitution in all of our negative pI variants even though it did not contribute to the decrease in pI. Variant CC3-m4, which has only the Q5V/A6E substitutions, had a 10◦C increase in its melting temperature compared to CC3 (Supplementary Table 2). Mutants CC3- m1hop, CC3-m2hop, CC3-m3hop, and CC3-m4hop all had lower pI values than the original CC3-hop and possessed a melting temperature at least 7◦C higher. Each regained at least 80% of their secondary structure after heat denaturation.

Neutralization

Clones CC3, CC3-hop, and all of the variants containing the hop tail were tested for their ability to neutralize CHIKV 181/25 (Table 2, Supplementary Figure 6). The hop tail had only a minor adverse impact on the sdAb's neutralization ability. However, the variant with the lowest pI, and the variants with the four highest pI values had reduced neutralizing activity. Due to the decreased binding ability of CC3-m1hop, the decreased neutralization was not surprising, however we had not

expected decreased neutralization for the variants with high pI. All four of the positively charged clones had a change in their CDR2, which did not significantly affect their binding to VLPs, but perhaps does affect their neutralizing activity.

Table 2. **Neutralization of Chikungunya virus strain 181/25 by CC3, CC3-hop, and variants.** Each measurement was done in duplicate. In addition, 2-3 biological replicates were performed; the average and standard deviation between sets of experiments are reported.

CHIKV SdAb	**$PRNT_{50}$ (ng/mL)**	**$PRNT_{90}$ (ng/mL)**
CC3	2.5 ± 0.07	31 ± 27
CC3-hop	5.4 ± 0.92	42 ± 22
CC3-M1-hop	12.3 ± 1.0	296 ± 21
CC3-M2-hop	4.6 ± 2.7	18 ± 2
CC3-M3-hop	3.9 ± 0.2	23 ± 2
CC3-M4-hop	3.8 ± 0.8	19 ± 16
CC3-M5-hop	4.4 ± 0.4	16 ± 2
CC3-M6-hop	8.9 ± 0.2	54 ± 14
CC3-M7-Hop	14.9 ± 3.5	146 ± 23
CC3-M8-Hop	21.1 ±2.3	209 ± 146
CC3-M9-hop	34.7 ± 6.8	149 ± 27
CC3-M10-hop	24.2 ± 2.0	169 ± 92

3.5 Conclusion

We found the anti-CHIKV sdAb CC3 to be broadly neutralizing, and constructed a series of CC3-

based variants to assess the correlation of pI and stability of this sdAb. Based on the combined results of binding, stability and neutralization assays, the CC3-m2hop, CC3-m3hop, and CC3-m4hop were found to be the best candidates for further study. These CC3-based constructs offer a specific site for modification (such as PEGylation) to provide better pharmacokinetics while possessing superior thermal stability and improved refolding, qualities that may be of value in cases where maintenance of the cold-chain of transport is difficult.

3.6 Supplemental information

List of sdAb sequences. Changes from wild type CC3 are highlighted in red; the hop tail is highlighted in green. After each sequence the theoretical pI and MW (in Da) are listed:

CC3-hop

MAEVQLQASGGGSVQAGGSLRLSCVTSQNLFEYYTMGWYRQVPGSQRERVALINNGG
STVAGSVEGRFTISRDHAKNSVYLQMNYLKPEDSAVYYCRAFGPADYWGQGTQVTVS
SAAAGAGGSGGAPASNRCSQGSCWNLEHHHHHH
Theoretical pI/Mw: 7.75 / 15700.25

CC3-m1hop

MAEVQLVESGGGSVQAGDSLRLSCVTSQNLFEYYTMGWYRQVPGSQRELVALINNGG
STVAGSVEGRFTISRDHAKNSVYLQMNYLQPEDSAVYYCRAFGPADYWGQGTQVTVS
SAAAGAGGSGGAPASNRCSQGSCWNLEHHHHHH
Theoretical pI/Mw: 6.12 / 15744.26

CC3-m2hop

MAEVQLVESGGGSVQAGDSLRLSCVTSQNLFEYYTMGWYRQVPGSQRERVALINNGG
STVAGSVEGRFTISRDHAKNSVYLQMNYLQPEDSAVYYCRAFGPADYWGQGTQVTVS
SAAAGAGGSGGAPASNRCSQGSCWNLEHHHHHH
Theoretical pI/Mw: 6.35 / 15787.29

CC3-m3hop

MAEVQLVESGGGSVQAGGSLRLSCVTSQNLFEYYTMGWYRQVPGSQRERVALINNGG
STVAGSVEGRFTISRDHAKNSVYLQMNYLQPEDSAVYYCRAFGPADYWGQGTQVTVS
SAAAGAGGSGGAPASNRCSQGSCWNLEHHHHHH
Theoretical pI/Mw: 6.63 / 15729.25

CC3-m4hop

MAEVQLVESGGGSVQAGGSLRLSCVTSQNLFEYYTMGWYRQVPGSQRERVALINNGG
STVAGSVEGRFTISRDHAKNSVYLQMNYLKPEDSAVYYCRAFGPADYWGQGTQVTVS
SAAAGAGGSGGAPASNRCSQGSCWNLEHHHHHH
Theoretical pI/Mw: 7.04 / 15729.29

CC3-m5hop
MAEVKLQASGGGSVQAGGSLRLSCVTSQNLFEYYTMGWYRQVPGSQRERVALINNGG
STVAGSVEGRFTISRDHAKNSVYLQMNYLKPEDSAVYYCRAFGPADYWGQGTQVTVS
SAAAGAGGSGGAPASNRCSQGSCWNLEHHHHHH

Theoretical pI/Mw: 8.40 / 15700.30

CC3-m6hop

MAQVKLQASGGGSVQAGGSLRLSCVTSQNLFEYYTMGWYRQVPGSQRERVALINNGG
STVAGSVEGRFTISRDHAKNSVYLQMNYLKPEDSAVYYCRAFGPADYWGQGTQVTVS
SAAAGAGGSGGAPASNRCSQGSCWNLEHHHHHH
Theoretical pI/Mw: 8.75 / 15699.31

CC3-m7hop

MAQVKLQASGGGSVQAGGSLRLSCVTSQNLFEYYTMGWYRQVPGSQRERVALINNGG
STVAGSVVGRFTISRDHAKNSVYLQMNYLKPEDSAVYYCRAFGPADYWGQGTQVTVS
SAAAGAGGSGGAPASNRCSQGSCWNLEHHHHHH
Theoretical pI/Mw: 9.00 / 15669.33

CC3-m8hop

MAQVKLQASGGGSVQAGGSLRLSCVTSQNLFEYYTMGWYRQVPGSQRERVALINNGG
STVAGSVKGRFTISRDHAKNSVYLQMNYLKPEDSAVYYCRAFGPADYWGQGTQVTVS
SAAAGAGGSGGAPASNRCSQGSCWNLEHHHHHH
Theoretical pI/Mw: 9.18 / 15698.37

CC3-m9hop

MAQVKLQASGGGSVQAGGSLRLSCVTSQNLFEYYTMGWYRQVPGSQRERVALINNGG
STVAGSVKGRFTISRDHAKNSVYLQMNYLKREDSAVYYCRAFGPADYWGQGTQVTVS
SAAAGAGGSGGAPASNRCSQGSCWNLEHHHHHH

