

DISSERTATION

DEVELOPMENT AND EFFICACY TESTING OF BROAD ALPHAVIRUS VACCINES AND
ANTIVIRALS AND CHARACTERIZATION OF ALPHAVIRUS NEUROINVASION

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ABSTRACT

DEVELOPMENT AND EFFICACY TESTING OF BROAD ALPHAVIRUS VACCINES AND ANTIVIRALS AND CHARACTERIZATION OF ALPHAVIRUS NEUROINVASION

Alphaviruses are mosquito-borne pathogens that cause worldwide disease and death in humans and animals. Several alphaviruses are select agents and are a legitimate biosafety and bioweapon concern. Additionally, several alphaviruses are emerging infectious diseases. Climate change and urbanization have expanded mosquito populations and increased human-mosquito interactions within this decade and will continue into future decades. As mosquito populations expand, naïve human populations are exposed to arthropod-borne viruses, including alphaviruses, and vector-borne diseases have surged. The increasing prevalence of arthropod-borne disease has highlighted the global need to develop measures that prevent or treat arthropod-borne disease infection. Currently, vaccines to prevent alphavirus infection are limited to investigational new drug status and no therapeutics are available to treat alphavirus disease. This dissertation will describe projects aimed at preventing or treating alphavirus infection and characterizing the process of alphavirus neuroinvasion.

To address the concern of potential outbreaks of an intentional or natural nature, alphavirus vaccines based on the ectodomain of alphavirus E1 were designed and tested. Cationic liposomes complexed with nucleic acid adjuvants and alphavirus E1 protein (lipid-antigen-nucleic acid complexes; LANACs) provided the best platform for alphavirus E1 vaccination. Interestingly, western equine encephalitis virus (WEEV) E1 (LANAC WEEV E1) protected against both mouse WEEV and eastern equine encephalitis virus (EEEV) challenge but not Venezuelan equine encephalitis virus (VEEV); whereas, VEEV E1 (LANAC VEEV E1) protected against both VEEV and EEEV challenge but not WEEV. LANAC VEEV E1 + WEEV E1 vaccination protected mice against EEEV, VEEV, and WEEV challenge. Mice immunized with LANACs (LANAC WEEV E1, LANAC VEEV E1 or LANAC VEEV E1 + WEEV E1) mounted strong humoral immune responses, but were lacking neutralizing antibody.

Hamsters immunized with LANAC WEEV E1 failed to mount humoral immune responses and were not protected from challenge.

Antibody derived from E1 vaccination binds infected cells and purified E1, but not intact virions. E1 antibody is non-neutralizing yet protective against CHIKV, EEEV, SINV, VEEV, and WEEV *in vitro*. *In vivo* we have demonstrated that antibody is protective against all three new world alphaviruses (NWAs). Antibody affects late stages of the viral life cycle and likely inhibits virus release or cell death.

Following a screen, conducted by our collaborators, of FDA approved and ex-US approved compounds for effectiveness against viral encephalitic and hemorrhagic fever viruses, we tested compounds for anti-alphavirus activity to develop therapeutics to treat alphavirus infection. Yield reduction assays identified four compounds that inhibited virus replication by two to four logs. These compounds were further tested *in vitro* for mechanism of action. Compounds P-75802, P-75803, P-75805, and P-75811 affected early stages of replication. Compound P-75802 was tested *in vivo* and was found to possess limited antiviral activity.

The process of alphavirus neuroinvasion, particularly following peripheral inoculation, is poorly defined. In the studies described here, encephalitic alphavirus neuroinvasion following EEEV, VEEV, and WEEV intranasal and subcutaneous inoculation were described relying on bioluminescent *in vivo* and *ex vivo* imaging, CLARITY imaging, and histopathology. We found that neuroinvasion occurs through two routes dependent on inoculation method. The cranial nerves, particularly the olfactory tract nerve, were involved in neuroinvasion following intranasal inoculation. Neuroinvasion from the periphery, footpad inoculation, occurred via a route independent of the olfactory tract. Neuroinvasion occurred in areas where the blood brain barrier is naturally absent including the hypothalamus, anteroventral third ventricle region (AV3V), area postrema, and the pineal body.

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DEDICATION

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CHAPTER ONE: LITERATURE REVIEW

Introduction

Arthropod-borne viruses (arboviruses) cause human disease worldwide. The twentieth century was marked with repeated campaigns to eradicate arboviruses, which resulted in brief respites from arthropod-borne diseases. Arboviruses still persist and are at the forefront of emerging and re-emerging diseases. Currently over one-third of the global population is at risk of arbovirus infection (Porretta et al., 2012). As arthropod populations continue to expand due to global climate change, arthropod-borne diseases are expected to rise and expand proportionately, as exemplified by the recent expansions of West Nile virus (WNV) (Garmendia et al., 2001) and chikungunya virus (CHIKV) (Kendrick et al., 2014). With the exception of yellow fever virus (YFV) and Japanese encephalitis virus vaccines, there are few options available to prevent or treat arbovirus infections. Arboviruses continue to spread into new areas, and the need for vaccines and therapeutics has become critical. A better understanding of the pathogenesis of arboviruses and what constitutes protective immune responses are needed to develop ways to prevent or inhibit arbovirus replication.

Arthropod-borne viruses

Arboviruses are a group of animal viruses that replicate in both vertebrates and arthropods and require transmission from one vertebrate to another by the bite of an infected, hematophagous arthropod. Arbovirus maintenance involves alternating transmission events from arthropod to vertebrate and vertebrate to arthropod, both occurring through hematophagous arthropod biting.

Arboviruses have three distinct transmission cycles: enzootic, epizootic, and epidemic classified based on both the vertebrate and vector species involved (Figure 1.1). Enzootic transmission cycles involve cyclic transmission from vertebrates, as reservoir hosts, to enzootic vectors. Epizootic transmission is infection of non-reservoir vertebrate species resulting from bridge vector biting. Lastly, epidemic transmission involves human to human transmission by human biting vectors. Though not defined by human infection, both enzootic and epizootic transmission cycles have the potential to cause

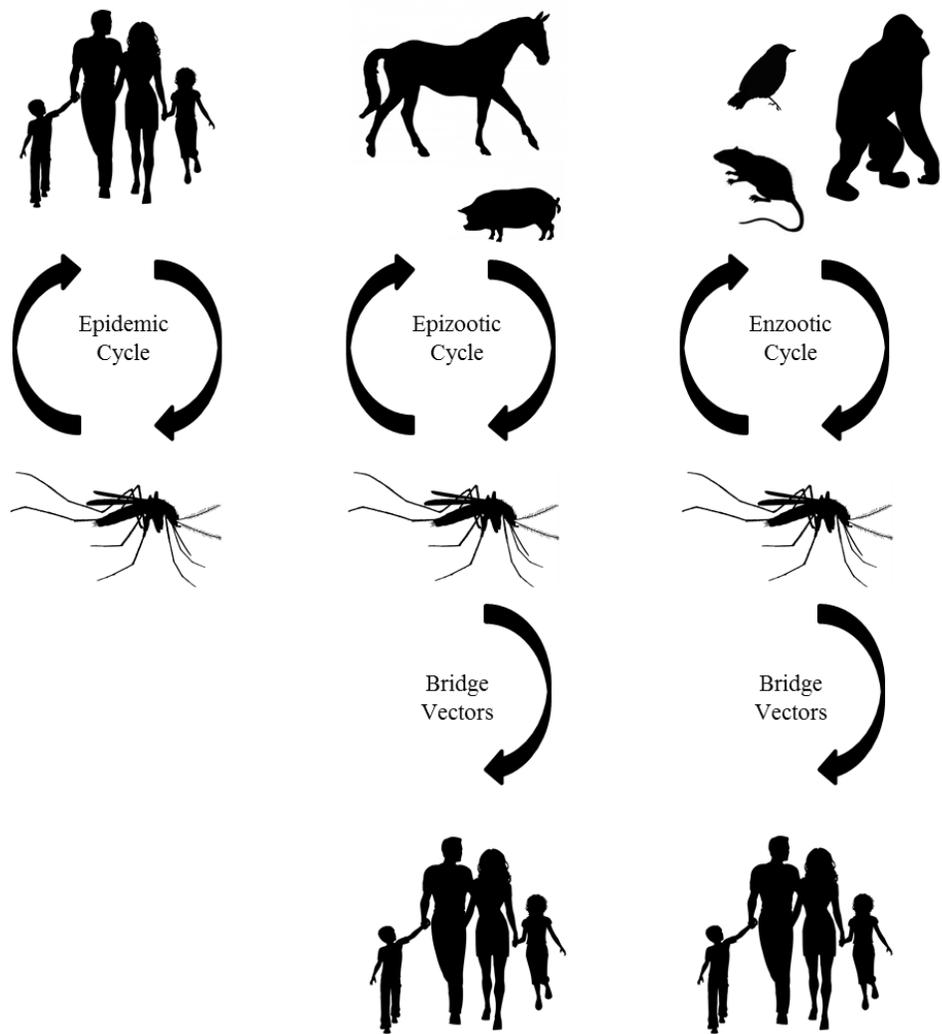


Figure 1. 1 Typical mechanisms of arbovirus transmission.

Adapted from (Weaver and Barrett, 2004). Left- Epidemic transmission, arbovirus transmission between human biting mosquitoes and humans. Middle- Epizootic transmission, arbovirus transmission between bridge vectors and non-reservoir vertebrate species. Right- Enzootic transmission, arbovirus transmission between reservoir vertebrates and vectors.

human spill over infections. Individual arboviruses, additionally, have differential tendencies to cause each transmission cycle. Arboviruses such as dengue virus, YFV, and CHIKV commonly adopt epidemic transmission cycles, whereas Venezuelan equine encephalitis virus (VEEV) is best known for epizootic transmission (Weaver and Barrett, 2004).

Outbreaks of human and animal disease matching that of arboviruses date back for centuries. However, the correlation between human disease and hematophagous arthropods was not made until the 19th century. In 1881, Carlos Finlay proposed that mosquitoes were necessary for the propagation of yellow fever epidemics (Finlay, 1937). In 1901 Walter Reed confirmed that yellow fever was transmitted among humans by *Aedes aegypti* mosquitoes (Reed et al., 2001), making yellow fever the first human disease linked to arthropod transmission by arthropod vectors. Yellow fever virus (YFV), was isolated 28 years later (Stokes et al., 1928). Since the isolation of YFV, approximately 550 arboviruses have been recognized and over 100 are attributable to human illness (Berge et al., 1975) (Table 1.1). Today, arboviruses are found in a variety of viral families including: *Togaviridae*, *Flaviviridae*, *Bunyaviridae*, *Reoviridae*, and *Rhabdoviridae* (Shope and Meegan, 1997).

Diseases caused by arboviruses range from febrile illness to hemorrhagic fever, hepatitis, encephalitis, and meningitis. Human arbovirus infections account for the loss of approximately one million disability adjusted life years annually (DALYs) (Labeaud et al., 2011). Arboviruses are globally distributed and are increasing in prevalence. The current emergence of CHIKV and Zika virus in the Americas and the 30 fold increase in dengue incidence in the past five decades demonstrate the danger arboviruses pose to human health (Gatherer and Kohl, 2015; Leparc-Goffart et al., 2014; WHO, 2012). Arboviruses were responsible for approximately 30% of emerging infectious diseases over the last decade and are continuing to rise disproportionately when compared to non-vector-borne disease (Jones et al., 2008). The alphaviruses are medically relevant arboviruses (Hollidge et al., 2010) and will be the focus of the work described herein.

Table 1. 1 Important arboviruses causing human disease.

Adapted from (Gubler, 2002). north (N). south (S).

Family/genus	Species	Vector	Host	Clinical symptoms	Geographical distribution	Epidemic
Togaviridae	Eastern equine encephalitis virus (EEEV)	Mosquito	Human, equine	Febrile illness, encephalitis	N/S-America	Yes
	Western equine encephalitis virus (WEEV)	Mosquito	Human, equine	Febrile illness, encephalitis	N/S-America	Yes
	Venezuelan equine encephalitis virus (VEEV)	Mosquito	Human, equine	Febrile illness, encephalitis	N/S-America	Yes
	Sindbis virus (SINV)	Mosquito	Human, bird	Febrile illness, arthritis	Africa, Asia, Australia, Europe	Yes
	Semliki Forest virus (SFV)	Mosquito	Human, rodent	Febrile illness, arthritis	Africa, Asia	Yes
	Chikungunya virus (CHIKV)	Mosquito	Human, primate	Febrile illness, arthritis	Africa, Asia, Europe	Yes
	O'nyong nyong virus (ONNV)	Mosquito	Human	Febrile illness, arthritis	Africa	Yes
	Ross River virus (RRV)	Mosquito	Human, marsupial	Febrile illness, arthritis	Australia	Yes
	Barmah Forest virus (BFV)	Mosquito	Human	Febrile illness, arthritis	Australia	Yes
Flaviviridae	Dengue 1–4 (DENV)	Mosquito	Human	Hemorrhagic fever	N/S-America, Asia, Australia, Africa	Yes
	Yellow fever virus (YFV)	Mosquito	Human	Hemorrhagic fever, hepatitis	N/S-America, Africa	Yes
	Japanese encephalitis virus (JEV)	Mosquito	Human	Febrile illness, encephalitis	Asia	Yes
	West Nile virus (WNV)	Mosquito	Human, equine	Febrile illness, encephalitis	N/S-America, Asia, Australia, Africa	Yes
	St.Louis encephalitis virus (SLEV)	Mosquito	Human	Encephalitis	N/S-America	Yes
	Murray valley encephalitis virus (MVEV)	Mosquito	Human, bird	Febrile illness, encephalitis	Australia	Yes
	Tick-borne encephalitis virus (TBEV)	Tick	Human	Encephalitis	Europe, Asia	No
Bunyaviridae	La Crosse virus (LACV)	Mosquito	Human	Encephalitis	N-America	No
	Rift valley fever virus (RVFV)	Mosquito	Human, ruminant	Hemorrhagic fever, encephalitis	Africa	Yes
	Bluetongue virus (BTV)	Midge	Ruminants	Febrile illness, cyanosis	N/S-America, Africa, Asia, Europe	Yes
Reoviridae	Colorado tick fever virus (CTFV)	Tick	Human	Febrile illness	N-America	No

Alphaviruses

Alphaviruses (family: *Togaviridae*) are arthropod-borne RNA viruses that cause pathology in humans and other animals. Depending on the alphavirus species, alphaviruses are maintained in nature through enzootic transmission cycles involving mosquitoes and birds and/or small rodents. Of the 31 recognized alphaviruses, approximately one-third are known to cause disease in humans or other vertebrates (King, 2012).

The alphaviruses are divided into seven complexes based on antigenic relationships (antibody cross-reactivity): Barmah Forest virus, Venezuelan equine encephalitis, Middelburg virus, Ndumu virus, Semliki Forest virus (SFV), eastern equine encephalitis, and western equine encephalitis (Powers et al., 2001).