Theoretical pI/Mw:
9.35 / 15757.44

CC3-m10hop

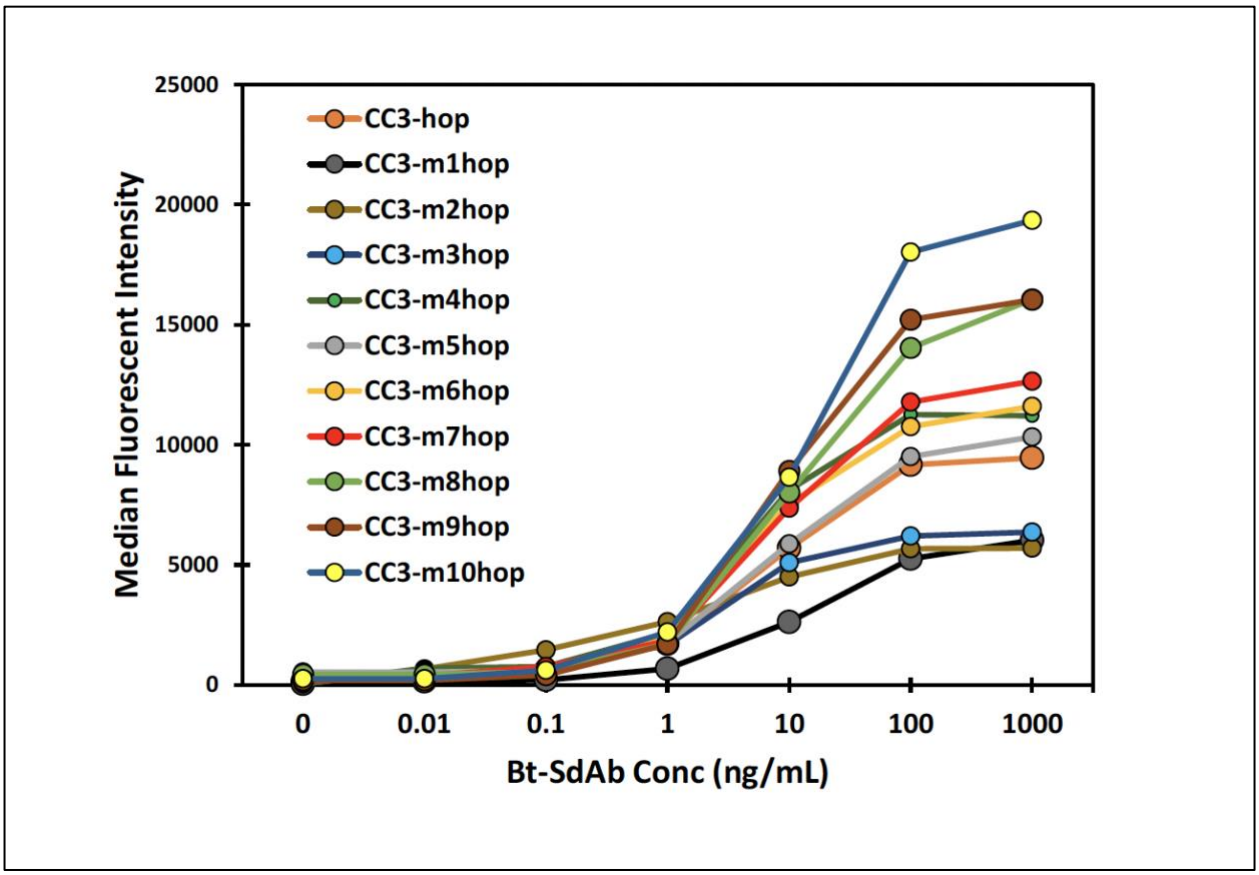
MAQVKLQASGGGSVQAGGSLRLSCVTSQNLFEYYTMGWYRQVPGSQRERVALINNGG
STVAGSVKGRFTISRDHAKNSVYLQMNYLKRADSAVYYCRAFGPADYWGQGTQVTVS
SAAAGAGGSGGAPASNRCSQGSCWNLEHHHHHH
Theoretical pI/Mw: 9.51 / 15699.40

Supplemental Table S1: Neutralization of CHIKV 181/25 by the five originally reported anti-CHIKV sdAb.

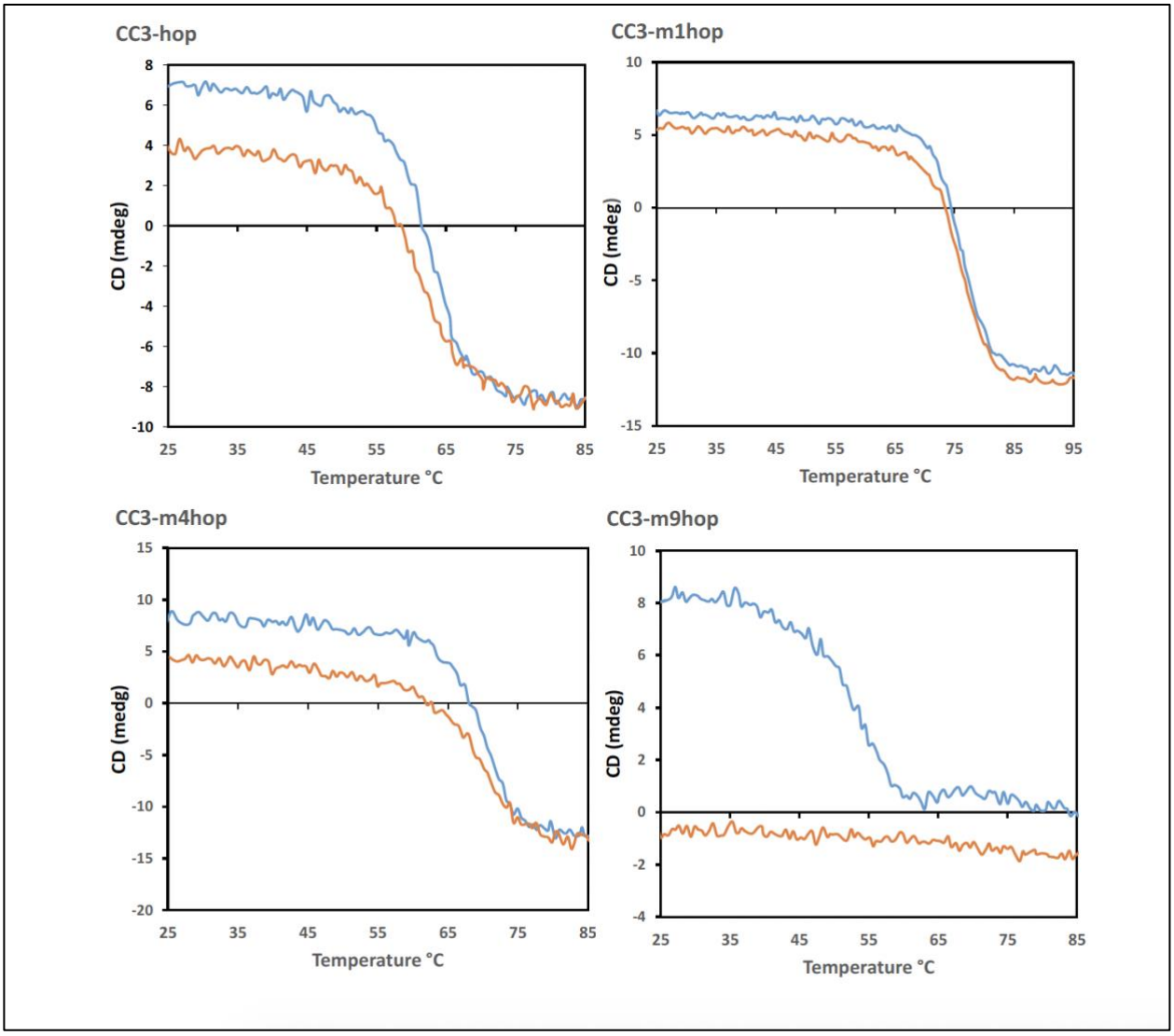
CHIKV SdAb	CHIKV PRNT50 (µg/mL)	CHIKV PRNT90 (µg/mL)
CC3	0.0024	0.0118
CA6	0.3106	1.4500
CH5	0.6300	2.2000
CH6	8.1000	>80
CD11-1	34.9000	>80

Supplemental Table S2. Yields, melting temperature and refolding of variants expressed without the hop tail

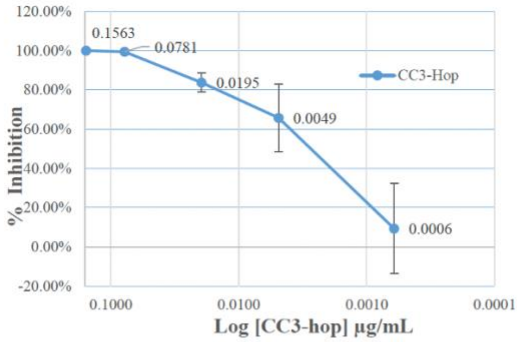
Clone	Theoretical pI	Yield mg/L	Tm (CD)	% refold
CC3	7.05	10	60	62
CC3-m1	5.91	15	75	95
CC3-m2	6.12	7.5	67	95
CC3-m3	6.35	9	71	91
CC3-m4	6.64	12.9	70	83
CC3-m5	7.86	15	59	62
CC3-m6	8.62	7.5	57	46
CC3-m7	9.00	7.2	56	45
CC3-m8	9.23	15.3	58	31
Cc3-m9	9.43	2.2	53	9
Cc3-m10	9.59	1.7	43	7

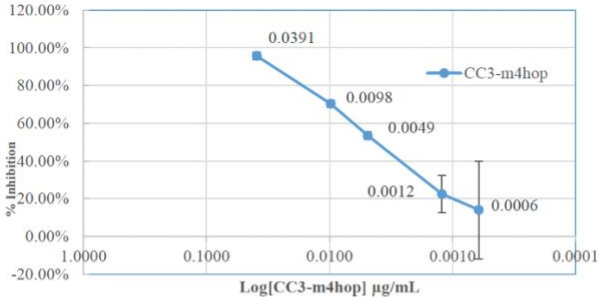


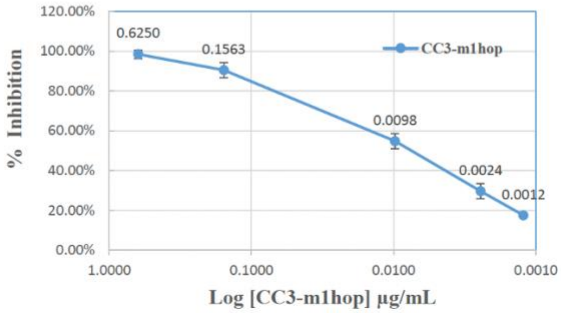
Supplemental Figure S1. MAGPIX direct binding data showing the ability of the CC3-hop variants to bind CHIKV VLPs immobilized on magnetic microspheres.

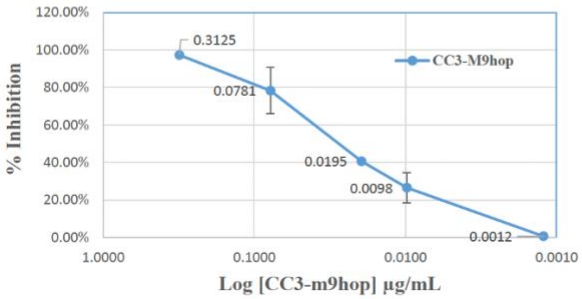


Supplemental figure S2. Circular Dichroism data showing melting (blue) and refolding (orange) curves for the CC3-hop (top left) and the indicated CC3-hop variants.









Supplemental figure S3. Neutralization curves for several representative CC3 derivatives were plotted by selecting data points within the range of neutralizing concentrations. All the data points were obtained from duplicate measurements and error bars represent standard deviation.

Virginia Tech's PRNT protocol:

Two-fold serial dilutions were prepared with 75µl of CC3 in sdAb diluent (e.g. RPMI-1640 media containing 25 mM HEPES, 1% BSA, 50 µg/mL Gentamicin, and 2.5 µg/mL Amphotericin B) starting at a concentration of 10µg/ml. Each dilution was mixed with 75µl of 1200 PFU/mL alphavirus stock, in triplicate, and incubated at 37°C for one hour. The

sdAb/alphavirus mixtures were then added to a monolayer of Vero 76 cells seeded in 24-well plates. Culture plates were incubated for one hour at 37°C, while being rocked every ten minutes, prior to addition of a 1.5% methylcellulose overlay. Plates were incubated at 37°C in a humidified 5% CO2 atmosphere for 2-4 days (dependent on alphavirus/virus strain). On the last day of incubation, cells were fixed with 10% formalin prior to being stained with crystal violet.

NRL PRNT protocol:

Twelve 2-fold serial dilutions of each sdAb were prepared. The starting concentration of sdAb was set at 20 μg/mL. Each dilution was incubated with ∼300 plaque forming units (PFU) of virus at 4° C overnight. Each sdAb-virus mix was then split and added to Vero/host cells seeded in 6-well culture plates for 1 1/2 hour incubation, followed by adding 0.6% agarose overlay in 1XBME (Thermofisher) to each well and incubating for 24 hrs at 37 °C in a humidified 5% CO2 atmosphere before the addition of the second 0.6% agarose overlay containing neutral red in 1xBME. After 24 hrs of neutral red staining, the transparent plaques were counted. 50% and 80% plaque reduction neutralization titer (PRNT50 and PRNT80) of each sdAb was calculated using XLfit dose response model. If needed, the starting concentration may be adjusted to include the dilutions ranging between PRNT50 and PRNT80.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

Author contributions

EG, JL, GA, EW, and PG designed the experiments. EG, JL, GA, EW, CB, and CG performed the experiments. EG and GA wrote the manuscript. All authors analyzed data and edited the

manuscript.

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CHAPTER FOUR: DEVELOPMENT OF CHIKV AND MAYV REFREACTORY *AEDES AEGYPTI*

Expression of camelid single-domain antibodies reduces alphavirus infection, dissemination, and transmission in transgenic Aedes aegypti

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Key words: Alphavirus, Mayaro virus, chikungunya virus, single-domain antibody, transgenic, *Aedes aegypti*, vector competence

4.1 Abstract

Chikungunya virus (CHIKV) and Mayaro virus (MAYV) are closely related alphaviruses that present with an acute febrile illness accompanied by an incapacitating polyarthralgia that can persist for years following initial infection. In conjunction with sporadic outbreaks throughout the sub-tropical regions of the Americas, increased global travel to CHIKV- and MAYV-endemic areas has resulted in imported cases of both viruses throughout the United States and

Europe, as well as imported CHIKV cases that led to autochthonous transmission in the United States. As CHIKV and MAYV have become increasingly prevalent across several continents within the last decade, a heavy focus has been placed on control and prevention programs. To date, the most effective means of controlling these arthritogenic infections is through mosquito control programs. However, these programs have crucial limitations in their effectiveness; therefore, novel approaches are necessary to control the spread of these crippling pathogens and lessen their disease burden. We have previously identified and characterized an anti-CHIKV single-domain antibody (sdAb) that potently neutralizes several alphaviruses. Given the close antigenic relationship between MAYV and CHIKV, we formulated a single defense strategy to combat both emerging arboviruses. To this end, we have generated transgenic *Aedes aegypti* mosquitoes that co-express two camelid-derived, anti-CHIKV sdAbs. Our data indicates significant reduction in CHIKV and MAYV infection, dissemination, and transmission within sdAb-expressing transgenic mosquitoes compared to wild-type controls; thus, providing a novel approach to controlling and preventing outbreaks of these pathogens that reduce quality of life around the globe.

4.2 Introduction

The widely distributed *Aedes* spp. mosquitoes, notably *Ae. aegypti* and *Ae. albopictus*, are vectors for various debilitating arboviral pathogens such as yellow fever virus (YFV), dengue virus (DENV), Zika virus (ZIKV), Rift Valley fever virus (RVFV), and chikungunya virus (CHIKV) (1). Although decades of research have been focused on combating these diseases through control and prevention programs, the primary method of reducing the spread of most arboviral diseases is limiting the interaction among mosquitoes and their human hosts. This is mainly achieved by educating the public on the use of personal protective equipment (e.g., long-sleeved clothing, screens and/or netting, etc.), the use of insect repellents, and ridding properties of standing water, which mosquitoes use for egg laying (e.g., tires, buckets, pools, etc.). While these tools have proven to be beneficial by hindering some of the interactions between mosquitoes and humans in more rural areas, some mosquito species, particularly *Ae. aegypti*, have evolved to live in close proximity to humans and are extremely anthropophilic (1), thus these measures are not nearly as effective when it comes to this infamous disease vector. Therefore, insecticides are used to reduce populations of mosquitoes in, and around, urban settings; however, the environmental impacts of these toxic chemicals have led to great concern and has even resulted in the restricted, or completely prohibited, use of some of the most aggressive insecticides (e.g., DDT). Since this time, development has focused on the production of insecticides that require fewer applications, have increased specificity, and do not bioaccumulate (2). These environmental concerns, the limited number of approved insecticides, the potency of available insecticides, and, most importantly, the development of insecticide

resistance have all played a major role in the emergence and/or re-emergence of many arboviral diseases (3).

Although, insecticide usage has been in practice for millennia, and has proven to be successful in limiting the spread of many diseases (2), insecticide resistance has become increasingly worrisome within the last century. Several studies have reported on the developed resistance of *Ae. aegypti* to a number of insecticides used for mosquito population control (4). This resistance has contributed greatly to the spread of vector-borne diseases and added to the importance of developing novel strategies to control mosquito populations and/or impede disease transmission.

In order to control mosquito populations and/or impede disease transmission, novel strategies have been explored over the last few decades, perhaps the most promising being genetic modification of these deadly mosquito vectors. Recent advances in this field have resulted in the release of genetically modified mosquitoes that carry lethal genes in an attempt to suppress natural mosquito populations and, thus, minimize their co-inhabitance with human hosts. While this strategy is effective, there are several concerns regarding the impacts reducing mosquito populations may have on the environment (5). As an alternative, scientists have developed control programs that focus on replacing natural disease-carrying mosquito populations with pathogen-refractory mosquito populations. For example, researchers from the World Mosquito Program have developed and released *Ae. aegypti* mosquitoes that carry a strain of *Wolbachia* as a means to replace wild-type mosquito populations (6). These stably transinfected *Ae. aegypti* mosquitoes were originally developed to combat DENV (7) and have since been shown to reduce infection with ZIKV (8) and CHIKV (9). Recently, field studies have suggested a 77% reduction in dengue virus incidence over a 27-month trial in Indonesia (10). These results provide great

potential for limiting the spread of arboviral diseases, in particular alphaviruses. Alongside these promising results, Mancini et al. reported on the *Wolbachia*-induced arboviral resistance of *Ae. albopictus* mosquitoes (11), an important implication given the vector competence of this species and its co-localization with the modified *Ae. aegypti* mosquitoes. Although this novel control strategy has had great success, there are limitations to these *Wolbachia*-transinfected mosquitoes. As reviewed by Yen et al., these limitations include the incomplete understanding of the mechanism by which *Wolbachia* blocks arboviral infections, *Wolbachia* density within the mosquitoes being affected by environmental temperatures and mosquito diets, and concern of the production of virus variants escaping the pathogen-blocking effects afforded by *Wolbachia* (12). Therefore, the development of alternative population replacement strategies is warranted to combat arboviruses.