Eastern equine encephalitis virus (EEEV), VEEV, and western equine encephalitis virus (WEEV) are classified as Category B agents by the National Institute of Allergy and Infectious Diseases due to biosafety concerns. Eastern equine encephalitis virus, VEEV, and WEEV infect laboratory animals via the intranasal route (Pamela, 2011). Intranasal infection occurs with relative ease owing to the relatively low dose required for infection and the ease of generating high titer virus. Intranasal infection is associated with a higher percentage of encephalitis and mortality in animals (Pamela, 2011). Accordingly, EEEV and VEEV are classified as select agents by Human and Health Services (HHS) and the United States Department of Agriculture (USDA) due to their ability to create self-sustaining epizootics and cause high mortality. Alphaviruses (CHIKV, EEEV, and Ross River virus (RRV)) are emerging infectious diseases (Hollidge et al., 2010). Unfortunately, there are no licensed vaccines or therapeutics to prevent or treat alphavirus infections.

Molecular virology of alphaviruses

Alphaviruses are enveloped arboviruses ranging in size from 60-70 nm in diameter. Virus particles consist of an icosahedral nucleocapsid enveloped in host derived membrane punctuated by copies of the viral glycoproteins, E2 and E1, which form heterodimeric, trimers on the surface of virions. The nucleocapsid is composed of alphavirus genomic RNA complexed with multiple copies of the viral

capsid protein. Capsid and the glycoproteins possess a one-to-one ratio resulting in a virion with 240 copies of capsid interacting with 80 heterodimeric, trimers (Strauss and Strauss, 1994).

The alphaviruses are single-stranded, positive-sense RNA viruses of approximately 11-12 kb in size. Alphavirus genomes possess both a 5' terminal cap and 3' terminal polyadenylated tail. Alphavirus genomic RNA structure resembles that of host mRNA and is translated by the host's translational machinery. The alphavirus genome is organized into two regions. The 5' end of the genome encodes four non-structural proteins which form the replication machinery and the 3' end of the genome generates a subgenomic RNA that encodes the structural proteins responsible for virion formation (Strauss and Strauss, 1994). The viral life cycle will be reviewed with respect to which viral proteins function in each process (Figure 1.2).

The alphavirus life cycle begins with entry into the host cell. Alphaviruses possess broad host cell tropism, and are capable of initiating infection in a wide variety of cell types. The alphavirus E2 glycoprotein is responsible for viral entry and the broad tropism of alphaviruses is likely related to the ability of E2 to either bind multiple receptors or to recognize ubiquitous receptors (Leung et al., 2011). Receptor-bound virus is endocytosed via a clathrin-mediated pathway. A low pH induced conformational change results in E2/E1 disassociation, exposure of the E1 fusion loop, and refolding of E1 into a homotrimer resulting in fusion of the virus envelope with the endosome membrane and release of the nucleocapsid into the cytoplasm (Kielian et al., 2010). The nucleocapsid quickly disassembles and the viral genome is released.

The alphavirus nonstructural proteins are immediately translated as one or two polyproteins, depending on the virus species (Strauss and Strauss, 1994). Polyprotein P123, containing nonstructural proteins (nsPs) 1-3 results from translation termination at an opal codon between nsP3 and nsP4. Polyprotein P1234, which includes nsP4, is produced by low-level readthrough (10-20%) of the opal codon (Strauss and Strauss, 1994). Viruses containing a sense codon as opposed to the opal codon between nsP3 and nsP4, produce only polyprotein P1234 (Myles et al., 2006). Following polyprotein

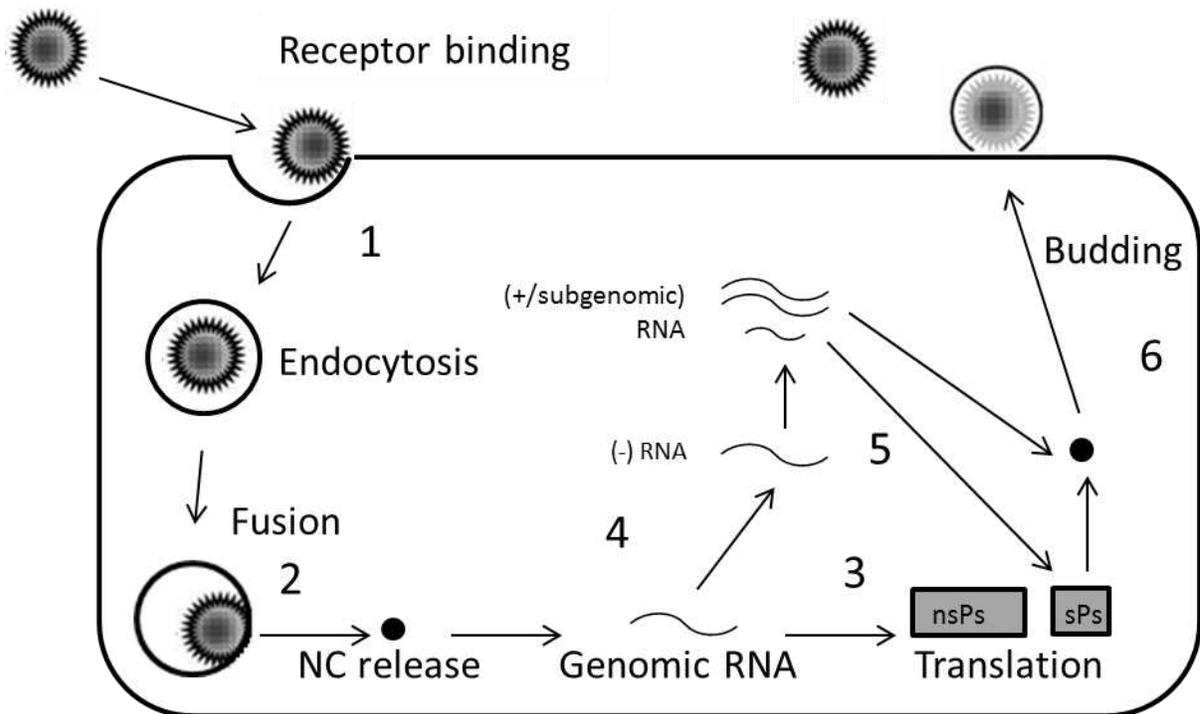


Figure 1. 2 Alphavirus life cycle.

Adapted from (Leung et al., 2011). 1) Alphavirus entry through receptor mediated endocytosis. 2) Fusion and nucleocapsid release. 3) Translation of non-structural proteins (nsPs). 4) Replication. 5) Translation of structural proteins (sPs). 6) Nucleocapsid assembly and budding.

translation, the individual nsPs are cleaved from both polyproteins in a structured manner that regulates that synthesis of negative- and positive- sense RNA.

The individual nsPs form replication complexes, at the plasma membrane within cytoplasmic vesicles (Spuul et al., 2011), which produce full, positive-sense copies of the genomic RNA in addition to 26S subgenomic RNA. All four nsPs are essential components of the replication complex: nsp1 functions in the capping and methylation of newly synthesized genomic and subgenomic RNA and anchors the replication complex to the plasma membrane; nsP2 functions as the viral helicase, contains the protease domain responsible for cleavage of all of the alphavirus nsPs, and functions in host cell transcriptional and translational shutoff in old world alphaviruses (OWAs) (Garmashova et al., 2007); the functions of nsP3 are still largely unknown but essential; and nsP4 functions as the viral RNA-dependent RNA polymerase and protein scaffold for assembly of the replication complex (Leung et al., 2011; Sawicki and Sawicki, 1998; Shirako et al., 2000).

Once transcribed, the 26S subgenomic RNA is translated to produce the alphavirus structural proteins. The structural proteins are produced as a polyprotein that is processed both co- and post-translationally. The nucleocapsid protein is translated first and contains a protease domain that allows for self-cleavage and release of nucleocapsid into the cytoplasm (Strauss and Strauss, 1994). Release of the nucleocapsid protein exposes a N-terminal signal sequence that results in the translocation of the remaining glycoprotein into the endoplasmic reticulum where PE2, 6K, and E1 are translated and post-translationally modified. PE2 and E1 dimerize in the ER membrane before transport to the cell surface. Before arriving at the cell surface, PE2 is cleaved by the cellular enzyme furin releasing E3 and activating the spike proteins (Sjoberg et al., 2011).

Virion assembly begins with cytoplasmic nucleocapsid recognizing an encapsidation signal on viral genomic RNA, resulting in the formation of the nucleocapsid core (Weiss et al., 1994). The nucleocapsid core is then trafficked to the plasma membrane where interactions between E2 glycoproteins and nucleocapsid proteins drive viral budding (Lopez et al., 1994).

Alphavirus expression systems

Since alphavirus genomic material is all that is required for initiation of infection, they are easily amenable to reverse genetic systems. Infectious clones of alphaviruses are traditionally generated by reverse transcription of the viral RNA genome and insertion of the resulting cDNA into a plasmid containing a bacterial origin of replication, antibiotic resistance marker, T7 or SP6 phage transcriptional promotor, and unique linearization site. Once assembled, recombinant virus can be generated by linearization of the plasmid, *in vitro* transcription of the linearized plasmid, and transfection of the generated RNA into cells (Foy and Olson, 2008). Recently, these systems have been engineered to bypass the linearization and *in vitro* transcription steps by incorporating ubiquitous mammalian transcriptional promoters and ribozymes (Steel et al., 2011).

Alphavirus expression systems (AESs) are useful tools for the expression of genes of interest (GOI) or RNAs during alphavirus infections. AESs consist of alphavirus infectious clones which have been manipulated to express a GOI. A variety of strategies have been used in the expression of GOI including: duplicated subgenomic promoters, fusion of GOI to viral proteins, and incorporation of self-cleaving GOI into viral open reading frames (Sun et al., 2014). Diverse proteins and sequences have been inserted into alphavirus genomes ranging from fluorescent and bioluminescent markers that track infection (Cook and Griffin, 2003; Phillips et al., 2013; Sun et al., 2014) to host molecules or siRNAs that influence the course of infection (Levine et al., 1996; Olson et al., 1996) each expanding our knowledge of alphavirus virology.

In vivo imaging has greatly advanced our understanding of infection processes dating back to 1995 when the technology was first used to track *Salmonella* replication in mice (Contag et al., 1995). *In vivo* imaging of intact, living animals allows for the streamlining of the steps required to understand the pathogenesis of infection as opposed to traditional methods, which require the euthanasia of several animals per time point and the assaying of organs for infectious agent (Patterson et al., 2011). Importantly, this technology can be extended beyond *in vivo* pathogenesis studies, and can be applied to vaccine and antiviral efficacy studies both *in vivo* and *in vitro*.

AESs expressing bioluminescent and fluorescent molecules have been verified as accurate markers of infection in *in vivo* imaging systems (Cook and Griffin, 2003; Phillips et al., 2014; Phillips et al., 2013) and, importantly, have been used to track the progress of infection *in vitro* and in animals (Phillips et al., 2014; Phillips et al., 2013; Teo et al., 2013). The studies described in this dissertation use recombinant CHIKV, EEEV, Sindbis virus (SINV), VEEV, and WEEV AESs expressing luciferase, GFP, or DsRed (Figure 1.3). Importantly, these viruses accurately recapitulate the replication kinetics and mortality of wild-type viruses (Figure 1.4 and demonstrated in (Sun et al., 2014), (Steel, 2014), and (Phillips et al., 2013)). These AESs served as an important tool and convenient system with which *in vivo* imaging and CLARITY techniques were utilized.

Alphavirus infections and protective host responses

Alphaviruses cause a wide range of disease in vertebrates, including humans. The genus alphavirus is divided into two groups based on the geographic origin and nature of the disease they cause. The OWAs, including SINV, o'nyong-nyong virus, and CHIKV, cause an arthralgic disease, while new world alphaviruses (NWAs), including EEEV, VEEV and WEEV, cause encephalitic disease.

The pathogenesis of OWAs and NWAs exhibit both commonalities and divergences with each other; however, a generalized sequence of infection and disease occurs. A stereotypical pathogenic sequence for alphaviruses includes: entry, replication at the entry site, transport to regional lymph nodes, replication in regional lymph nodes, systemic viral release from regional lymph nodes, and viremia and infection of extraneural and neural sites.

New world alphaviruses

New world alphavirus infections range from mostly asymptomatic to symptomatic depending on the alphavirus species. Disease is biphasic with an acute phase characterized by fever, headache, and malaise and a secondary phase involving central nervous system (CNS) involvement. Central nervous system involvement manifests as: somnolence, confusion, seizures, ataxia, and/or coma. Mortality rates associated with NWAs reach 75% (Fields et al., 2013). Long term deficits have been reported.

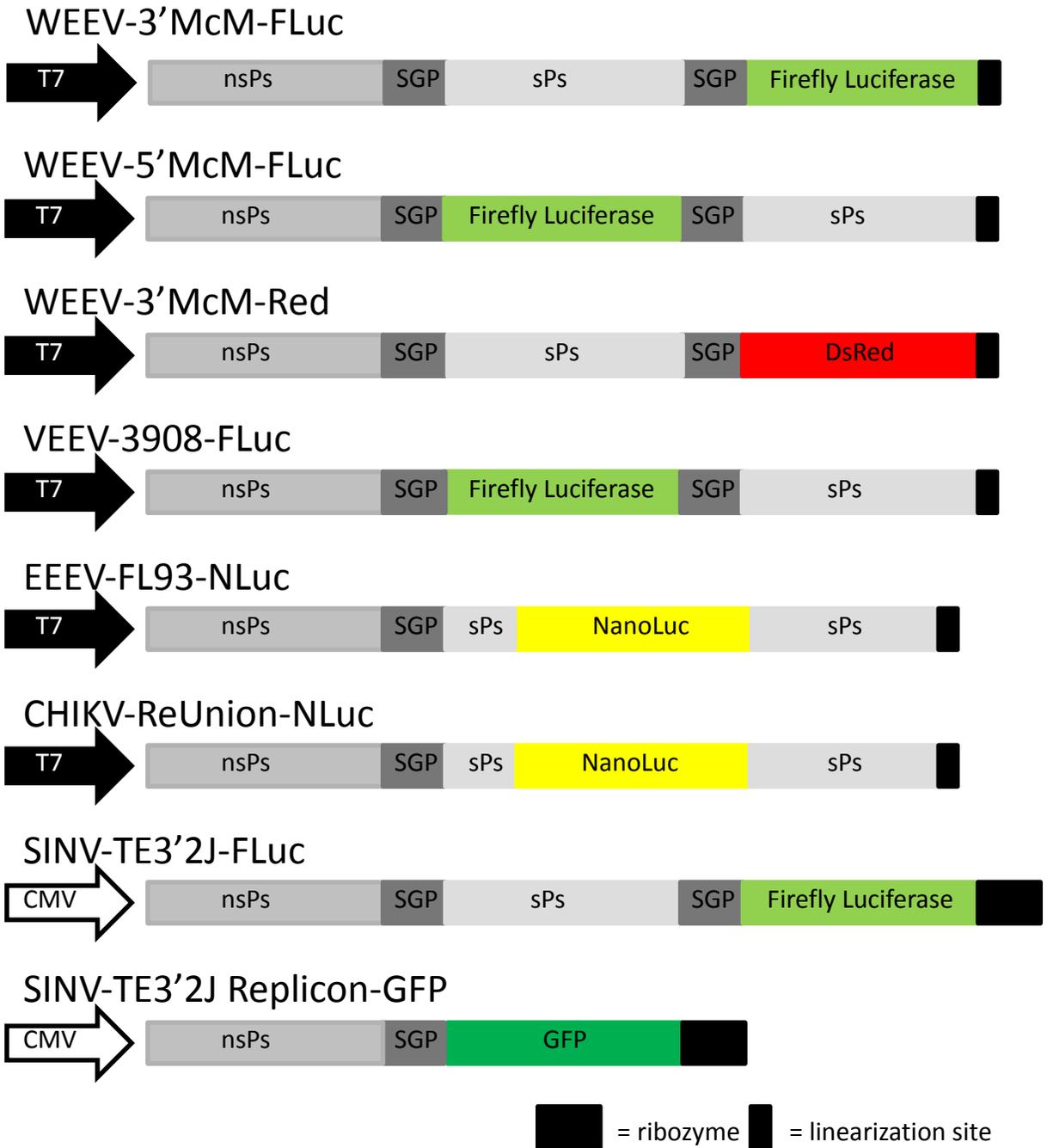


Figure 1. 3 Diagram of AESs.

Illustration of the genome of each AES used in these studies. non-structural proteins (nsPs) subgenomic promotor (SGP) structural proteins (sPs).

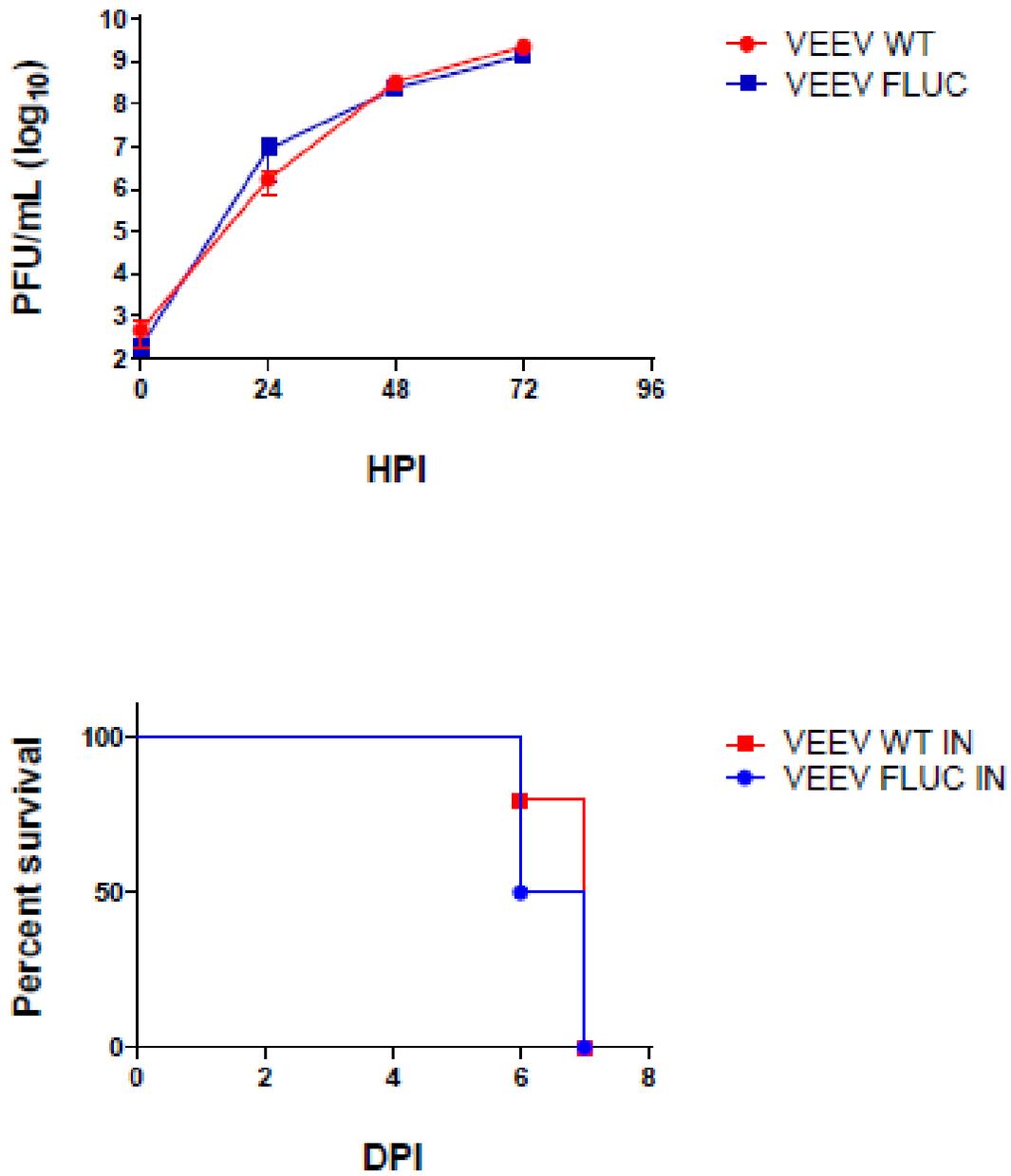


Figure 1. 4 VEEV-3908-FLuc resembles VEEV WT.

Top- VEEV-3908-FLuc kinetics in Vero cells (0.1 MOI). Bottom- mean time to death (10⁴ PFU IN)

Histopathological findings in fatal cases of NWA human infection document lesions in the brain, lymph nodes, spleen, and lung. For EEEV and WEEV, the brain is the primary site of pathology with vasculitis, hemorrhage, and meningitis. In VEEV infections, lymphoid organs are additionally affected. Major histological findings in the lymphoid organs include lymphocyte degeneration, lymphoid depletion, and follicular necrosis (Steele and Twenhafel, 2010).

Old world alphaviruses

The majority of OWA infections are asymptomatic. Depending on the infecting alphavirus species, disease is characterized by fever, malaise, rash, arthralgia and myalgia with recovery in less than one week. Skin manifestations occur primarily on the trunk and limbs and are typically maculopapular. Alphavirus polyarthralgia is usually symmetrical and can affect peripheral and large joints. Some patients develop chronic or recurrent manifestations of disease. OWA mortality rates are low at 0-1% (Suhrbier et al., 2012).

Old world alphavirus have not been as fully characterized histopathologically as NWAs; however, lymphoid organs, tendons, joints, bones, and muscles are all affected. Inflammation of the tendons, joint effusion and bone marrow edema comprise common joint pathology. Bone resorption and diffuse hemorrhage have been documented in a small proportion of cases (Suhrbier et al., 2012).

Sequelae

Up to sixty percent of patients who recover from alphavirus acute clinical disease (NWA or OWA) are left with debilitating defects, sequelae, ranging from arthritic to neurological in nature (Borgherini et al., 2008; Steele and Twenhafel, 2010). These sequelae account for the majority of alphavirus morbidity DALYs and are thought to be due to inflammation resulting from the persistence of virus and/or its products within respective tissues.

Protective host responses

IFN- α/β and antibody act synergistically to control and promote recovery from alphavirus infection. The sensitivity or resistance of any particular alphavirus to IFN- α/β is a direct correlate of the rapidity of the antibody response and is predictive of outcome. Accordingly, alphavirus antibody

responses have long been implicated as the component of the immune response dictating the outcome of infection.

Alphavirus animal models

A broad range of vertebrates are susceptible to alphavirus disease, and experimental infections of mice, non-human primates, and other species have all been conducted. Alphavirus animal models are typically split into NWA and OWA models, with animal models of encephalitic and arthritic disease both available. Several well characterized animal models are available for alphavirus-induced encephalitis and arthralgia; however, many aspects regarding the mechanisms and features of disease require modern characterization. Mouse models are the most heavily relied on and will be primarily discussed here.

New world alphavirus models

Encephalitic mouse models exist for the NWAs EEEV, VEEV, and WEEV. Additionally, mouse neurovirulent SINV and RRV have occasionally been utilized to characterize alphavirus-induced encephalitis. Fifty percent lethal doses (LD50s) range from 10-1000 plaque forming units (PFU) depending on virus, route of inoculation, and age of the mouse (Steele and Twenhafel, 2010). Both aerosol infections (relevant to biological warfare) and natural infections have been described (Pamela, 2011). Mice infected with encephalitic alphaviruses exhibit lethargy, piloerection, huddling, loss of coordination, and paralysis. Typically, mouse models of NWA infection fail to reproduce the vascular component of disease that is seen in human infections (Steele and Twenhafel, 2010). Mice typically succumb between two and seven days post infection (DPI).

Outbred CD-1 mouse models of NWA infections have been developed and used to characterize infection using traditional and modern methods. Logue et al. (2009) characterized virulence differences of WEEV strains in CD-1 mice. Comparisons of mortality and mean time to death (MTD) following subcutaneous (SC) challenge with 10^3 PFU identified three different virulence groups from the six strains analyzed. The McMillan (McM) strain, isolated from a human encephalitis case in 1942, was the most virulent of the strains tested with 100% mortality and a MTD of 4 days. Another strain, Montana-64, exhibited intermediated virulence. Isolated in 1967 from a horse brain, Montana-64 exhibited 70%

mortality and a MTD of 6-7 days. The least virulent group demonstrated 0-20% mortality and a MTD of 7-9 days. Both the McM and Montana-64 CD-1 mouse models have been further utilized in neurovirulence, neuroinvasion, and vaccine studies (Mossel et al., 2013; Phillips et al., 2014; Phillips et al., 2013). The CD-1 outbred mouse model has successfully been adapted to a variety of alphavirus infections including those using VEEV strain 3908 and EEEV strain FL93-939 (FL93) (Guerbois et al., 2013; Phillips et al., 2014). Importantly, virus strain, age of the mouse, route of infection, and virus titer have all been shown to influence the course of alphavirus infections.

In addition to mice, hamsters, non-human primates, horses, birds, and other animals have all been infected with NWAs. Bird and horse infections have served as natural or reservoir infection models and horse infections have facilitated the development of EEEV, VEEV, and WEEV horse vaccines. Hamsters and recently hatched chickens serve as sensitive, highly susceptible NWA infection models with LD50 values below one PFU (Steele and Twenhafel, 2010). The hamster model of NWA infection has also been touted for its ability to reproduce the vascular component of human disease that is lacking in mouse models (Paessler et al., 2004; Steele and Twenhafel, 2010). Lastly, NWA infections of non-human primates, particularly cynomolgus macaques, serve as the closest model for human infections with similar pathology and mortality when compared to human cases (Steele and Twenhafel, 2010). Unfortunately, none of the current animal models has been able to successfully replicate the long-term neurological sequelae often seen in human patients.

Old world alphavirus models

Of the OWAs animal models, mouse models of RRV, Semliki Forest virus, SINV, and CHIKV are the most thoroughly characterized. Mice infected with arthralgic alphaviruses exhibit lethargy, piloerection, huddling, loss of muscle tone, swelling of the inoculation site, and severe hind limb disease and paralysis (Taylor et al., 2015). Age-dependent susceptibility has been the prevailing difficulty with the development of arthralgic OWA animal models. Often immune-deficient or immunologically immature mice have had to be utilized; however, a variety of juvenile and adult mouse models have been developed that more accurately mimic OWA human infections (Taylor et al., 2015). As with the NWAs,

mouse models are still unable to perfectly recapitulate human disease (Suhrbier et al., 2012). Collagenosis and chronic arthralgia are still lacking in any of the available mouse models. Chikungunya mouse models will be discussed further as the focus of OWAs in this dissertation.

The pathogenesis of OWA arthritic disease in humans is still largely not understood; however, suitable acute CHIKV models are available. Ziegler et al. (2008) subcutaneously infected newborn and 14-day-old CD-1 and ICR mice with CHIKV strain LR 2006-OPY. Clinically and histologically, newborn and 14-day-old CD-1 mouse SC CHIKV infection resembled human infection. Importantly, the authors reaffirmed the concept of age-dependent susceptibility in these studies documenting that 14-day-old mice were significantly less susceptible to CHIKV in terms of viremia and histopathology. Zeigler et al. (2011) extended this system using four to six-week-old mice and *in vivo* imaging to better elucidate areas of viral replication; though, age-dependent susceptibility prevented disseminated infection in the majority of infected mice. Lastly, by moving the inoculation site from a site dorsal to the thoracic spine to the ventral side of each hind foot, Gardner et al. (2010) was able to model human viremia and rheumatic disease in adult, wild-type mice. Long-term CHIKV infection mouse models still remain elusive.

Though considerable progress has been made with regard to CHIKV mouse models, non-human primate models remain the most recapitulative of human disease. Experimental infections of macaques have demonstrated a clinical picture of CHIKV infection resembling that of human disease including acute, sub-acute, and chronic phases of infection. Similarly mirroring human disease, a spectrum of disease was observed upon non-human primate CHIKV infection ranging from sub-clinical to severe and was dependent on the amount of inoculated virus. Notably, non-human primate models are currently the only available model for long term CHIKV infection, and have implicated macrophages as reservoirs of CHIKV infection (Labadie et al., 2010).

Alphavirus antibodies

Due to the importance of alphavirus antibody in recovery from alphavirus infection, alphavirus antibody has long been used as a surrogate marker of protection. Alphavirus virions primarily consist of three viral structural proteins: nucleocapsid, E2 and E1. Antibodies generated following alphavirus infection or vaccination are primarily directed towards the immunogenic glycoproteins E2 and E1.

Alphavirus E2, which functions in virus entry, is associated with neutralizing antibody and is less conserved than E1. E2 contains four to eight unique epitopes, depending on alphavirus species, that elicit neutralizing and hemagglutination inhibiting antibodies (Mendoza et al., 1988). Alphavirus E1 directed antibodies are typically hemagglutination inhibiting and non-neutralizing, but are often cross-reactive since E1 is more conserved among alphaviruses than E2 due to its role in viral fusion. Four to six epitope sites have been identified for E1 (Mendoza et al., 1988).

Neutralizing and non-neutralizing antibodies directed towards both E2 and E1 have been reported to be highly protective in challenge models. Accordingly, unaided virus neutralization is not the mechanism by which all alphavirus antibodies protect (Griffin, 1995). Interestingly, the ability of antibody to bind to the surface of infected cells is correlated with protection or recovery from alphavirus challenge. Antibodies that protect well when administered prophylactically do not necessarily protect when given therapeutically and vice a verse (Mendoza et al., 1988). For this reason, more than one antibody mechanism is likely; however, the precise mechanisms by which antibody protects or promotes recovery from alphavirus disease are not known (Griffin, 1995). Description of the mechanism through which non-neutralizing antibodies protect has been particular elusive. Enhanced uptake by non-permissive macrophages, inhibition of virion maturation, alteration of intracellular virus replication, inhibition of virus budding, and restoration of cellular ion flux have all been implicated (Griffin, 1995; Mendoza et al., 1988).

For alphavirus vaccine development, E2 neutralizing antibodies have primarily been the standard indicator of protection with 80% plaque reduction neutralization titers (PRNT80) of >20-40, depending on virus species, indicative of vaccine responders with antibody titers sufficient for prevention of

infection (Reisler et al., 2012). However, as information has become available on the safety and immunogenicity of the currently available investigational-new-drug (IND) inactivated and attenuated alphavirus vaccines, alternative vaccines have been developed utilizing a variety of vaccine platforms and alphavirus antigens. Importantly, in recent years there has been a push for broad or universal vaccines that offer protection from all strains of a given virus (Dorans, 2009; He et al., 2015) or even all viruses within a particular group. Current IND status alphaviruses vaccines only produce antibodies reactive to the viral vaccination species and sometimes do not even protect against all strains within that species. Additionally, antibodies derived from alphavirus infection or vaccination are known to interfere with the production of novel alphavirus antibodies resulting from a secondary infection or vaccination, an occurrence termed antibody interference (Reisler et al., 2012). Antibodies directed towards alphavirus E1 are known to be cross-reactive against many alphavirus species, alphavirus E1 directed vaccines may offer pan-alphavirus protection (Hunt and Roehrig, 1985; Wust et al., 1989).