Alphaviruses are arboviruses belonging to the *Togaviridae* virus family that infect millions of people annually. Infections with alphaviruses can result in a range of debilitating disease symptoms. Most notably, alphaviruses that reside in the Semliki Forest serocomplex; a serological group within the *Alphavirus* genus, typically result in a febrile illness that is accompanied by incapacitating polyarthralgia that can last for years following infection (13, 14). Both CHIKV and Mayaro virus (MAYV) are medically-relevant members of this serocomplex and have become increasingly prevalent throughout the Americas within the last decade. Since 2004, CHIKV has been reported in over 100 countries (15) and some scientists suggest this could also be the case for MAYV if this virus imitates the evolutionary and epidemiological patterns of other arboviruses (16, 17). In conjunction with sporadic outbreaks throughout the sub-tropical regions of the Americas, increased global travel to these endemic areas has resulted in imported

cases of both these viruses throughout the United States and Europe, as well as imported cases that facilitated autochthonous CHIKV transmission in the United States (18-27). Thus, there is a tremendous need for novel approaches to control these viruses.

Several past studies have been conducted to understand cross-protective immunity among alphaviruses, particularly those within the Semliki Forest serocomplex (28-34). These studies have shown that antibodies generated to one alphavirus are also capable of neutralizing closely related alphaviruses. In particular, antibodies developed against CHIKV can neutralize and cross-protect against infection with MAYV (28, 33). Our previous studies have characterized a camelid-derived anti-CHIKV single-domain antibody (sdAb) that has potent cross-protective activity against other medically-relevant alphaviruses, including MAYV (35). Camelid sdAbs have many advantages when compared to conventional mammalian antibodies, including increased thermostability, ease of production, and small size (~15 kDa) when compared to conventional mammalian antibodies (Figure 1).

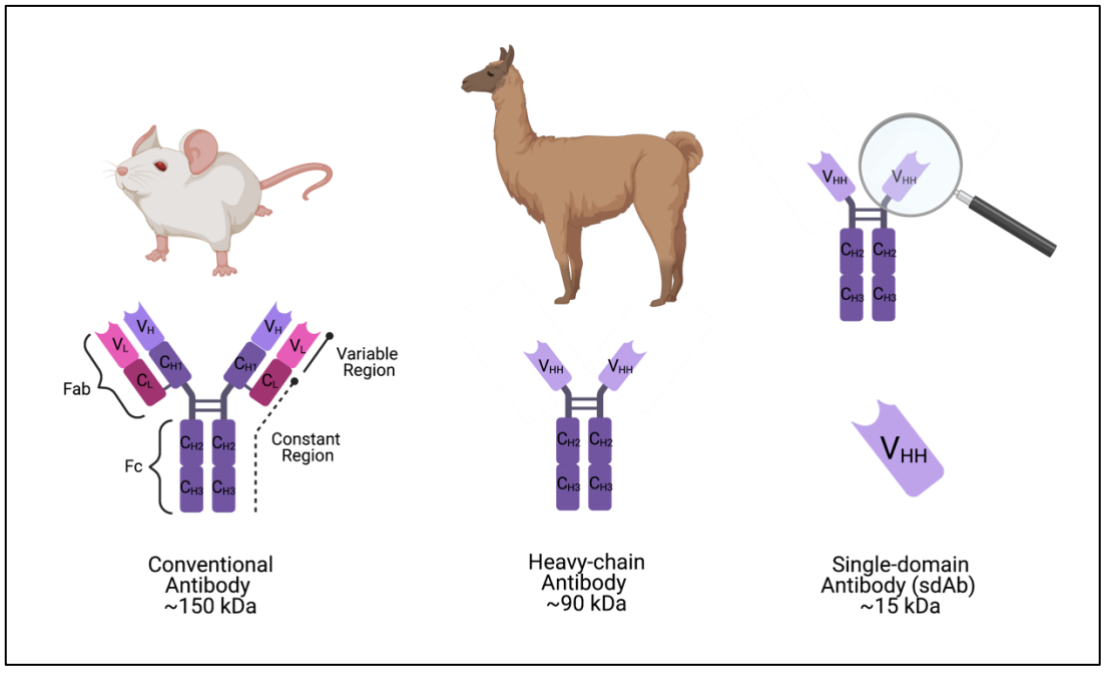

Figure 1. Representation of conventional, heavy-chain, and single-domain antibodies. Conventional mammalian antibodies (*left*) are comprised of a basic structure consisting of two heavy and two light chains forming Fab and Fc regions. Alongside conventional antibodies, camelids also produce heavy-chain antibodies (*middle*) that are composed of only two heavy chains. The antigen binding domain of camelid heavy-chain antibodies, or the V_HH region, retains full functionality and is known as a single-domain antibody (sdAb; *right*).

With cross-protective immunity and these sdAb benefits taken into account, we designed and developed transgenic *Ae*. *aegypti* mosquitoes that express two anti-CHIKV sdAbs targeting both CHIKV and MAYV. We demonstrated potent neutralization of CHIKV and MAYV in the presence of two anti-CHIKV sdAbs *in vitro*. We also observed a significant reduction of CHIKV and MAYV infection, dissemination, and transmission within our transgenic mosquitoes compared to wild-type controls. This is the first development of transgenic mosquitoes that targets multiple alphaviruses. We believe these results provide a proof of concept for an alternative population replacement program to control these pathogens that reduce quality of life around the globe.

4.3 Methods and Materials

Virus propagation and cell culture

The CHIKV and MAYV strains used in this study were obtained from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) at the University of Texas Medical

Branch (Galveston, TX), with the exception CHIKV SL-15649 (ECSA lineage), a kind gift from Dr. Mark Heise (36). The viruses obtained from WRCEVA include MAYV 12A (genotype L), MAYV TRVL-4675 (genotype D), and CHIKV H-20235 (Asian lineage). African green monkey kidney (Vero) cells were used to propagate viruses. Vero cells were purchased from American Type Culture Collection (ATCC; Manassas, VA) and maintained in culture with Dulbecco's Modified Eagle Medium (DMEM; Sigma Aldrich; St. Louis, MO) supplemented with L-glutamine, 5% fetal bovine serum (FBS), and 1% gentamycin and kept at 37°C with an atmosphere of 95% humidity and 5% CO_2. Vero cells were grown to ~85% confluency before being infected with the respective viruses at an MOI of 0.01 in viral diluent (RPMI-1640 media with 25 mM HEPES, 1% BSA, 50 μg/mL Gentamicin, 2.5 μg/mL Amphotericin B). The cells were infected for 1 hour at 37°C, with rocking every 10-15 mins to ensure the entire monolayer had contact with the virus suspension. After 1 hour of incubation, Vero cell maintenance media was added to the flask. Once 50-75% of cells demonstrated cytopathic effects (CPE), the cellular supernatant was collected and clarified by centrifugation at 4°C before storage at -80°C. Virus stocks were titrated by Vero cell plaque assay (described below) before use in subsequent experiments.

Cloning AE_CA6CC3

Construction of AE_CA6CC3 was performed by inserting synthesized genes containing the Ae CpA promoter, Dfurin 1 sequence, P2A sequence, and the sdAb sequences into piggyBac [polyUb GFP] via GeneArt Gibson Assembly cloning (ThermoFisher Scientific, Waltham, MA) per manufacturer's protocol. Genes were synthesized by GENEWIZ, Inc. (South Plainfield, NJ). All primers for cloning, and subsequent Sanger sequencing, were designed using SnapGene (San

Diego, CA) software and obtained from Integrated DNA Technologies. Inc. (IDT; Coralville, IA).

Mosquito rearing

Aedes aegypti LVP-IB12 eggs were obtained from BEI resources (Manassas, VA; MRA-735**).** Mosquitoes were reared and housed at 26°C, relative humidity 75%, and 12:12 (light/dark) photoperiod. Larvae were fed Nishikoi fish food (Essex, England) and adult mosquitoes were provided with a 10% sucrose solution administered through cotton balls. Mosquitoes were provided defibrinated sheep's blood (Colorado Serum Company; Denver, CO) using artificial membrane feeders.

PRNTs

Purified sdAb samples were serially diluted in viral diluent (RPMI-1640 media with 25 mM HEPES, 1% BSA, 50 µg/mL Gentamicin, 2.5 µg/mL Amphotericin B) to the appropriate concentrations (10 µg/ml-0.01 µg/ml). Diluted sdAbs were then mixed with an equal volume of the respective virus at 1000 PFU/ml. Following a 1.5 hour incubation at 37ºC, sdAb/virus mixtures were then added to confluent monolayers of Vero cells. After a one hour incubation period, an overlay containing 1.5% methylcellulose was added. Plaques were visualized following formalin fixation and staining with crystal violet three days post-infection.

Plaque assays

Virus titration was performed on Vero cells by plaque assay. Serial ten-fold dilutions of each sample were made in viral diluent (RPMI-1640 media with 25 mM HEPES, 1% BSA, 50 µg/mL Gentamicin, 2.5 µg/mL Amphotericin B) and then added to confluent monolayers of Vero cells. After a one hour incubation period, an overlay containing 1.5% methylcellulose was then added.

Plaques were visualized following formalin fixation and staining with crystal violet three days post-infection.

Mosquito transformations

All transformations were carried out by adapting protocols previously described (37). PiggyBac donor plasmid (500 ng/µl; piggyBac [polyUb GFP]) that contains an eGFP transformation marker driven by the *Ae. aegypti* polyubiquitin promoter and was co-injected with an *in vitro* transcribed piggyBac mRNA (300 ng/ul) into less than 1 hour old embryos of *Ae. aegypti* LVP-IB12. The piggyBac-hsp70-transposase was used as a template for *in vitro* transcription using the mMessage mMachine T7 Ultra kit (Thermofisher), followed by MEGAclear (Thermofisher) column purification. Surviving G_0 females were mated to LVP-IB12 males in pools of 20-25 mosquitoes. Each G_0 male was mated individually with 5 LVP-IB12 females in individual cages. G_1 larvae were screened for green fluorescence using a Leica M165 FC fluorescence microscope. Positive G_1 individuals were out-crossed to Liverpool mosquitoes to ensure that all transgene cassettes were stably inherited to the G_2 generation.

Western blots

Midguts from WT, AE1, and AE5 mosquitoes (n=30/group) were dissected 16 hours after a bloodmeal and protein sample were extracted with ice-cold radioimmunoprecipitation assay buffer (RIPA buffer; 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.25% Na-deoxy- cholate, 1% NP-40, 1 mM EDTA) containing one cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail tablet (MilliporeSigma, Burlington, MA). The entire samples were then homogenized and centrifuged at 4ºC. Clarified supernatants were transferred to a fresh microcentrifuge tube, pulse-sonicated twice for 10 seconds each, and mixed with SDS loading buffer. All samples were then

placed in a heating block at ~90-95ºC for 10 minutes. Samples were run on a 4-20% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were then stained with Ponceau S stain to visualize protein transfer. Following destaining, membranes were probed with AffiniPure Goat Anti-Alpaca IgG, VHH domain primary antibody (128-005-232; Jackson ImmunoResearch, West Grove, PA) at a concentration of 15 µg/ml overnight at 4ºC. The secondary antibody used was rabbit anti-goat HRP (HAF017; R&D Systems, Minneapolis, MN) at a dilution of 1:1000 per the manufacturer's recommendation. Images were generated by applying Prometheus™ ProSignal™ Pico chemiluminescent ECL reagents (20-300B; Genesee Scientific, San Diego, CA) to the blots and visualized using an Azure c400 gel imaging system (Azure Biosystems, Inc, Dublin, CA).

Vector competence experiments

Vector competence experiments were carried out by adapting protocols previously described (38). Female mosquitoes 5-7 days old (n=50) were separated into cartons and sucrose starved for 16 hours before infection to promote blood feeding. Mosquitoes were fed virus-spiked bloodmeals containing defibrinated sheep's blood (Colorado Serum Company; Denver, CO) using artificial membrane feeders and only fully engorged mosquitoes were separated into new containers. Pre- and post-infection blood samples were collected for use as back-titer calculations. Seven days post-infection, mosquitoes were cold-anesthetized and midguts, legs/wings, and saliva were collected in viral diluent (RPMI-1640 media with 25 mM HEPES + 1% BSA + 50 µg/mL Gentamicin +2.5 µg/mL Amphotericin B). Saliva was collected through forced salivation for 30 minutes in immersion oil and then mixed with viral diluent. Samples were stored at -80 °C until subjected to plaque assay.

Statistics

Data from the preliminary vector competence experiments were analyzed via a two-tailed Fisher's exact test using the GraphPad QuickCalc 2x2 contingency table. All other vector competence experiments were analyzed by Kruskal Wallis test with multiple comparisons.

4.4 Results

CA6 and CC3 display potent virus neutralization *in vitro*

We previously identified several anti-CHIKV sdAbs that neutralize CHIKV *in vitro* with two of these (clones CC3 and CA6) being the most potent at neutralizing CHIKV (39). We then further analyzed the ability of CC3 to neutralize a breadth of medically-relevant alphaviruses (35). We found CC3 also has potent neutralizing capacity against MAYV (35). We then performed plaque reduction neutralization tests (PRNTs; Figure 2A) to demonstrate that CA6 also has anti-MAYV activity and to confirm the anti-MAYV activity of CC3 (Figure 2C). We hypothesized that using both neutralizing sdAbs would reduce the probability of producing viral escape variants, thus we performed a series of combination PRNTs (Figure 2B) to evaluate the level of CHIKV and MAYV neutralization afforded in the presence of both CC3 and CA6 (Figure 2D). These data suggest CC3 and CA6 maintained the ability to potently neutralize both CHIKV and MAYV.

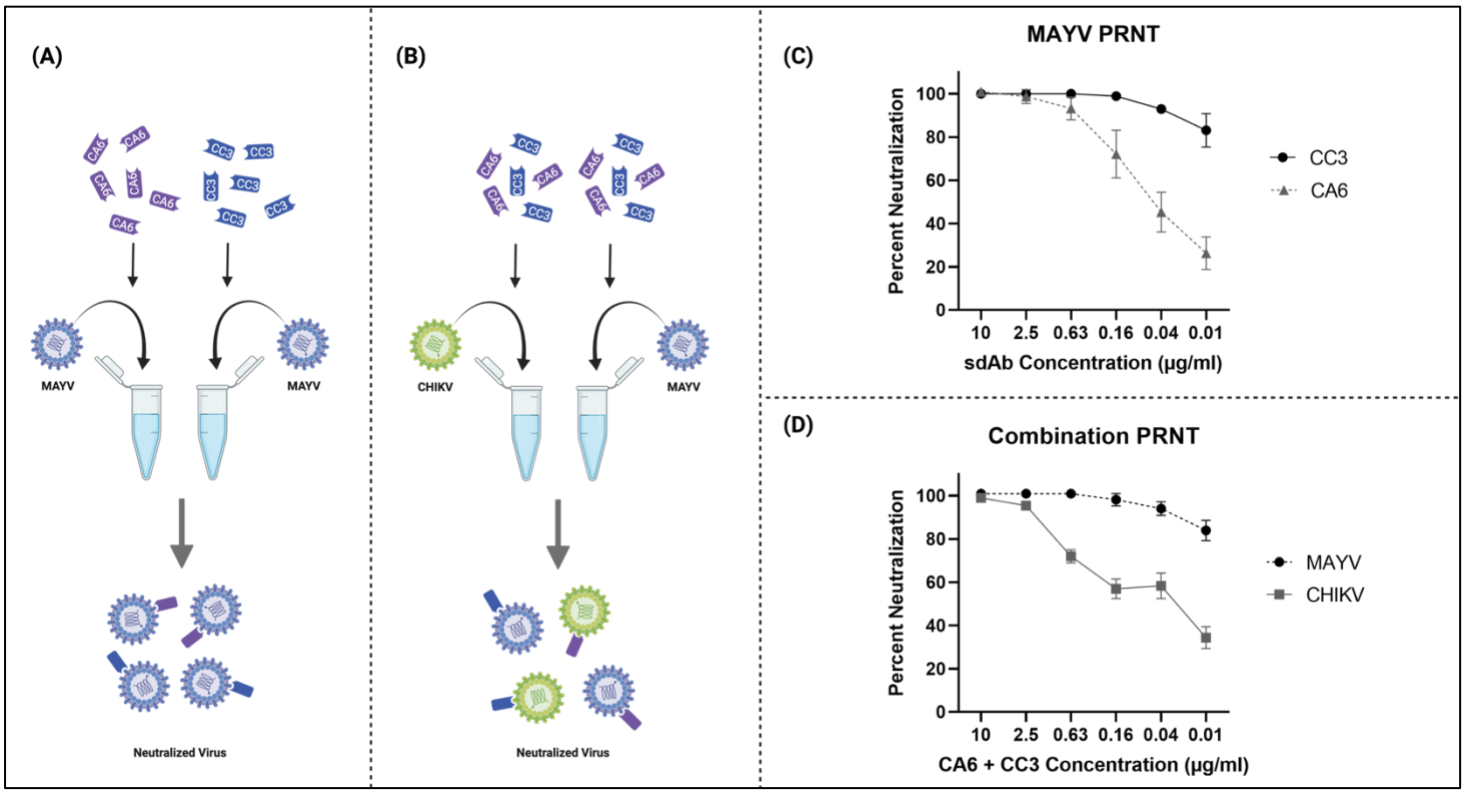

Figure 2. General schematic of PRNT methods and virus neutralization data. (A) Schematic representing PRNT method of MAYV neutralization via CA6 (dark purple) and CC3 (blue). (B) Schematic representing combination PRNT method of CHIKV (green) and MAYV (light purple) neutralization via samples mixed with both CA6 (dark purple) and CC3 (blue). (C) PRNT data provides percent neutralization of MAYV by CC3 (black, solid line) and CA6 (gray, dashed line). (D) Combination PRNT data provides percent neutralization of MAYV (black, dashed line) and CHIKV (gray, solid line) by mixed samples of both CA6 and CC3. Data points are representative of six replicates obtained in two independent experiments. Error bars represent SD.