Vaccines

In 1796, Edward Jenner demonstrated that smallpox could be prevented by previous inoculation with the less virulent cowpox virus. Unfortunately, since the theory of spontaneous generation had yet to be rejected, the principles gleaned from Jenner's cowpox experiments were left untouched for a nearly a century before the next vaccine appeared. The next vaccine to significantly impact human health was Louis Pasteur's attenuated rabies vaccine in 1885. The science of vaccine development took off in the 1930's with the development of useful diphtheria, tetanus, anthrax, cholera, plague, tuberculosis, and yellow fever virus vaccines; the latter facilitated by the development of tissue culture techniques. The modern vaccine era has led to vaccines for polio, measles, mumps, and rubella. Today, vaccine usage prevents three million deaths worldwide each year. Modern vaccines are divided into inactivated, attenuated, DNA, recombinant vectored, and subunit formulations (Hilleman, 2000).

Alphavirus vaccines

Regrettably, there are no licensed alphavirus vaccines available for human use in the United States (US). However, IND status vaccines against some alphaviruses are available to at risk individuals through the special immunizations program (SIP). However, poor safety profiles and limited immunogenicity restricts their broad use. Additionally, several multivalent EEEV/VEEV/WEEV inactivated and attenuated vaccines are available for use in horses. All of these vaccines have gone through extensive testing and demonstrated acceptable safety profiles and response rates.

Five alphavirus IND status vaccines have been administered to laboratory workers and other at risk personnel at the US Army Medical Research Institute of Infectious Diseases (USAMRIID) to prevent CHIKV, EEEV, VEEV, or WEEV infections.

Venezuelan equine encephalitis virus IND vaccination, as part of the SIP, involves primary vaccination with the TC-83 live-attenuated vaccine and a booster vaccination using the C-84 inactivated vaccine if PRNT80 titers are <20 following the primary TC-83 dose. The TC-83 vaccine has been in use since 1963 is protective and induces long-lasting antibodies. However, 15-30% of recipients experience febrile illness and viral shedding, permitting for potential viral reversion to virulence and mosquito transmission. Moreover, approximately 25% of vaccinees fail to seroconvert (Erwin-Cohen et al., 2012). The inactivated C-84 vaccine, derived from the TC-83 vaccine strain, has a better safety profile with no reported febrile reactions, but provides only limited protection and requires repeated boosters. When given as a series, C-84 safely boosts antibody derived from primary TC-83 vaccination and boosts vaccine response rates to over 90% (Edelman et al., 1979). Twenty-eight days post vaccination with the TC-83/C-84 series, PRNT80 titers of >20 are indicative of immunity. Though the current VEEV vaccine regimen has proven efficacy, demonstrated by a reduction in laboratory-acquired infections, it is unable to meet the reaction ($<5\%$) and seroconversion response ($>95\%$) rates required for approval (Plotkin et al., 2013).

Inactivated EEEV and WEEV vaccines are also available through the SIP. The WEEV vaccine, TSI-GSD-210, is given as three doses on days 0, 7, and 28 and has a response rate of 41% with adverse

events occurring uncommonly. Similarly the EEEV vaccine, TSI-GSD-104, is given twice on days 0 and 28 and has response and reaction rates of 77% and <1%, respectively (Plotkin et al., 2013).

Lastly, a CHIKV IND vaccine has also been given as part of the SIP. The vaccine, TSI-GSD-218, is a live, attenuated vaccine that is well tolerated and highly immunogenic, resulting in 98% seroconversion in vaccinees following one immunization. Transient arthralgia is observed in approximately 8% of recipients (Edelman et al., 2000). With seroconversion rates nearing 100%, recipients are considered immune four weeks after vaccination. Reversion to virulence and the possibility of mosquito transmission has been considered and deemed unlikely to occur.

The vaccines offered through the SIP fluctuate and as of January 14, 2014, USAMRIID is only offering EEEV and VEEV vaccines through the SIP. Unfortunately, the safety profiles and reaction rates of the discussed alphavirus IND status vaccines have prevented their expanded use and licensure. None of the alphavirus IND status vaccines is able to protect against more than one alphavirus species, and experience has demonstrated that prior alphavirus immunization decreases the ability of individuals to mount immune responses to subsequent alphavirus species. As mosquito populations and range expand yearly due to climate change and as regions where multiple alphaviruses are circulating become more common, the need for broad alphavirus vaccines has increased, and driven the development of the next (second) generation of vaccines. Importantly, vaccines targeting one virus have become obsolete and future second generation vaccines will require extended coverage to multiple alphaviruses.

Alphavirus inactivated and attenuated vaccines

A decreased emphasis has been placed on the development of second generation alphavirus attenuated and inactivated vaccines due to safety and immunogenicity concerns, respectively; however, several recent attempts have been made using novel approaches.

Next-generation, attenuated vaccines must rely on more than a few unstable, attenuating mutations to disallow reversion to wild-type and to ensure safety. Likewise, attenuated vaccines should be non-permissive to mosquito replication. The current TC-83 vaccine strain meets none of these requirements: it relies on only two attenuating mutations, is known to revert to virulence in vaccinees, and

retains the ability to replicate in mosquitoes as indicated by its isolation from mosquitoes following its use to halt the 1972 Texas VEEV outbreak (Pedersen et al., 1972).

Next-generation, attenuated alphavirus vaccines have focused on internal ribosome entry site (IRES)-based attenuation or gene deleted mutants. The IRES-based candidates rely on the replacement of the alphavirus subgenomic promoter with mammalian IRES elements. The resulting viruses are highly attenuated, safe, deficient in invertebrate replication, and protective in a variety of animal models. IRES-based attenuation strategies have been applied to CHIKV (Roy et al., 2014), EEEV (Pandya et al., 2012), and VEEV (Volkova et al., 2008) vaccine development. Gene-deleted mutant attenuation relies on site directed mutagenesis of alphavirus genomes. The VEEV gene deletion mutant, V3526, is attenuated by deletion of the PE2 furin cleavage site and mutation of amino acid 253 of E1. V3526 elicits protection in mice, hamsters, and non-human primates towards many VEEV strains, is unable to revert to virulence, and has limited transmission potential in mosquitoes (Carossino et al., 2014). Alternatively, alphavirus replicon particles, gene deletion mutants lacking the majority of the structural polyprotein, have been developed and used for vaccination against a broad array of pathogens (Davis et al., 2002; Herbert et al., 2013), including alphaviruses (Chang et al., 2014).

The primary difficulty with the current inactivated alphavirus vaccines is their limited cross-reactivity and requirement for repeated vaccination. These problems are typified in both the EEEV (TSI-GSD-104) and WEEV (TSI-GSD-210) vaccines. These vaccines have low rates of seroconversion, require frequent booster vaccinations, and are poorly cross-reactive to heterologous strains (Plotkin et al., 2013).

Next-generation, inactivated alphavirus vaccines have depended on the addition of adjuvants to vaccine formulations. An inactivated RRV vaccine has recently been formulated and tested in humans. The RRV vaccine is formalin and UV-light inactivated and adjuvanted with aluminum hydroxide and given at a dose of 2.5 μg at weeks 0, 3, and 24 in humans. This formulation is well tolerated with 92% of recipients seroconverting (Aichinger et al., 2011). Similarly, adjuvanted, CHIKV and VEEV inactivated

vaccines have been formulated and tested with comparable seroconversion and reactivity rates in mice (Tiwari et al., 2009).

Considerable progress has been made in the development of next-generation, attenuated and inactivated alphavirus vaccines. These new vaccine formulations address the safety and immunogenicity concerns that arose from usage of IND status vaccines. Although improvements have been made these vaccines are unable to protect against more than a single alphavirus species, and are associated with high manufacturing costs (Barrett and Stanberry, 2008; Pamela, 2011).

Alphavirus DNA vaccines

Due to the inability of the alphavirus IND status vaccines to be licensed, several alphavirus DNA vaccines have been formulated. DNA immunization offers inherent benefits for the development of next-generation alphavirus vaccines including: safety, cost effectiveness, and a diminished need to propagate and inactivate pathogen.

Nagata et al. (2005) showed that DNA vaccines may be a promising vaccine approach against WEEV by expressing the 26S region from WEEV strain 71V-1658 as part of a DNA vaccine plasmid. The vaccine plasmid, pVHX-6, was administered intraepidermally (5 µg x 4 doses, 2 weeks apart) to BALB/c mice via gene gun. Two weeks following the final boost vaccination, mice were challenged with homologous or heterologous strains of WEEV. Complete protection was observed for 71V-1658 challenge; whereas, mice challenged with heterologous WEEV strains, Fleming and CBA87, were not completely protected with 62% and 50% survival, respectively. Importantly, this study established that DNA vaccines could be applied to alphaviruses. Guaci et al. (2010) further investigated which structural proteins were responsible for WEEV protection. Using the pVHX-6 DNA vaccine plasmid, three additional DNA vaccines encoding different portions of the 71V-1658 structural reading frame were constructed. A pE3-E2-6K-E1 vaccine provided the best level of protection slightly outperforming the original pVHX-6 vaccine, a 6K-E1 vaccine provided similar levels of protection as the pVHX-6, and lastly, an E3-E2 vaccine provided no protection.

DNA vaccination has also been examined for protection against VEEV and CHIKV. Similar to the WEEV DNA vaccine, the initial VEEV DNA vaccine expressed the full 26S structural gene (VEEV_{WT}) of VEEV strain Trinidad and was administered by particle-mediated epidermal delivery. The vaccine has been tested for efficacy in mice, guinea pigs, and macaques. Mice were fully protected from homologous subcutaneous challenge and 80% protected against homologous intranasal challenge (Riemenschneider et al., 2003), guinea pigs were completely protected from homologous subcutaneous challenge (Riemenschneider et al., 2003), and macaques were partially protected from intranasal challenge after VEEV DNA vaccination (Dupuy et al., 2010). Following these studies, the construct was codon optimized with (VEEV_{COCAP}) or without (VEEV_{CO}) the capsid gene and given to mice and macaques via intramuscular electroporation (IM EP). Both constructs resulted in increased survival in mouse studies and decreased clinical signs in macaque studies (Dupuy et al., 2011). Whether this difference was due to codon optimization or delivery method is unknown. Lastly, as was seen with the WEEV DNA vaccination study, the authors found that VEEV_{CO} vaccination elicited better immune responses than VEEV_{COCAP} possibly due to the transcription and translation inhibiting effects of capsid (Dupuy et al., 2011; Gauci et al., 2010). The CHIKV DNA vaccine also consisted of the envelope proteins and was protective in a mouse challenge model and drove neutralizing antibody titers to relevant levels in a macaque model system (Mallilankaraman et al., 2011).

Alphavirus DNA vaccination offers many advantages compared to traditional attenuated and inactivated vaccines, and can provide protection similar to that of TC-83 and C-84. Importantly, DNA vaccines do not require propagation and inactivation of pathogen and have improved safety profiles; however, many factors still prevent their human usage and licensure. The biggest hurdle for the licensing of alphavirus DNA vaccines is their limited cross protection. Current alphavirus DNA vaccines are only semi-efficacious at protecting against challenge with heterologous strains of virus, and none have been tested for the ability to protect against different alphavirus species. Presently, broad alphavirus vaccines are sought; therefore these alphavirus DNA vaccines would require expanded coverage before further consideration. Electroporation of alphavirus DNA vaccines, as opposed to gene gun delivery, allows for

the accommodation of multiple vaccines in a single administration and may present a feasible method for increasing the reactivity of current alphavirus DNA vaccines.

Recombinant vectored vaccines

Recombinant vectored vaccines are alternatives to attenuated and live-inactivated vaccines. This vaccine platform offers the advantages of improved safety; single dose, fast acting protection; inexpensive manufacturing; and are typically incapable of mosquito transmission (Barabe et al., 2007). A variety of recombinant vectored vaccines against alphaviruses have been tested in mice and macaques. Prominent among tested recombinant vectored vaccines are adenovirus-vectored vaccines, alphavirus-vectored vaccines, and measles-vectored vaccines.

Recombinant adenoviruses are an alternative recombinant vectored approach for alphavirus vaccine development. Recombinant adenovirus vaccines have many advantages: they are safe for human use, have no potential for reversion to virulence, mimic natural infection, stimulate mucosal immunity, and are cost effective. Additionally, regarding the use of recombinant adenoviruses for alphavirus vaccination, they are incapable of mosquito replication and transmission. Several recombinant adenovirus-vectored alphavirus vaccines are reported in the literature. Phillpotts et al. (2005) developed VEEV vaccines consisting of defective type 5 adenovirus and the E3E26K structural genes of VEEV strain Trinidad. The vaccine, delivered IN, established a mucosal immune response and protected against homologous challenge, but was less effective against heterologous challenge. Wu et al. (2007) employed a similar approach for WEEV. Ad5-WEEV vaccinated mice generated neutralizing antibody and were completely protected from lethal homologous challenge. Passive transfer of Ad5-WEEV immune serum to naïve mice provided partial protection from WEEV infection, highlighting the importance of humoral immunity during alphavirus infection.

The attenuation of chimeric alphaviruses was first realized during studies using SINV/RRV chimeric viruses to study interactions between non-structural and structural regions of alphavirus genomes (Kuhn et al., 1996). Since this discovery, chimeric alphaviruses have been used as alphavirus-vectored vaccine constructs for CHIKV, EEEV, VEEV, and WEEV. Alphavirus-vectored vaccine

constructs typically possess the non-structural genes of SINV, a non-pathogenic human alphavirus, and the structural genes of a pathogenic human alphavirus. A SINV/VEEV alphavirus-vectored vaccine, SIN-83, containing SINV non-structural genes, with a mutation in nsP2, and the structural genes of VEEV strain TC-83 was highly protective in a lethal mouse model and did not cause detectable illness in adult or newborn mice (Paessler et al., 2003). Wang et al. (2007) also constructed alphavirus-vectored vaccines against North American (NA) and South American (SA) strains of EEEV. These vaccines relied on SINV for the non-structural genes while the structural genes were derived from either EEEV strain FL93 (NA) or BeAr436087 (SA). Both constructs were highly attenuated in mice, but resulted in the development of high titer antibodies and protection from lethal challenge. Significantly, SINV/SAEEEEV was able to fully cross-protect mice against NA EEEV challenge while providing a better safety profile; however, this cross-protection was not observed in non-human primates, though SINV/NAEEEEV retained 82% protection against NA EEEV strains in non-human primates (Roy et al., 2013). Using a similar approach, three chimeric CHIKV alphavirus-vectored vaccines were constructed and tested: SIN/CHIK, SAEEEE/CHIK, and VEE/CHIK (Wang et al., 2008). All vaccines were immunogenic and resulted in the production of neutralizing antibody and minimization of viremia in challenged mice. The CHIKV chimeras containing VEEV and EEEV backbones were slightly more immunogenic than the chimera containing a SINV backbone. Due to the documented attenuation of SA EEEV strains, the authors propose SAEEEE/CHIK as the most promising of these vaccine candidates and are conducting further studies. Lastly, WEEV alphavirus-vectored vaccines have also been formulated. Atasheva et al. (2009) developed three WEEV SINV-vectored vaccines. The most efficacious vaccine, SIN/EEE/WEE, provided better protection in mice than an adenovirus vaccine (Barabe et al., 2007). SIN/EEE/WEE contained SINV non-structural protein genes, the amino-terminal domain of EEEV capsid, the carboxy-terminal domain of WEEV McM capsid, and WEEV McM envelope glycoproteins. This vaccine was highly immunogenic and offered complete protection against IN heterologous challenge.