Generation of construct and transgenic *Ae. aegypti* colonies

After defining combinatory virus neutralization *in vitro*, we proposed and constructed a piggyBac transposon-based plasmid, herein referred to as "AE_CA6CC3", to determine the ability of these sdAbs to be co-expressed and neutralize virus *in vivo*. AE_CA6CC3 was designed to express both CC3 and CA6 under the control of the *Aedes aegypti* carboxypeptidase A promoter (AeCpA) (40) to provide bloodmeal-upregulated, gut-specific gene expression. Together with this inducible expression, green fluorescent protein (GFP), constitutively expressed via the *Ae. aegypti* polyubiquitin promoter (Ae polyUb) (41), was also included in the design to aid in screening and selection of transgenic mosquitoes.

To generate AE_CA6CC3, and to co-express the sdAbs from AeCpA, a single coding sequence was designed *in silico* that encompassed a Dfurin 1 cleavage site (R-Q-K-R) (42) and *Porcine teschovirus*-1 2A (P2A) self-cleaving peptide (43) inserted between the CA6 and CC3 sequences (39) (Figure 3A). The entire transcript was then codon-optimized for *Drosophila* and synthesized by GENEWIZ, Inc. (South Plainfield, NJ). The gene synthesis product was then cloned into a piggyBac [polyUb GFP] plasmid vector via GeneArt Gibson Assembly cloning (ThermoFisher Scientific, Waltham, MA). The final construct was subjected to Sanger sequencing analyses to confirm the successful construction of AE_CA6CC3.

Subsequent transgenesis procedures consisted of embryonic microinjection of *Ae. aegypti* eggs and G_0 outcrossing to wild-type (WT) mosquitoes. At G_2 multiple transgenic lines were identified (n=5) to move forward with experiments based on strong GFP expression (Figure 3B). Upon further observations, one of the transgenic lines was removed from experimentation due to an apparent male-linked insertion of our construct. The resulting transgenic lines (n=4; AE1,

AE2, AE4, and AE5) were then subjected to further rearing until preliminary vector competence studies were performed.

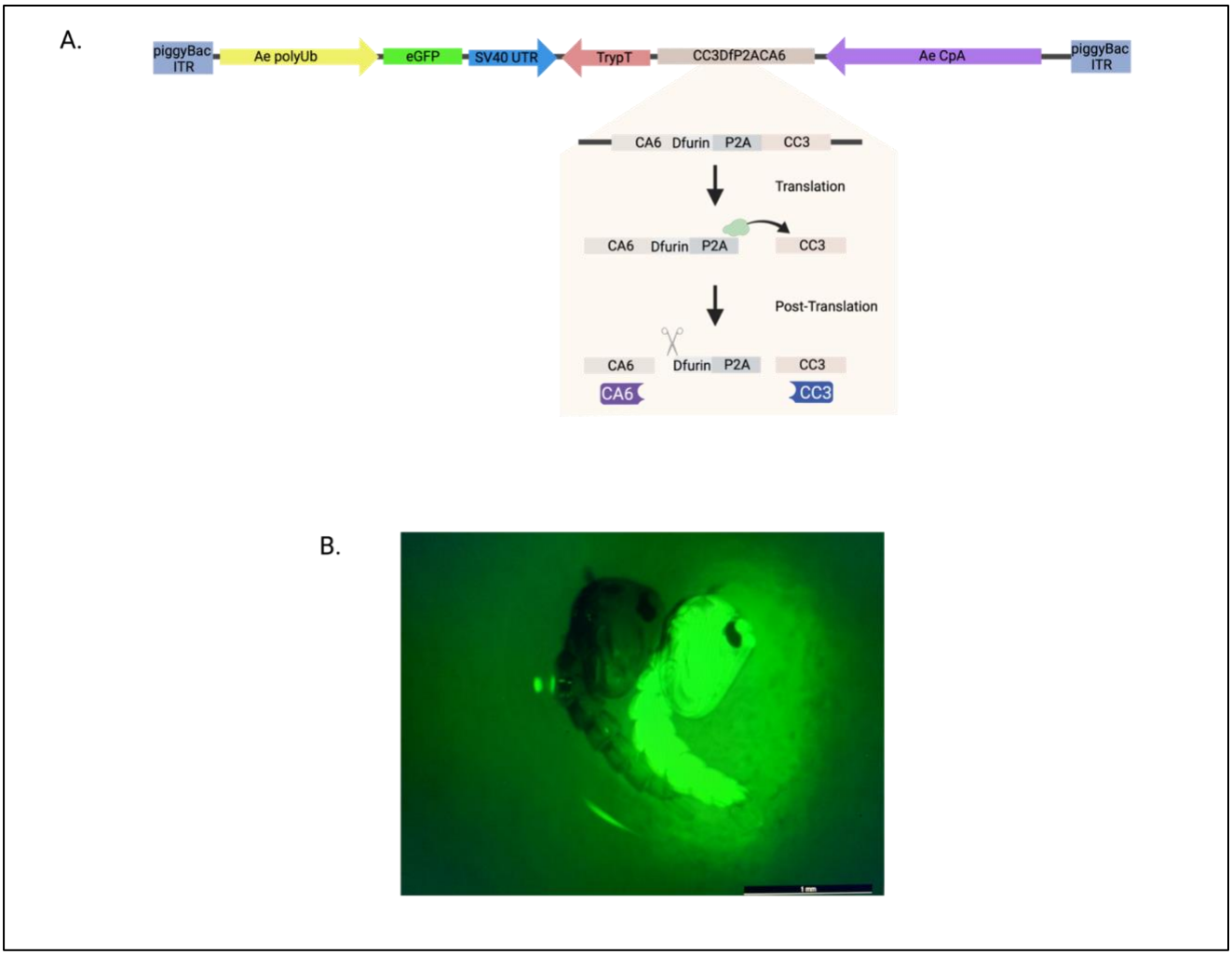

Figure 3. Schematic of construct design and transgenic GFP expression. (A) AE_CA6CC3 construct design indicating fusion peptide of CA6, Dfurin 1, P2A, and CC3. During translation, ribosomal skipping/translocation is induced (indicated by green ribosome and horizontal arrow) resulting in co-translational

cleavage of the polyprotein. Post-translational processing then removes the residual P2A sequence from the upstream gene (CA6; indicated by scissor icon). (B) Image of WT (*left*) and transgenic (*right*) pupae expressing GFP.

sdAb-expressing transgenic mosquitoes are refractory to alphavirus infection

As the overall goal of this study was to develop transgenic *Ae. aegypti* mosquitoes that are refractory to both CHIKV and MAYV, we performed preliminary vector competence experiments to determine (1) which virus strains should be used and (2) which transgenic lines provided the most resistance to CHIKV and MAYV. Because data from vector competence studies are not available for these viruses (specifically in *Ae. aegypti* LVP) (44), preliminary studies consisted of a small-scale vector competence experiment comparing two strains of CHIKV (e.g., CHIKV H-20235 and CHIKV SL-15549) and two strains of MAYV (e.g., MAYV 12A and MAYV TRVL-4675) in WT *Ae. aegypti* LVP mosquitoes. These experiments were conducted by spiking the bloodmeal with each virus and evaluating virus infection 7 days later. Based on CHIKV H-20235 and MAYV 12A transmission rates (83% and 75%, respectively), these strains were selected for downstream analyses (Table 1).

Table 1. Preliminary screening of vector competence of WT *Ae. aegypti* LVP mosquitoes for CHIKV and MAYV strains.

CHIKV H-20235 (representing the Asian lineage), CHIKV SL-15649 (representing the ECSA lineage), MAYV 12A (representing the L genotype), and MAYV TRVL-4675 (representing the D genotype) were used in these studies. Data are presented

as the number of virus positive/number tested with rates of infection, dissemination, and transmission indicated by percentages in parentheses.

Virus	Infection (Midgut)	Dissemination (Legs/Wings)	Transmission (Saliva)
CHIKV H-20235 (Asian)	12/12 (100%)	12/12 (100%)	**10/12 (83%)**
CHIKV SL-15649 (ECSA)	11/12 (92%)	9/12 (75%)	3/12 (25%)
MAYV 12A (L)	12/12 (100%)	12/12 (100%)	**9/12 (75%)**
MAYV TRVL-4675 (D)	11/12 (92%)	11/12 (92%)	5/12 (42%)

Following the selection of appropriate virus strains, each of the four transgenic lines was subjected to a preliminary vector competence analysis to determine which lines showed the most resistance to CHIKV and MAYV. In these studies, female mosquitoes from each transgenic line and WT mosquitoes were exposed to the respective virus and infection, dissemination, and transmission rates were calculated. These data indicated the AE1 and AE5 transgenic colonies were the most refractory to CHIKV and MAYV when compared with WT mosquitoes (Table 2). While P values for infection, dissemination, and transmission were not each significant for the viruses tested, AE1 and AE5 were selected because the P values of their transmission rates represented significant or near significant changes, suggesting a trend towards a reduction in transmission potential to human hosts. Specifically, AE1 transmission was highly reduced for CHIKV and MAYV (P=0.002 and P=0.004, respectively) and AE5 had a highly significant reduction (P=0.008) in transmission for CHIKV and a near significant reduction (P=0.056) for MAYV. Thus, these two transgenic lines were used in the subsequent experiments.

Table 2. Preliminary vector competence of transgenic and WT *Ae. aegypti* mosquitoes for CHIKV H-20235 and MAYV 12A.

Data are presented as the number of virus positive/number tested with two-tailed P values of infection, dissemination, and transmission compared to WT. P values are indicated in parentheses. All two-tailed P values were calculated via 2x2 contingency table via Fisher's exact test.

Virus	Transgenic Line	Infection	Dissemination	Transmission
CHIKV H-20235	**AE1**	20/20 (1.000)	17/20 (0.231)	**2/20 (0.002)**
	AE2	20/20 (1.000)	20/20 (1.000)	8/20 (0.343)
	AE4	20/20 (1.000)	19/20 (1.000)	9/20 (0.527)
	AE5	20/20 (1.000)	18/20 (0.487)	**3/20 (0.008)**
	WT	20/20 (N/A)	20/20 (N/A)	12/20 (N/A)
MAYV 12A	**AE1**	16/20 (0.106)	9/20 (0.019)	**4/20 (0.004)**
	AE2	20/20 (1.000)	20/20 (0.231)	14/20 (1.000)
	AE4	18/20 (0.487)	12/20 (0.155)	10/20 (0.333)
	AE5	18/20 (0.487)	10/20 (0.041)	**7/20 (0.056)**
	WT	20/20 (N/A)	17/20 (N/A)	14/20 (N/A)

Transgenic *Ae. aegypti* express CA6 and CC3

To evaluate expression of CA6 and CC3 *in vivo*, transgenic *Ae. aegypti* mosquito lines AE1 and AE5 were subjected to Western blot analyses. Midguts from each transgenic line, and WT mosquitoes, were dissected 16 hours post-bloodmeal. Purified CC3 and CA6 sdAbs (a kind gift from Dr. Ellen Goldman) were used as a positive control (Figure 4). These data indicated expression of the sdAbs in the midgut tissues of mosquitoes from AE1 and AE5, but not WT mosquitoes; however, banding patterns may suggest incomplete cleavage of the sdAbs based on the presence of bands at both ~30 and 15 kDa (Figure 4).

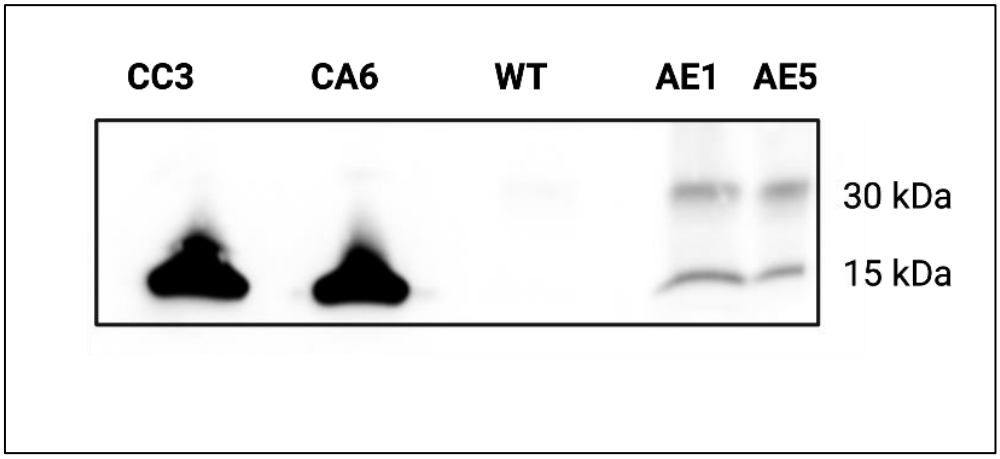

Figure 4. Western blot analysis to probe for the presence of sdAbs in AE1 and AE5. Western blots were carried out using dissected midgut tissues from 16 hour post-bloodmeal WT, AE1, and AE5 mosquitoes (n=30/group). Purified CC3 and CA6 samples were used as controls. The presence of an approximate 15 kDa band confirms the analysis of the control antibodies. The presence of an approximate 15 kDa band indicates expression of the sdAbs in the transgenic, but not WT, mosquito

midgut tissues. The presence of an approximate 30 kDa band in transgenic midgut tissues may indicate either incomplete cleavage or aggregation of the sdAbs.

AE1 and AE5 are refractory to both CHIKV and MAYV

We next sought to assess more thoroughly the infectivity of CHIKV and MAYV in these transgenic colonies. To this end, we performed vector competence experiments with AE1 and AE5 to compare midgut, legs/wings, and saliva viral titers to those of WT mosquitoes. AE1 and AE5 had significant reductions in CHIKV titers in the midgut (AE1 $p<0.0001$; AE5 $p<0.0001$), legs/wings (AE1 $p=0.016$; AE5 $p=0.008$), and saliva (AE1 $p<0.0001$; AE5 $p<0.0001$) when compared to WT (Figure 5). Similarly, we observed significant reductions in MAYV replication in midguts (AE1 $p<0.0001$; AE5 $p=0.0008$), legs/wings (AE1 $p=0.0008$; AE5 $p=0.0025$), and saliva (AE1 $p=0.0003$; AE5 $p=0.0024$) (Figure 5).

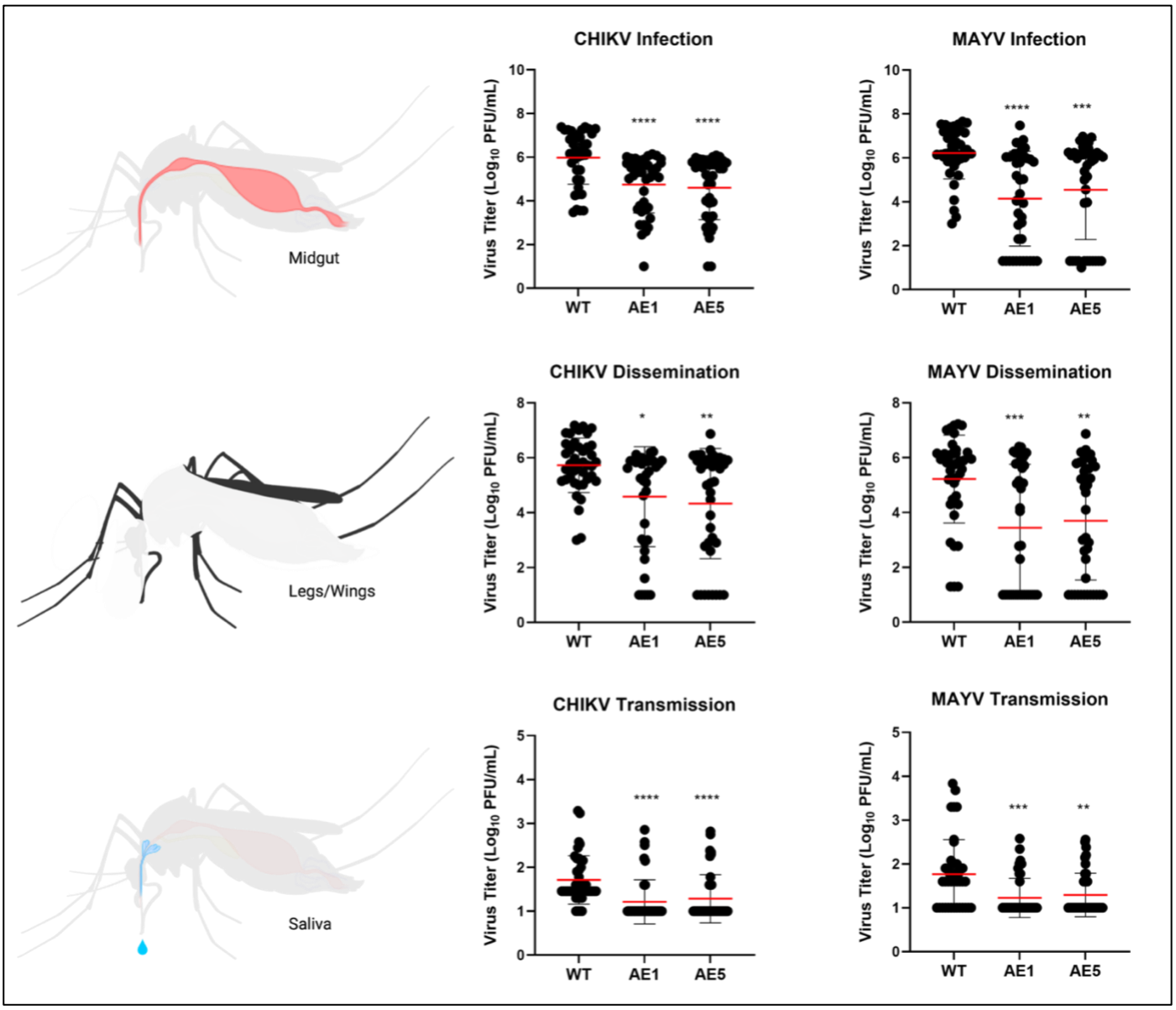

Figure 5. Vector competence analysis of WT and transgenic mosquitoes for CHIKV and MAYV. Plots depict viral titers in WT, AE1, and AE5 mosquitoes. To evaluate the replication of CHIKV and MAYV, we measured viral titers from dissected midguts (representative of infection), legs and wings (representative of dissemination), and saliva (representative of transmission). Viral titers were

measured via plaque assay on Vero cells 7 days post-infectious bloodmeal. Data points are representative of 40 replicates obtained in two independent experiments. Red horizontal bars represent the mean with SD. *P <0.05, **P < 0.01, ***P <0.001, ****P<0.0001.