Recombinant measles virus vaccines expressing alphavirus-like particles are a viable alternative to traditional means of alphavirus vaccine development. As one of the safest and most effective human

vaccines available, the measles virus vaccine is produced at low cost and elicits life-long immunity with a single dose. Recombinant measles viruses have been adapted to vaccine manufacturing and have demonstrated efficacy against arboviruses (Brandler et al., 2012; Brandler et al., 2010). A recombinant measles vaccine expressing the CHIKV structural polyprotein (MV-CHIKV) has been developed and tested in mice, non-human primates, and humans with promising results (Brandler et al., 2013; Ramsauer et al., 2015). Vaccination with MV-CHIKV in phase 1 human trials resulted in 44%, 92%, and 90% seroconversion in low, medium, and high dose groups, respectively. Booster vaccination resulted in 100% seroconversion regardless of dosage group. Seroconversion was independent of anti-measles immunity and resulted in cross neutralizing antibody responses. No serious vaccine-related adverse events were recorded. MV-CHIKV vaccine will be further tested in larger phase 2 human trials.

Recombinant vectored vaccines are a promising approach to alphavirus vaccine development. Significantly, recombinant vectored vaccines are inexpensive, cross-reactive, and do not allow for mosquito replication. The largest obstacles to the further development of recombinant vectored vaccines are pre-existing immunity, which has been highly reported with adenovirus-vectored vaccines, and residual virulence of the attenuated viruses. Overcoming these difficulties, some recombinant vectored vaccines are entering larger human trials and hold potential as future alphavirus vaccines.

Alphavirus subunit vaccines

Lastly, alphavirus subunit vaccines are alternatives to the current IND status vaccines. Since alphavirus E2 and E1 are highly immunogenic and antibodies directed towards both are highly protective in animal models, these proteins have been investigated for use in alphavirus subunit vaccines. Subunit vaccines, like DNA and recombinant vaccines, offer the advantages of a diminished need for virus propagation and a better safety profile when compared to traditional inactivated and attenuated vaccines.

Western equine encephalitis virus E2 and E1 have been expressed and purified from *Escherichia coli* (*E. coli*) and used in vaccination studies of mice (Das et al., 2004; Das et al., 2007). While both antigens stimulated humoral and cell-mediated immune responses, they demonstrated little or no ability to protect mice from WEEV challenge. *Escherichia coli* derived E1 immunization resulted in 25%

protection towards homologous WEEV challenge and 0% protection towards heterologous challenge; whereas; *E. coli* derived E2 vaccination resulted in 37.5% protection towards homologous challenge and 12.5% protection towards heterologous challenge. Significantly, the low levels of protection stimulated by antigen vaccination could be due to the lack of glycosylation of *E. coli* expressed proteins.

To assess the utility of baculovirus-expression systems for the expression of alphavirus antigens, Hodgson et al. (1999) expressed, purified, and tested the immunogenicity of baculovirus-derived VEEV antigens. Six VEEV expressing baculovirus constructs were tested: C-E3-E2-6K-E1, E3-E2-6K-E1, E3-E2-6K-E1(T), C, E3-E2, and 6K-E1. Mice immunized with baculoviruses-expressing VEEV C-E3-E2-6K-E1, E3-E2-6K-E1, E3-E2-6K-E1(T), or 6K-E1 antigens mounted antibody responses and were protected from lethal VEEV challenge demonstrating that baculovirus-expressed alphavirus proteins are effective antigens capable of protecting mice from lethal challenge.

Toth et al. (2011) expressed and purified recombinant WEEV glycoproteins in the baculovirus expression system. A variety of constructs were tested for protein production including E1, E1ecto, E26KE1, and E2E1chim each under three different baculovirus promoters P_{ie1} (immediate early), $P_{p6.9}$ (late), and P_{polh} (very late). All WEEV proteins were efficiently expressed though the timing of expression and nature of the construct both impacted production. In later studies, E1ecto was used to immunize mice and prophylactic efficacy was assessed (Phillips et al., 2014). The most efficacious WEEV E1ecto-based vaccine consisted of liposomes, dsRNA (0.1 mg/mL), CpG DNA (0.1 mg/mL), and WEEV E1ecto (50 μ g/mL) (lipid-antigen-nucleic acid complexes: LANACs) delivered as a 200 μ L dose at days 0 and 14. This LANAC WEEV E1 formulation protected 100% of mice from homologous, lethal WEEV challenge regardless of challenge route: SC, IN, or mosquito. Significantly, LANAC WEEV E1 vaccination was, additionally, completely protective against heterologous strains of WEEV and highly protective against EEEV. Antibody derived from LANAC WEEV immunization was not neutralizing. Importantly, *in vivo* imaging of vaccinated animals utilizing recombinant luciferase-expressing WEEV showed that vaccination completely prohibited viral replication in the absence of neutralizing antibody. Passive

transfer of serum from vaccinated mice completely protected naïve mice from lethal disease, demonstrating that E1 antibody is sufficient for protection.

Like all of the vaccine approaches discussed here, subunit vaccines have inherent advantages and disadvantages. High rates of cross-reactivity, the lack of virus propagation, and safety profiles rivaling inactivated vaccines are among the most prominent advantages offered by subunit alphavirus vaccination. Whereas, the high costs of antigen expression and purification and the need for adjuvants are key disadvantages to subunit vaccine approaches.

Therapeutics for viral diseases

Interest in the development of therapeutic compounds for the treatment of viral diseases has increased markedly in recent years; the pace of development now rivals the pace at which antibiotics were developed 30 years ago. Contributing factors to the increased demand for therapeutics include: the emergence and re-emergence of viruses, increasing numbers of chronically infected individuals, and viral diseases that have proven non-permissive to vaccine approaches (Littler and Oberg, 2005). Additionally, the threat of bio-warfare has fueled therapeutic drug discovery (Littler and Oberg, 2005).

To date, there are still only a few U.S. Food and Drug Administration (FDA) approved drugs to treat viral infection (<100). Moreover, the current viral therapeutic arsenal predominantly targets herpes, hepatitis, human immunodeficiency, and influenza virus infections. These therapeutics are not always efficacious or well-tolerated and, importantly, the emergence of viral resistance is limiting their use. Accordingly, there is a high demand for the refinement and expansion of the available therapeutics with which to treat viral infections.

History of viral therapeutic development

Unlike the early discovery of antibacterials in the 1930's, the first therapeutics to treat viral diseases were slow to appear and did not enter clinical use until the 1960's. The first two compounds were Methisazone for the prophylaxis and treatment of smallpox and idoxuridine for the treatment of herpetic keratitis (Bauer, 1985).

The discovery of Methisazone occurred as a chance event. In 1951, after devising a method to detect antiviral activity in chicken embryos, Browlee and Hamre conducted their first antiviral tests with p-aminobenzaldehyde thiosemicarbazone (Brownlee and Hamre, 1951). Fortuitously, this antibacterial compound was active against vaccinia, causing a 70% reduction in the infecting dose of virus. Further optimization by Thompson et al. (1953), Bauer (1955), and Bauer and Sadler (1960) identified 1-methylisatin 3-thiosemicarbazone (Methisazone) as the lead compound for investigation in man. In 1962, Methisazone was used to treat a seven month old boy suffering from infantile eczema, perhaps marking the first time an antiviral drug was used in man (Turner et al., 1962). Over the next few years, Methisazone was used in the treatment of eczema vaccinatum, vaccinia gangrenosa, alastrim, and smallpox each with marked success (Bauer, 1985).

At the same that the Methisazone trials were being conducted, another therapeutic compound, idoxuridine (IDU), was being studied. Unlike the serendipitous discovery of Methisazone, idoxuridine resulted from a directed research program aimed at developing DNA synthesis inhibitors for the treatment of neoplasia. While IDU failed as an antineoplastic agent, Kaufman (1962) tested it for effect against DNA viruses and demonstrated that IDU could cure herpetic keratitis in 1962.

Importantly, the discovery of these therapeutics for the treatment of viral disease came before the advent of cell culture techniques and were, consequently, developed by experiments in animal models. The advent of cell culture methods in the early 1940s and 1950s fueled virology and the search for further therapeutic compounds.

The next breakthrough in therapeutic development came with the detection of the broad spectrum interferons (IFNs). Discovered by Isaacs and Lindenmann (1957), the IFNs are an early local defense mechanism. Twenty years after its discovery, the role of IFN in combating viral infection and inducing an antiviral state was confirmed. The IFNs have subsequently been used for the treatment of hepatitis B and hepatitis C virus infections. Despite its capacity as a broad spectrum viral therapeutic, the discovery and application of IFN in human and animal therapy has been anticlimactic.

Between 1960 and 1980, therapeutic development for the treatment of viral diseases greatly expanded and focused primarily on the herpesviruses. Cytarabine, trifluorothymidine, vidarabine, and acyclovir, all synthetic nucleosides, resulted from the attack on herpesviruses. Importantly, vidarabine was the first therapeutic to successfully treat herpes simplex encephalitis (Whitley et al., 1977). Outside of the attack on herpesviruses, amantadine hydrochloride became the first therapeutic drug directed towards an RNA virus (Davies et al., 1964) and ribavirin was the first broad spectrum therapeutic discovered after IFN (Sidwell et al., 1972).

In 1983, the virus that would become known as human immunodeficiency virus (HIV) was first isolated. With this discovery, the direction of therapeutic development would be permanently altered. Four years after the discovery of HIV, the FDA approved the first HIV therapeutic, azidothymidine (AZT), only 25 months after the first reports of its anti-HIV activity. The next 25 years would result in an additional 24 HIV therapeutics being approved for use (De Clercq, 2010). Despite an abundance of HIV therapeutics, a cure for HIV has been elusive due primarily to therapeutic resistance and viral latency. Currently, combinatorial therapy, or highly active anti-retroviral therapy (HAART), offers the most promise for a HIV cure.

Today, HIV and herpesvirus therapeutics still dominate the arsenal of drugs to treat viral infections. However, therapeutic drugs are available for some of the hepatitis and influenza viruses.

Classification of therapeutic drugs

Therapeutic drugs to treat viral infections are split into two major categories: antivirals, which target the virus, and immunomodulators, which augment the host's response to infections. Typically, virus-targeted therapeutics are highly specific, whereas therapeutics that target the host response act more broadly. Regardless of whether a therapeutic is targeting the host or virus, therapeutic drugs must have the following characteristics: they must be able to reach the target organ of the virus, must be chemically and metabolically stable, must specifically inhibit virus function without affecting host functions, should be readily absorbed, and should not be toxic, carcinogenic, allergenic or mutagenic (Saxena et al., 2010).

Immunodulatory therapeutics- Interferon

The IFNs are a class of broad spectrum therapeutic agents that protect cells from infection with viruses of all kinds. The IFNs are broken into three major classes IFN α , β , and γ . Of the IFNs, IFN- α has been used extensively as a viral therapeutic; whereas IFN- β and IFN- γ have been used to a lesser extent. The IFNs, as part of the innate immune defense, are produced after the detection of pathogen associated molecular patterns within the body. After their induction, the IFNs induce an antiviral effect through the production of multiple proteins with actions ranging from immunological to anti-proliferative to antiviral in nature (Friedman, 2008). Due to the multifactorial nature of interferon, little is known regarding the mechanism of action of the interferons (Friedman, 2008). The clinical application of IFN involves the exogenous application of IFN after symptom onset and diagnosis. Because IFN is not administered until after symptom onset, IFN has proven relatively ineffective for the treatment of acute viral infections; however, IFN therapy is often conducive to recovery and viral clearance in the case of severe acute infections and persistent viral infections (Main and Handley, 1992). Despite positive indications in the treatment of persistent infections, IFN therapy was slow in coming to use and it was not until recently that IFN has established itself as a potent antiviral agent. The interferons are currently used in the treatment of a variety of infections including hepatitis B, C, and D; human herpesvirus-8; and human papilloma virus infections (Friedman, 2008). In future years, IFN therapy, alone or in combination with other therapeutics, will likely be used in the treatment of a variety of additional infectious diseases (Main and Handley, 1992).

Immunodulatory therapeutics- Passive antibody therapy

Passive antibody therapy or serum therapy has been used to treat infections since the 1890's and consists of the administration of immune serum from one individual or animal to another who lacks protective antibody. Antibody therapy results in protection by a variety of mechanisms, both direct and indirect, including: pathogen neutralization, inhibition of pathogen attachment, complement activation, opsonization, and the generation of antioxidants (Casadevall and Scharff, 1995). For antibody therapy to be successful both accurate and rapid identification of the pathogen are necessary. Though antibody

therapy has been in use since the late 1800's, its use was limited in the early 1900's due to the advent of chemotherapy. Today, antibody therapy is still used for the treatment of botulism, rabies, diphtheria, and tetanus among other non-infectious illnesses (Casadevall et al., 2004). Importantly, with the increasing resistance of pathogens to antivirals and the inefficiency of treating immunocompromised patients with chemotherapy, antibody therapy is expected to be reconsidered (Casadevall et al., 2004).

Antiviral therapeutics- Ribavirin

Ribavirin is a broad spectrum therapeutic agent inhibiting a range of DNA and RNA viruses *in vitro* including influenza, measles, and Lassa fever among others. *In vivo*, ribavirin antiviral activity is limited to RNA viruses. Since its synthesis in 1972, the mode of action underlying the antiviral activity of ribavirin has been sought. At present there are five proposed mechanisms of action for ribavirin inhibition, depending on the virus studied: 1) indirect immunomodulation, 2) lethal mutagenesis, 3) inhibition of RNA capping, 4) direct inhibition of viral RNA polymerases, and 5) depletion of intracellular GTP pools. The multiple mechanisms of action of ribavirin have been a dual edged sword preventing the development of significant ribavirin resistance but thwarting the development of more efficacious derivatives at the same time. Unfortunately, these factors have led to ribavirin failing to live up to its original hype. Today, ribavirin is only used in monotherapy against Lassa fever and severe respiratory syncytial virus infection. However, ribavirin has recently found a niche in the treatment of chronic hepatitis C infection in combination therapy with IFN- α . In the future, ribavirin may be the most useful as a combinatorial drug, enhancing the effects of more potent antivirals while limiting the development of resistance to them.

Therapeutic drug development

Therapeutic drug development for viral diseases is a billion dollar industry in which individual drugs take approximately 10-15 years and one billion dollars to reach the market. The process of therapeutic drug development is a multiple step process involving: target identification/screening, lead generation and optimization, preclinical and clinical studies, and registration of the drug.