4.5 Discussion

CHIKV and MAYV have become increasingly prevalent throughout several continents within the last decade, highlighting the need for novel approaches to control the spread of these crippling pathogens. The overall goal of this study was to develop a single control strategy to co-target these emerging alphaviruses. Thus, we generated transgenic mosquitoes that are highly refractory to both CHIKV and MAYV replication and significantly reduce the transmission potential of these viruses.

Previous groups have generated mosquitoes expressing small RNAs and antibody fragments for resistance to ZIKV and DENV (45-47); however, our approach is different because we used camelid-derived sdAbs to co-target two distinct alphaviruses. While the use of sdAbs against viruses is not novel, as Wu et al. reviews the several studies have focused on using these for their antiviral properties (48), our design is the only *in vivo* expression system of sdAbs in a mosquito vector. Alongside these differences, our studies evaluated viral titers (via plaque assay) in comparison to other studies quantifying the presence of viral mRNA. This is relevant because it provides a more thorough measure of infectious virus and in turn, potential for transmission to human hosts. Interestingly, our *in vitro* data suggested the sdAbs more potently neutralized MAYV compared to CHIKV, however, our data suggests these sdAbs had a greater impact on CHIKV *in vivo*. Although, it should be noted that these data share the same trend observed in the

preliminary transgenic vector competence experiments (Table 2) where CHIKV transmission was more significantly reduced compared to MAYV, suggesting a greater effect on CHIKV. The fact that our transgenic lines provide significant reductions with both CHIKV and MAYV at infection, dissemination, and transmission levels is particularly important because these viruses co-circulate throughout South America (49-53), thus this strategy could potentially limit the spread of both pathogens simultaneously.

While these data are promising, future studies are needed to determine the potential for these viruses to escape from sdAb-mediated neutralization within the transgenic mosquitoes. As mentioned, we hypothesized that expressing two sdAbs in our construct design would reduce the probability for these viruses to generate antibody escape variants; however, this hypothesis needs to be tested in future studies. In addition, the stability of sdAb expression by these transgenic mosquitoes should also be evaluated. As environmental factors such as temperature and mosquito diets affect *Wolbachia* density in transinfected mosquitoes (12), there could be similar effects on sdAb expression in these transgenic mosquitoes. Future studies should also assess whether these mosquitoes are refractory to more distantly related alphaviruses since we previously showed substantial neutralization by sdAb CC3 to a range of alphaviruses (35). Finally, in order to produce field-release-ready mosquitoes the transgene insertion sites for AE1 and AE5 need to be determined.

Altogether, these results provide support for this novel alternative mosquito population replacement strategy to combat these emerging alphaviruses. As this is the first development of transgenic *Ae. aegypti* mosquitoes that are specifically refractory to CHIKV and MAYV, this study is particularly significant because it highlights the ability to co-target two emerging

arboviruses that are crippling public health and obliterating quality of life around the globe within a single defense strategy.

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CHAPTER FIVE: CONCLUSION

5.1 Conclusion

Alphaviruses are arthropod-borne viruses belonging to the *Togaviridae* family that infect millions of people annually and are major public health threats due to their infections resulting in a range of debilitating disease symptoms. A hallmark of these symptoms is the development of incapacitating polyarthralgia that can persist for years following initial infection (1, 2). To date, the most effective means of controlling these arthritogenic, arboviral infections is through mosquito control programs. However, the programs have crucial limitations in their effectiveness (3-5); therefore, novel approaches are necessary to control the spread of these crippling pathogens. Given the close phylogenetic and antigenic relationship between MAYV and CHIKV, our research has focused on harnessing cross-reactive immunity between these two emerging alphaviruses.

Despite its history of small sporadic outbreaks, MAYV has the potential to cause large devastating outbreaks through peridomestic, human-amplified transmission (6, 7). Therefore, identifying and understanding the factors that can influence the epidemic potential of MAYV is important to forecasting its spread and disease burden. We hypothesized that prior CHIKV immunity may affect the outcome of MAYV disease and/or limit its emergence and epidemic potential in humans. While several studies have reported on cross-protection among alphaviruses (8-14), our work evaluated the level of cross-protective effects provided to MAYV via wild-type CHIKV infection as well as vaccination with several CHIKV vaccine candidates (15-17). The data obtained in this study has shown anti-CHIKV neutralizing antibodies have cross-reactive

and cross-neutralizing capabilities with MAYV. We have also provided support that prior CHIKV immunity can confer cross-protective effects against MAYV as we observed strong cross-protection against MAYV in murine models pre-exposed to wild-type CHIKV. In fact, immunity to CHIKV infection completely prevented the development of MAYV viremia. Alongside these results, our data indicated that naturally acquired CHIKV-specific human antibodies from patients in Trinidad can strongly cross-neutralize MAYV infection *in vitro*. This has important implications for MAYV's epidemic potential since the absence, or even significant reduction, of MAYV viremia in CHIKV-immune persons may lead to a prevention in subsequent transmission. This is particularly crucial for many parts of Asia and Latin America because these are strong CHIKV-endemic regions (18-23). Our data indicated moderate, but significantly reduced, cross-protection in murine models vaccinated with CHIKV candidates, thus this highlights the need for a specific defense strategy against MAYV.

Focusing on antibody-mediated cross-protection, we characterized the potency of an anti-CHIKV camelid-derived single-domain antibody, termed CC3, to neutralize a breadth of medically-relevant alphaviruses, including MAYV, Ross River virus (RRV), Venezuelan equine encephalitis virus (VEEV), and Western equine encephalitis virus (WEEV). These data revealed robust broadly-neutralizing activity against MAYV and RRV ($PRNT_{50}$ values <0.625 μg/ml). VEEV and WEEV, both New World alphaviruses, were included in these studies in order to determine the scope of CC3's cross-reactivity. Interestingly, CC3 provided a degree of neutralizing activity against VEEV; albeit $PRNT_{50}$ values (>4.0 μg/ml) were significantly less than those of MAYV and RRV. In addition, CC3 did not neutralize WEEV. Altogether, these data further supported antibody-mediated cross-protection among alphaviruses, particularly Old World alphaviruses.

As CHIKV and MAYV have become increasingly prevalent across several continents within the last decade, and increased global travel to these endemic areas has resulted in imported cases of both these viruses into new geographical regions (24-33), the necessity for an effective defense measure is highlighted. Given the strong evidence of antibody-mediated cross-protection among CHIKV and MAYV, we postulated that a single defense strategy could be formulated to combat both of these emerging arboviruses. First, we evaluated the neutralizing activity afforded by a combination of anti-CHIKV sdAbs (CA6 and CC3) against CHIKV and MAYV *in vitro*. We found that this combination of sdAbs provided potent neutralization against both viruses. We hypothesized that by expressing CA6 and CC3 *in vivo* in transgenic *Aedes aeygpti* mosquitoes we could simultaneously target both CHIKV and MAYV. Thus, we developed and evaluated a series of transgenic colonies for their ability to express the sdAbs and reduce CHIKV and MAYV replication. Our experimentation resulted in two transgenic colonies, AE1 and AE5, that express the sdAbs and highly impact viral replication. Both AE1 and AE5 had significant reductions in CHIKV titers in the midgut (AE1 $p<0.0001$; AE5 $p<0.0001$), legs/wings (AE1 $p=0.016$; AE5 $p=0.008$), and saliva (AE1 $p<0.0001$; AE5 $p<0.0001$) when compared to WT mosquitoes. Further, significant reductions in MAYV replication in midguts (AE1 $p<0.0001$; AE5 $p=0.0008$), legs/wings (AE1 $p=0.0008$; AE5 $p=0.0025$), and saliva (AE1 $p=0.0003$; AE5 $p=0.0024$) was also observed. This study is particularly significant because it highlights the ability to co-target two emerging arboviruses that are crippling public health and obliterating quality of life around the globe.

In summary, we have further investigated the cross-reactivity among Old World alphaviruses, characterize the antibody-mediated cross-neutralization of alphaviruses, and developed a single

defense strategy to combat both CHIKV and MAYV. This work moves the field forward because it provides support for developing defense strategies that target multiple emerging arboviruses simultaneously.

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