Though the process is different for each therapeutic developed, critical steps in therapeutic drug development include: demonstration of the ability of the drug to inhibit the virus *in silico*, *in vitro*, and *in vivo*, description of the mechanism of action of the therapeutic drug, optimization of the drug, determination of the drugs toxicity *in vitro* and *in vivo*, and registration of the drug.

Prospects for future antiviral development

Due to the high costs associated with drug development, the number of viral diseases amenable to antiviral development has historically been restricted. Predominantly, viral diseases for which therapeutics have been developed are long in duration, high in prevalence, and are typically not vaccine preventable. As opposed to “one-bug-one-drug” antivirals, broad-spectrum therapeutics continue to hold promise. Today, improved technologies for therapeutic development, the emergence/re-emergence of viral diseases, and the threat of bioterrorism are each extending the list of targets for therapeutic drug development.

Historically, therapeutic development has been plagued by a myriad of difficulties ranging from poor economy in screening to the development of resistance after a drug has been implemented. Modern technologies are rapidly improving the efficiency with which drugs are developed and administered. High throughput screening and intelligent drug design have replaced the once serendipitous trial and error identification of compounds; whereas, combinatorial drug regimens have reduced the development of drug resistance while increasing antiviral efficacy and limiting toxic side effects. Unfortunately, even with these advances in drug development, we still require significant progress before effective measures to combat viral disease are attained.

Alphavirus therapeutics

Similar to the current state of alphavirus vaccines, there are currently no alphavirus therapeutics approved for the treatment of alphavirus infections and supportive care is indicated. Supportive care for non-neuroinvasive infections includes: fluid management and the administration of analgesics. For neuroinvasive infections, anticonvulsants, management of intracranial pressure, and respiratory

management are, additionally, indicated. The development of alphavirus therapeutics is urgently needed due to the re-emergence of CHIKV and the threat that the encephalitic alphaviruses pose as bioweapons.

Despite the lack of any approved alphavirus therapeutic, many chemotherapeutic agents have been reported as effective inhibitors of alphavirus replication *in vitro* or in small animal models. Both antiviral and immunomodulatory agents have been identified. Potential antiviral therapeutics include: ribavirin, chloroquine, and siRNAs; while, IFN- α , poly(I:C), and antibody are potential immunomodulatory therapeutics (Parashar and Cherian, 2014). Unfortunately, many of these potential therapeutics have failed to show efficacy when used in human clinical studies (Parashar and Cherian, 2014). Today, the prevention and treatment of alphavirus disease is limited to mosquito control to prevent infection and supportive care following infection; however, recent advances in therapeutic drug development along with a rise in global alphavirus infection suggest that an alphavirus therapeutic might be on the horizon.

Viral infections of the central nervous system

Viral infections of the CNS are important causes of morbidity and mortality worldwide. Neurotropic viruses have been identified in most viral families including the herpesviruses, enteroviruses, retroviruses, coronaviruses, rhabdoviruses, alphaviruses, flaviviruses, bunyaviruses, arenaviruses, and paramyxoviruses (Koyuncu et al., 2013). Symptoms of neuroinvasive virus infection include but are not limited to paralysis, dementia, seizures, coma, and death. Moreover, survivors of CNS infection typically develop neurological sequelae following acute infection recovery.

Routes to the central nervous system

In order to invade the CNS, neurotropic viruses must overcome several physical layers of protection; this process is known as neuroinvasion. Neuroinvasion occurs through two predominant routes: through the blood and through the peripheral nerves.

Hematogenous routes of neuroinvasion

Spread of virus from the blood into the CNS is considered the hematogenous route of neuroinvasion. Several mechanisms of hematogenous spread have been identified all of which involve usurping the blood brain barrier (BBB) (Johnson, 1982). The mechanisms are: entry through infection of

the brain microvascular endothelium, invasion via inflammation-induced breakdown of the BBB, and entry through infection of leukocytes (Johnson, 1982).

The BBB serves as a vascular-tissue barrier preventing the movement of harmful substances, including viruses, from the blood into the CNS. The BBB is more effective than other vascular-tissue barriers at prohibiting the movement of substances due to tight junctions formed between brain microvascular endothelial cells at this interface. The glia limitans, consisting of astrocyte foot processes and the parenchymal basement membrane, forms an additional barrier between the blood and CNS at the BBB. Together, these barriers prohibit the entry of most bacteria, viruses, and large proteins into the CNS while allowing for the maintenance of homeostasis within the CNS (Johnson, 1982).

Entry through infection of the brain microvascular endothelium

A variety of viruses are able to gain access to the CNS via infection of brain microvascular endothelial cells. Hematogenous spread of viruses into the CNS via brain microvascular endothelial cells involves several steps: 1) entry into the host, 2) growth in extraneural tissues, 3) maintenance of viremia, 4) infection of brain microvascular endothelium, and 5) exit of progeny virus into the CNS and viral disruption of BBB integrity (Johnson, 1982). Many viruses are proposed to gain access to the CNS through infection of brain microvascular endothelial cells, including WNV and human cytomegalovirus (Johnson, 1982).

Invasion via inflammation-induced breakdown of the blood brain barrier

Alternative to the direct viral infection of the brain microvascular endothelium, viruses can gain access to the CNS through systemic viral replication and immune-mediated perturbation of the BBB (Koyuncu et al., 2013; Schafer et al., 2011). Neuroinvasion following systemic viral replication occurs through the expression and secretion of pro-inflammatory cytokines. Local production of cytokines in the CNS disrupts the tight junctions of the BBB and allows for virus to slip into the CNS. Viruses capable of neuroinvasion through the immune-mediated route include: influenza A virus, mumps virus, measles virus and VEEV (Johnson, 1982; Koyuncu et al., 2013; Schafer et al., 2011).

Entry through infection of leukocytes

Another hematogenous route of invasion into the CNS is through the infection of circulating leukocytes. This mechanism of CNS entry is often referred to as “Trojan horse” entry since virus is hidden within leukocytes and transported into the CNS (Koyuncu et al., 2013). Canine distemper virus, HIV, simian immunodeficiency virus, and enterovirus 71 all enter the CNS by infecting circulating leukocytes (Johnson, 1982).

Trans-neural routes of neuroinvasion

Spread of virus into the CNS directly through peripheral or olfactory nervous tissue is another mechanism of neuroinvasion. Peripheral and olfactory nervous system tissue, unlike CNS tissue, is in direct contact with peripheral tissues and is more amenable to viral infection. Mechanisms of trans-neural neuroinvasion involve the direct infection of sensory, motor, and olfactory nerves.

Direct infection of sensory and motor nerves

Direct infection of peripheral nerves is one means by which viruses can enter the nervous system (Johnson, 1982; Koyuncu et al., 2013). Sensory nerve endings are access points through which viruses can gain access to the peripheral nervous system. Infections resulting from sensory nerve ending infection are typically limited to the peripheral nervous system though CNS invasion is seen rarely. Viruses entering the peripheral nervous system via the invasion of sensory nerve endings include the alphaherpesviruses (Johnson, 1982; Koyuncu et al., 2013). Alternatively, some neurotropic viruses enter the CNS through the invasion of motor neurons at neuromuscular junctions followed by retrograde neuronal spread to the CNS (Johnson, 1982; Koyuncu et al., 2013). Rabies virus and poliovirus both use neuromuscular junctions as access points to invade the CNS (Johnson, 1982; Koyuncu et al., 2013).

Direct infection of olfactory nerves

Viral invasion of the CNS through the olfactory nerves is an additional route of trans-neural neuroinvasion (Johnson, 1982; Koyuncu et al., 2013). Though the olfactory nerves are well protected from insult, their intimate association with the CNS serves as a conduit through which neurotropic viruses

can reach the CNS. Viruses capable of olfactory route neuroinvasion include CHIKV, Nipah virus, and vesicular stomatitis virus.

Neuropathology

Regarding pathology, the neurotropic viruses cause a variety of neurological diseases including meningitis, encephalitis, and myelitis or any combination thereof (Swanson and McGavern, 2015).

Symptoms correlate with disease type and affected regions of the brain. Importantly, as detailed above, neuropathology following viral nervous system invasion is not restricted to virus-induced pathology; many viruses cause inflammation-induced pathology (Koyuncu et al., 2013).

Alphavirus neuroinvasion

Many alphaviruses readily cause encephalitis upon aerosol or intranasal inoculation making them potential bioterror weapons and necessitating the need for vaccines and therapeutics. Detailed characterization of the virus-host relationship is needed for the development of these measures. Unfortunately, for many arboviruses (including alphaviruses), the mechanism of neuroinvasion is uncharacterized.

The alphaviruses most commonly associated with neuroinvasion are the NWAs and include EEEV, VEEV, and WEEV; however, OWAs such as CHIKV, SINV, and SFV have occasionally been associated with neurotropic infection.

Alphavirus neuroinvasion following intranasal delivery

Alphavirus neuroinvasion following intranasal delivery occurs through the olfactory pathways, regardless of infecting virus species or animal model. Immunohistochemistry and *in vivo/ex vivo* imaging studies have shown that alphaviruses enter the CNS after the development of a high titer viremia, which seeds the olfactory sensory neurons and allows access to the olfactory bulbs and CNS (Phillips et al., 2013; Schafer et al., 2011). Following entry into the CNS via the olfactory bulbs, viral replication induces pro-inflammatory cytokine release and BBB opening, allowing for a second seeding of virus into the CNS (Schafer et al., 2011).

Alphavirus neuroinvasion following peripheral delivery

Contrary to alphavirus intranasal delivery, no common route of neuroinvasion following peripheral or subcutaneous delivery has been defined and conflicting routes of neuroinvasion have been reported. For example, Gorelkin (1973) reported an endothelial route for VEEV strain Trinidad neuroinvasion: whereas, Charles et al. (1995) reported an olfactory route tract for VEEV strain Trinidad, and Honnold et al. (2015) reported EEEV neuroinvasion occurring through an endothelial route. Proposed routes of neuroinvasion following peripheral inoculation include: infection of brain endothelium (SINV (Johnson, 1965) and VEEV (Gorelkin, 1973)), infection of leukocytes (CHIKV (Couderc et al., 2008) and EEEV (Vogel et al., 2005)), the olfactory tract (SINV (Cook and Griffin, 2003) and VEEV (Charles et al., 1995)), and retrograde axonal transport (SINV (Cook and Griffin, 2003)). Considering the ambiguity in routes of alphavirus neuroinvasion following peripheral delivery, more studies are required.

Summary and specific aims

Alphaviruses continue to represent a threat to human and veterinary health worldwide. The re-emergence of alphaviruses (such as CHIKV) and the ease of encephalitic alphavirus weaponization complicates the public health burden imposed by alphaviruses.

As a part of studies involved in this dissertation, we sought to develop novel alphaviruses prophylactics for the prevention of alphavirus infection. Currently, there are only limited unlicensed, alphavirus vaccines available against some of the encephalitic alphaviruses. Unfortunately, these vaccines have significant side effects and none of the available vaccines are able to cross protect against other alphaviruses. We have developed alphavirus E1 glycoprotein based vaccines to provide protection from lethal challenge with multiple alphaviruses. These vaccines may provide an avenue for the development of broad alphavirus vaccines for use in humans.

Antibodies towards alphaviruses are protective against disease in multiple animal models; however, the mechanism by which these antibodies provide protection is often unknown. For neutralizing antibodies, blockade of cellular receptor-virion interactions imparts protective activity. Less is known

regarding the activity of non-neutralizing antibodies. We have characterized the activity of protective, non-neutralizing alphavirus E1 antibodies *in vitro* and *in vivo*.

Currently, treatment for alphavirus disease is limited to supportive care as no therapeutics are available. Another goal of these studies was to develop effective alphavirus therapeutics. Using recombinant luciferase-expressing alphaviruses, we have tested a drug library for alphavirus specific compounds and identified several lead candidates.

Lastly, we sought to describe the pattern of alphavirus neuroinvasion into the CNS following peripheral or intranasal virus installation. These studies contribute to previous studies in delineating two clear routes of neuroinvasion following alphavirus infection. This work has enhanced our knowledge of virus-host interactions and will potentially help in the development of alphavirus therapeutics and vaccines.

The work described in this dissertation has contributed to the development of broad alphavirus vaccines, enhanced our understanding of non-neutralizing antibodies, supplemented antiviral efficacy testing, and identified previously unknown routes for alphavirus entry into the CNS. These advancements will potentially help in efforts to prevent and treat alphavirus infection, reducing the global burden of alphavirus disease.

CHAPTER TWO: VACCINES PROTECTIVE AGAINST MULTIPLE ALPHAVIRUSES

Introduction

Alphaviruses are mosquito-borne pathogens that cause disease and death in humans and other vertebrates. Geographically, alphaviruses are divided into NWAs and OWAs. New world alphaviruses cause an encephalitic disease and are important biodefense and biosafety threats. As viral agents of encephalitis, NWAs pose the most significant threat to human health among alphaviruses, with fatality rates as high as 70% (Zacks and Paessler, 2010). Medically important NWAs include EEEV, VEEV, and WEEV. Old world alphaviruses cause acute flu-like diseases that often lead to long-term arthritis. Chikungunya virus, a medically important OWA, is currently undergoing a dramatic expansion, spreading into the western hemisphere where over one million human cases were confirmed in 2014 (Powers, 2015). Sequelae are present in approximately 60% of alphavirus infection survivors (Steele and Twenhafel, 2010).

Prophylactics to prevent human alphavirus infection are currently unavailable for use in the United States. Investigational-new-drug status vaccines are available against some alphaviruses, but safety concerns and poor immunogenicity limit their usage (Barrett and Stanberry, 2008). Of the unlicensed vaccines currently used under IND status, none protect against more than one alphavirus species. Furthermore, antibody interference typically precludes vaccination against a second alphavirus species (Barrett and Stanberry, 2008). With the spread of alphaviruses into new areas and the presence of multiple alphaviruses in a single location, the need for a pan-alphavirus vaccine is critical.

Alphaviruses have two envelope proteins, E2 and E1, which form trimeric, heterodimer spikes on the surface of the virion. Both E2 and E1 are responsible for virus entry participating in viral binding and fusion, respectively. E2 binds with a cellular receptor resulting in virus internalization through receptor mediated endocytosis. Once internalized, E1 mediates virus fusion to the host cell under low pH conditions. Vaccines directed towards either of these alphavirus envelope glycoproteins are highly protective in laboratory models (Khan et al., 2012; Phillips et al., 2014; Roy et al., 2013; Swayze et al.,

2011). Although E2 is the major neutralizing antigen, E1 is the better candidate for a pan-alphavirus subunit vaccine, because E1 is highly conserved among alphaviruses (Netolitzky et al., 2000).

Cationic liposomes coupled to nucleic acids (CLNCs) are effective activators of innate immunity (Logue et al., 2010; Troyer et al., 2009). CLNCs combined with antigen (lipid-antigen-nucleic acid-complexes: LANACs) induce both adaptive and innate immunity. The LANAC associated nucleic acids (Toll-like receptor (TLR)-3 and 9 agonist) trigger innate immunity, while LANAC associated pathogen antigen triggers an adaptive immune response. LANACs, when used as vaccines, have conferred protection against a variety of infectious agents (Firouzmand et al., 2013; Jones et al., 2010; Phillips et al., 2014).

In this study, we examined the protective potential of LANACs containing recombinant WEEV and/or VEEV E1. Vaccination studies, described herein, establish the protective utility of LANAC alphavirus E1 vaccination against the NWAs.

Methods

Virus strains

Recombinant luciferase-expressing VEEV plasmid (VEEV-3908-FLuc) was obtained from Dr. Scott Weaver (University of Texas Medical Branch) and virus was generated as follows: MluI linearized VEEV-3908-FLuc plasmid was purified by QIAprep Spin MiniPrep Kit (Qiagen) and genomic RNA was *in vitro* transcribed using a T7 RNA Polymerase MAXIscript kit (Life Tech). Vero cells (4×10^6 cells in 400 μ L) (ATCC) were electroporated with genomic RNA using an ECM 630 electroporator (BTX Harvard Apparatus). Two pulses (450 V, 1200 Ω , and 150 μ F) were administered. Forty-eight hours post electroporation virus was recovered from supernatant. EEEV-FL93-NLuc plasmid was obtained from Dr. William Klimstra (The University of Pittsburgh) and rescued as described (Sun et al., 2014). pWEEV-5'McM-FLuc was generated by duplication of the subgenomic promoter sequence immediately downstream of nsP4. MfeI linearized pWEEV-5'McM-FLuc was purified by QIAprep Spin MiniPrep Kit (Qiagen) and infectious clone genomic RNA was *in vitro* transcribed using a T7 RNA Polymerase MAXIscript kit (Life Tech). Vero cells (4×10^6 cells in 400 μ L) (ATCC) were electroporated with

genomic RNA using an ECM 630 electroporator (BTX Harvard Apparatus). Two pulses (450 V, 1200 Ω , and 150 μ F) were administered. Forty-eight hours post electroporation virus was recovered from supernatant. All recombinant viruses were used without further passage after rescue. EEEV strain FL93-939 and VEEV strain Trinidad were obtained from Dr. Richard Bowen (Colorado State University (CSU)). EEEV FL93-939 and VEEV Trinidad stocks were produced by infecting Vero cells at a multiplicity of infection (MOI) of 0.1 PFU/cell. Cell culture supernatants were collected 24 hours post-infection (HPI). All viral stocks were titered by plaque assay on Vero or BHK15 cells as described previously and stored at -80C (Mirchamsy and Rapp, 1968).

Animal studies

The animal use in this study was approved by the Institutional Animal Care and Use Committee at CSU. Mice were handled in compliance with the PHS Policy and Guide for the Care and Use of Laboratory Animals. All experiments were conducted in a biosafety level 3, and in a CDC Select Agent-approved facility. Outbred, 4- to 6- week old female CD-1 mice (Charles River Laboratories) and Syrian hamsters (Harlan) were allowed to acclimate to the facility for three to five days. Subcutaneous (SC) inoculations were administered in the right rear footpads. Intranasal (IN) inoculations were performed by installing inocula into each nostril of lightly anesthetized animals. Inocula were back-titered to confirm dosages. Animals were observed twice daily for signs of morbidity. Moribund animals were euthanized by CO₂ inhalation, and the day of euthanasia was taken as the day of death to calculate mean times to death (MTD). Survivorship was followed for 14-28 days.

Preparation of LANAC E1 vaccines

Vaccine was prepared fresh on the day of vaccination as described. Cationic liposomes (100 mM DOTIM lipid + cholesterol) in 10% sucrose were provided by Dr. Steven Dow (CSU). Cationic- lipid- nucleic acid- complexes (CLNCs) were prepared by diluting cationic liposomes 1:5 in sterile Tris- buffered 5% dextrose water and adding dsRNA and CpG DNA (InVivoGen), to a final concentration of 0.1 mg/mL each, causing spontaneous formation of liposome nucleic-acid complexes. Recombinant, WEEV McMillan (Toth et al., 2011) and/or VEEV Trinidad E1 (produced in a baculovirus-insect

expression system and provided by Dr. Donald Jarvis) were then added at a final concentration of 50 $\mu\text{g}/\text{mL}$ (10 $\mu\text{g}/200\ \mu\text{L}$ dose) (mice) or 250 $\mu\text{g}/\text{mL}$ (50 $\mu\text{g}/200\ \mu\text{L}$ dose) (hamsters). Antigens were expressed at Expression Systems LLC. in Davis, CA. Jarvis et al. characterized and purified baculovirus expressed antigens. After allowing antigen to associate with the liposome nucleic-acid complexes for 15 minutes LANAC complexes were used to vaccinate animals. Each dose of vaccine consisted of 200 μL of LANAC delivered via SC injection dorsal to the cervical spine. This priming dose was followed by an identical boost vaccination two weeks later. Control animals received no vaccination treatment.

Infections and imaging

Nine weeks after booster vaccination, CD-1 mice and Syrian hamsters were challenged either by the SC or IN route with EEEV, VEEV, or WEEV. Subcutaneous and intranasal infections were performed with a dose of 1×10^4 PFU of virus in a volume of 20 μL . Starting 24 HPI, mice were imaged using an IVIS 200 system (Perkin Elmer). Imaging was performed and quantified as described previously (Phillips et al., 2013; Sun et al., 2014).

Plaque reduction neutralization titer

Vero cells were seeded in 6-well plates. Plaque reduction neutralization titers (PRNTs) were measured by incubating virus with serial dilutions of homologous immunized mice sera, inoculating samples onto seeded cells, and incubating the plates at 37C for 1 hr. After aspiration of inocula, cells were washed and overlaid with Tragacanth gum overlay (Mirchamsy and Rapp, 1968) and incubated at 37C for 2 days, plaques were visualized by crystal violet staining. Controls consisting of virus not pre-treated with serum and treated with neutralizing sera were included with each assay. An 80% PRNT (PRNT80) was used as the neutralization endpoint and expressed as the reciprocal of the lowest dilution of test sera able to neutralize 80% of input virus.

Enzyme-linked immunosorbent assay

For enzyme-linked immunosorbent assay (ELISA), microtiter plates were coated overnight at 4C with 100 ng/well of recombinant WEEV E1 antigen in phosphate buffered saline (PBS). Coated plates were washed twice with PBS containing 0.25% Tween 20 and twice more with PBS. Plates were blocked

with 200 μ L SuperBlock T20 (Thermo Scientific) for one hour at room temperature (RT) and then washed. Two-fold serially diluted sera were added and incubated at RT for 1 hour. After washing, alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) was added for 1 hour. Color was developed with p-nitrophenyl phosphate and read at an absorbance of 405 nm. Endpoint titers were calculated as the reciprocal of the greatest dilution that was 0.200 optical density units greater than that for the negative control.

Statistics

Kaplan-Meier (log rank test) analyses were applied to survival curves using Prism version 6.00 for Windows (Graphpad). Quantitative analyses of bioluminescence were conducted using Living Image 3.0 software (PerkinElmer). Quantitative analyses of bioluminescence and antibody geometric mean titers were performed by Mann-Whitney test.

Results

Protective efficacy of LANACs containing WEEV E1 in mice

We assessed the ability of WEEV E1 LANACs to protect immunized mice against the following viruses: EEEV FL93-939 and VEEV Trinidad. A diagram of LANAC alphavirus E1 is shown (Figure 2.1). Nine weeks post booster vaccination, protective efficacy was assessed by challenging vaccinated mice by either the SC or IN route.

Immunization with LANAC WEEV E1 provided 76.4% protection against SC EEEV FL93-939 infection ($p < 0.0001$) and 52.9% protection against IN EEEV FL93-939 infection ($p < 0.0001$) (Figure 2.2).

Conversely, LANAC WEEV E1 immunization did not significantly increase survival of mice following IN infection with VEEV 3908 ($p = 0.0502$). Immunization resulted in 0% protection against IN VEEV 3908 infection (Figure 2.3). LANAC WEEV E1 immunization significantly increased survival following SC VEEV 3908 infection (Figure 2.3) ($p = 0.0031$).

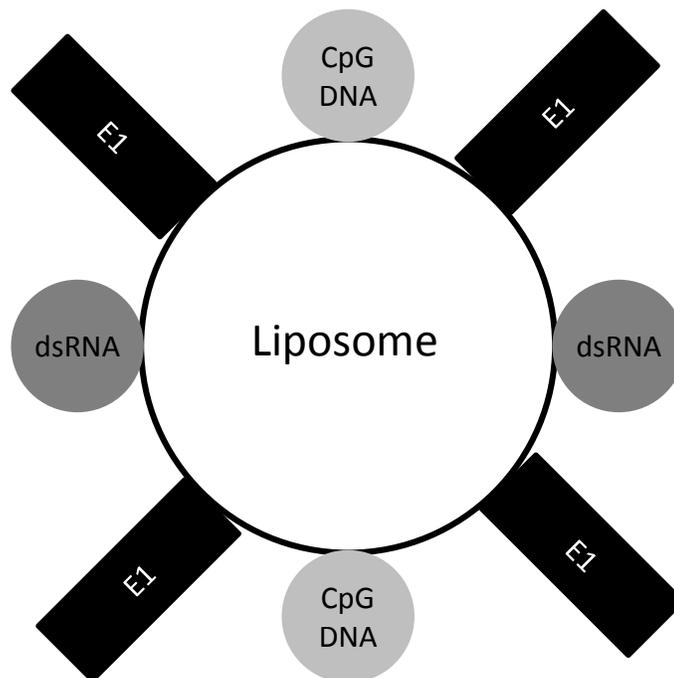


Figure 2. 1 Diagram of LANAC alphavirus E1.

Illustration of LANAC alphavirus E1 vaccine used in these studies. E1 was either VEEV E1 or WEEV E1 or both. CpG DNA- TLR-9 agonist. dsRNA-TLR-3 agonist.

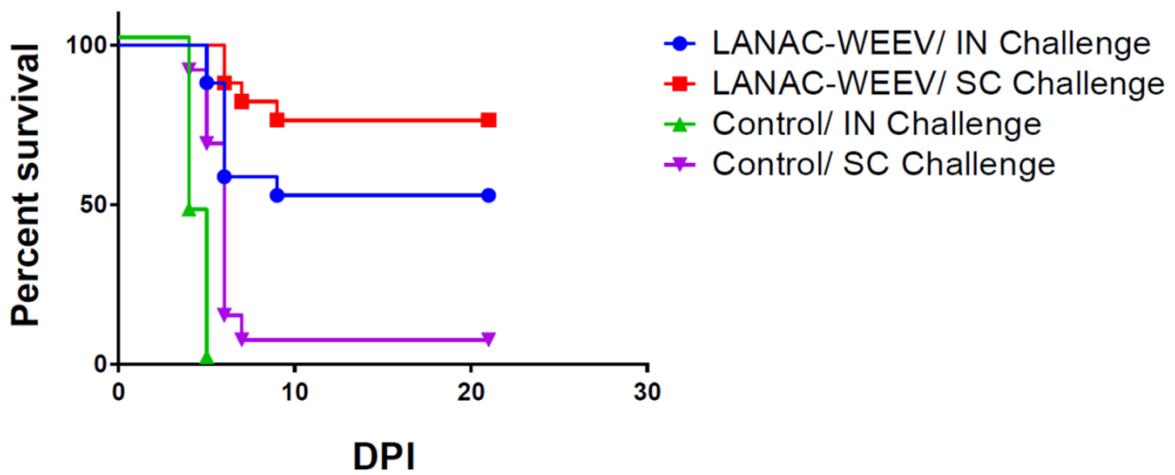


Figure 2. 2 LANAC WEEV E1 protection against IN or SC challenge with EEEV.

Mice were prime-boost immunized with LANAC WEEV E1 and challenged with 10^4 PFU EEEV FL93-939. The differences in survival among mice immunized with LANAC WEEV E1 or control mice were statistically significant ($p < 0.0001$ for both IN and SC challenge).

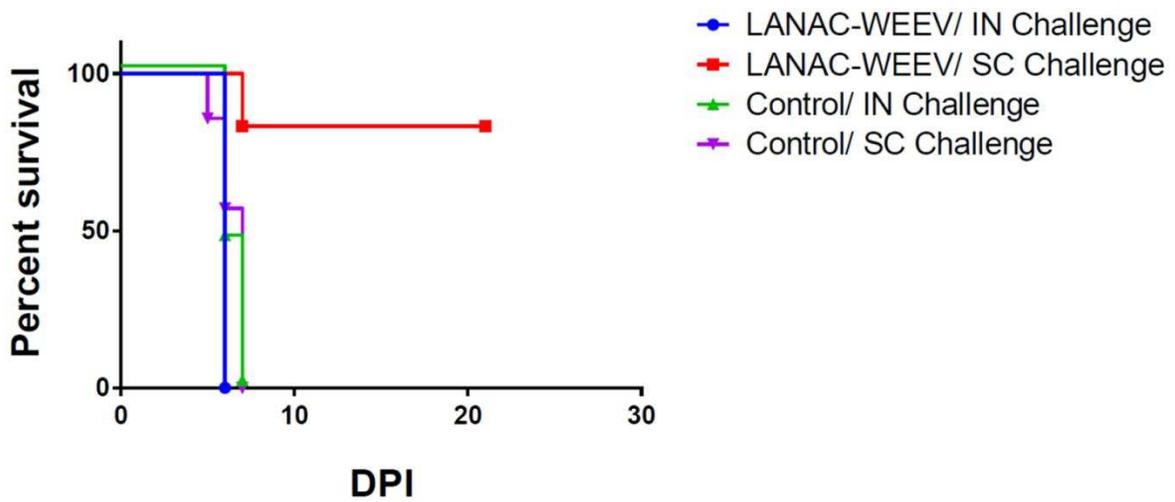


Figure 2. 3 LANAC WEEV E1 protection against IN or SC challenge with VEEV.

Mice were prime-boost immunized with LANAC WEEV E1 and challenged with 10^4 PFU VEEV Trinidad. The differences in survival among mice immunized with LANAC WEEV E1 or control mice were statistically significant for SC infections ($p=0.0031$), but not for IN infections ($p=0.0502$).

Protective efficacy of LANACs containing VEEV E1 in mice

We assessed the ability of VEEV E1 LANACs to protect immunized mice against the following viruses: VEEV-3908-FLuc, EEEV FL93-939, and WEEV-5'McM-FLuc. Nine weeks post booster vaccination, protective efficacy was assessed by challenging vaccinated mice by either the SC or IN route.

LANAC VEEV E1 immunization significantly increased survival of mice following SC infection with EEEV FL93-939. Immunization resulted in 100% protection against SC EEEV FL93-939 infection ($p=0.0002$) (Figure 2.4). LANAC VEEV E1 immunization also conferred 52.9% protection against IN EEEV FL93-939 challenge ($p=0.0002$) (Figure 2.4).

Similarly, immunization with LANAC VEEV E1 provided 100% protection against SC VEEV-3908-FLuc infection ($p=0.0004$) and 85.7% protection against IN VEEV-3908-FLuc infection ($p<0.0001$) (Figure 2.5).

LANAC VEEV E1 immunization partially protected mice (14.3% survival) from lethal IN WEEV-5'McM-FLuc challenge ($p=0.0004$) (Figure 2.6). Mean time to death was extended from four to six days following IN WEEV-5'McM-FLuc challenge in immunized mice.

Protective efficacy of LANACs containing VEEV and WEEV E1 in mice

We assessed the ability of VEEV E1 + WEEV E1 LANACs to protect immunized mice against the following viruses: VEEV-3908-FLuc, EEEV-FL93-NLuc, and WEEV-5'McM-FLuc. Nine weeks post booster vaccination, protective efficacy was assessed by challenging vaccinated mice by either the SC or IN route.

LANAC VEEV E1 + WEEV E1 immunization fully protected mice from lethal SC and IN EEEV-FL93-NLuc challenge ($p=0.0004$) (Figure 2.7).

Similarly, LANAC VEEV E1 +WEEV immunization fully protected mice against lethal IN VEEV-3908-FLuc challenge ($p=0.0004$) (Figure 2.8).

Immunization with LANAC VEEV E1 + WEEV E1 provided 100% protection against IN WEEV-5'McM-FLuc infection ($p=0.0003$) (Figure 2.9).

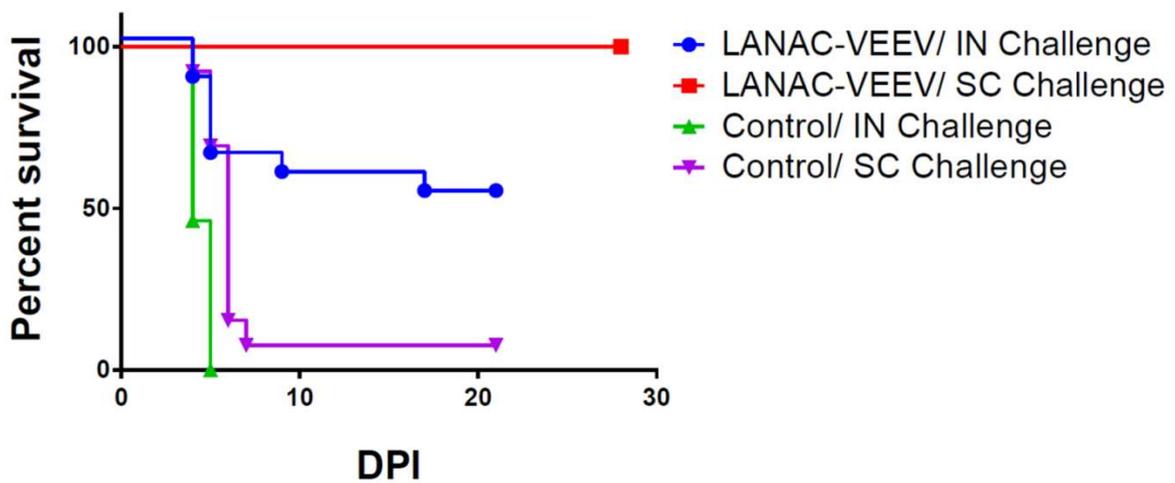


Figure 2. 4 LANAC VEEV E1 protection against IN or SC challenge with EEEV.

Mice were prime-boost immunized with LANAC VEEV E1 and challenged with 10^4 PFU EEEV FL93-939. The differences in survival among mice immunized with LANAC VEEV E1 or control mice were statistically significant ($p=0.0002$ for both IN and SC challenge).

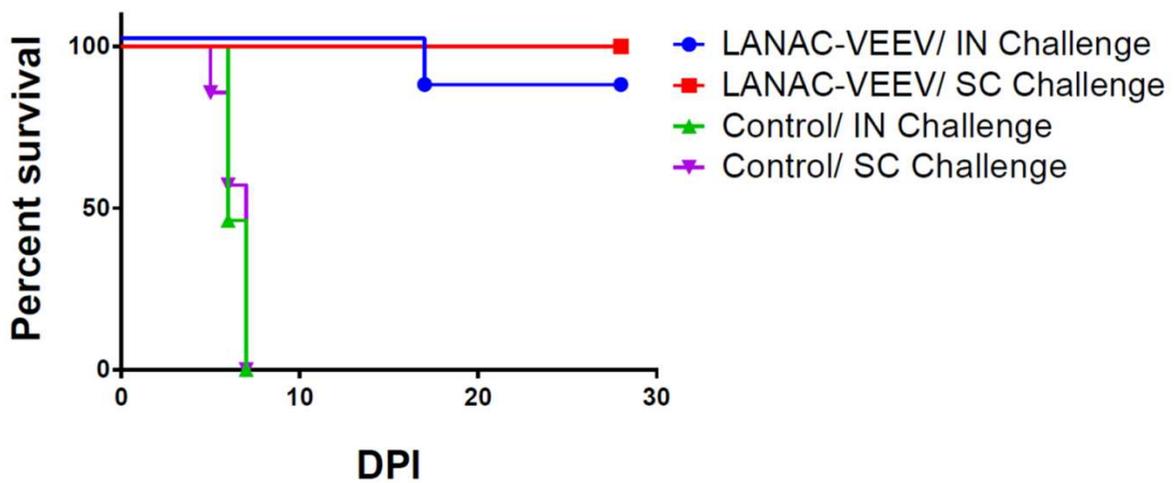


Figure 2. 5 LANAC VEEV E1 protection against IN or SC challenge with VEEV.

Mice were prime-boost immunized with LANAC VEEV E1 and challenged with 10^4 PFU VEEV-3908-FLuc. The differences in survival among mice immunized with LANAC VEEV E1 or control mice were statistically significant ($p=0.0004$ for IN and $p<0.0001$ for SC).

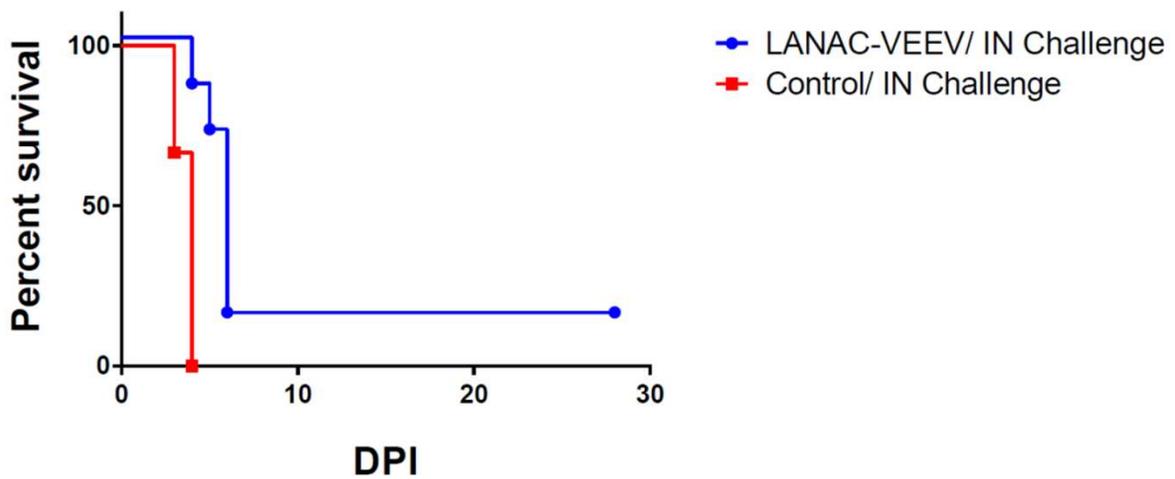


Figure 2. 6 LANAC VEEV E1 protection against IN challenge with WEEV.

Mice were prime-boost immunized with LANAC VEEV E1 and challenged with 10^4 PFU WEEV-5' McM-FLuc. The differences in survival among mice immunized with LANAC VEEV E1 or control mice were statistically significant ($p=0.0004$).

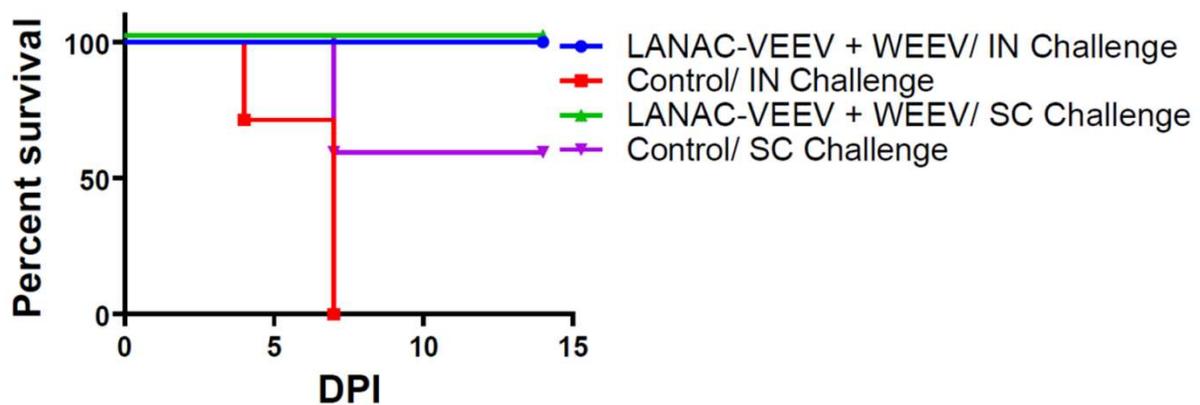


Figure 2. 7 LANAC VEEV E1 + WEEV E1 protection against IN or SC challenge with EEEV.

Mice were prime-boost immunized with LANAC VEEV E1 + WEEV E1 and challenged with 10^4 PFU EEEV-FL93-NLuc. The differences in survival among mice immunized with LANAC VEEV+ WEEV E1 or control mice were statistically significant for IN infections ($p=0.0004$), but not for SC infections ($p=0.0597$).

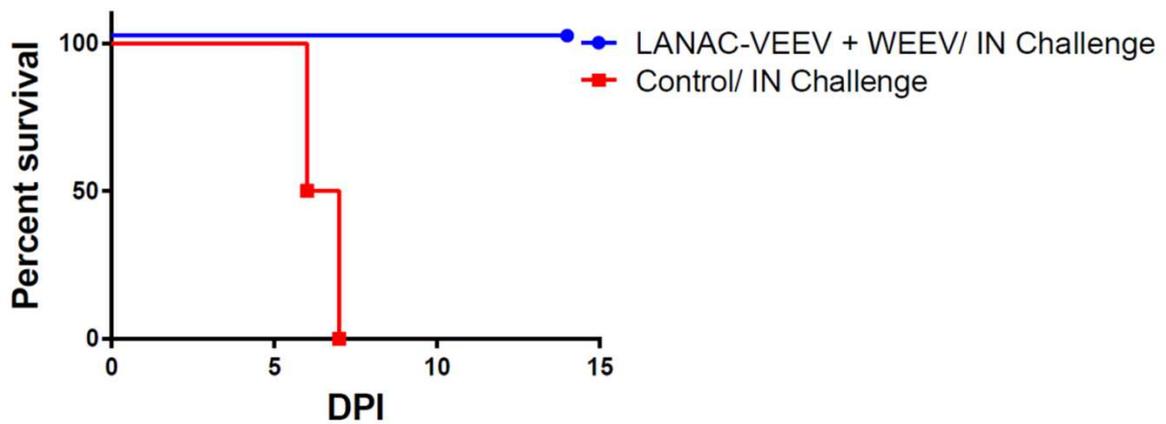


Figure 2. 8 LANAC VEEV E1 + WEEV E1 protection against IN challenge with VEEV.

Mice were prime-boost immunized with LANAC VEEV E1 + WEEV E1 and challenged with 10^4 PFU VEEV-3908-FLuc. The differences in survival among mice immunized with LANAC VEEV E1 + WEEV E1 or control mice were statistically significant ($p=0.0004$).

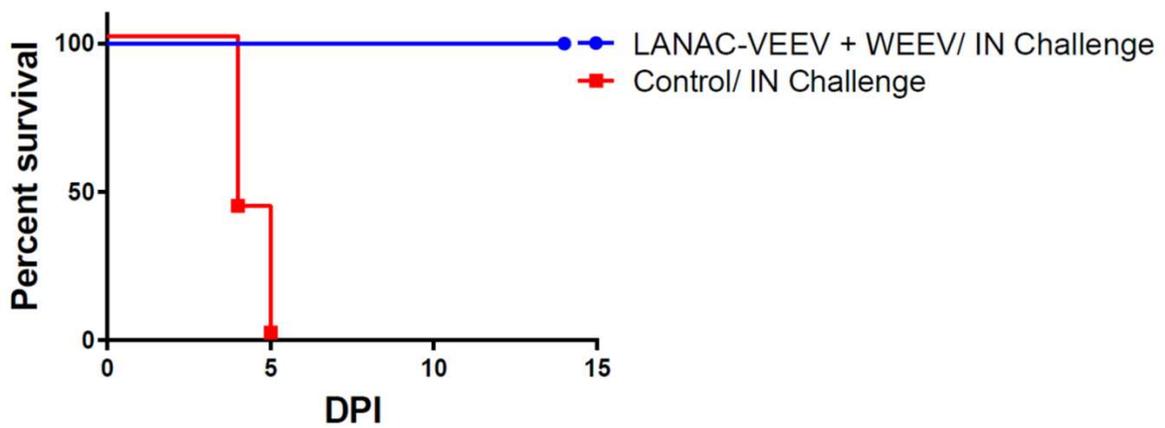


Figure 2. 9 LANAC VEEV E1 + WEEV E1 protection against IN challenge with WEEV.

Mice were prime-boost immunized with LANAC VEEV E1 + WEEV E1 and challenged with 10^4 PFU WEEV-5' McM-FLuc. The differences in survival among mice immunized with LANAC VEEV E1 + WEEV E1 or control mice were statistically significant ($p=0.0003$).

Bioluminescence imaging to gauge vaccine efficacy in mice

To assess the ability of LANAC VEEV E1 + WEEV E1 immunization to inhibit EEEV-FL93-NLuc, VEEV-3908-FLuc, and WEEV-5'McM-FLuc viral replication, we used *in vivo* imaging to examine immunized and unimmunized mice for luciferase expression upon IN infection. Imaging revealed a reduction in bioluminescence, indicative of decreased viral replication, in immunized mice as compared to unimmunized mice at all time points following EEEV, VEEV (p=0.0002), and WEEV challenge (Figures 2.10-2.13). Importantly, LANAC VEEV E1 + WEEV vaccinated mice challenged with EEEV exhibited detectable virus replication and developed EEEV neutralizing antibody; whereas LANAC VEEV E1 + WEEV vaccinated mice challenged with VEEV and WEEV did not develop neutralizing immunity (Table 2.1).

Protective efficacy of LANACs containing WEEV E1 in hamsters

LANAC WEEV E1 immunization did not significantly change survival or mean time to death in hamsters challenged with WEEV-5'McM-FLuc (Figure 2.14). Additionally, hamsters produced significantly lower titers of E1 antibody than mice (p=0.0014) (Figure 2.15).

Discussion

We present a new LANAC alphavirus E1 formulation for the prevention of EEEV, VEEV, and WEEV infections. LANACs containing recombinant alphavirus E1 antigen induce a strong humoral immune response.

Of particular significance, is the cross protective effect of both VEEV and WEEV E1 antigen (Figures 2.2 and 2.5). We chose these baculovirus-derived protein antigens for our LANAC formulations for several reasons. First, regarding the baculovirus expression system, previous studies using bacterial expression systems have demonstrated the importance of E1 glycosylation in initiating a protective immune response to alphavirus infection (Das et al., 2004). Phillips et al. (2014) and Hodgson et al (1999) have, however, demonstrated protective immune response towards glycosylated baculovirus-derived

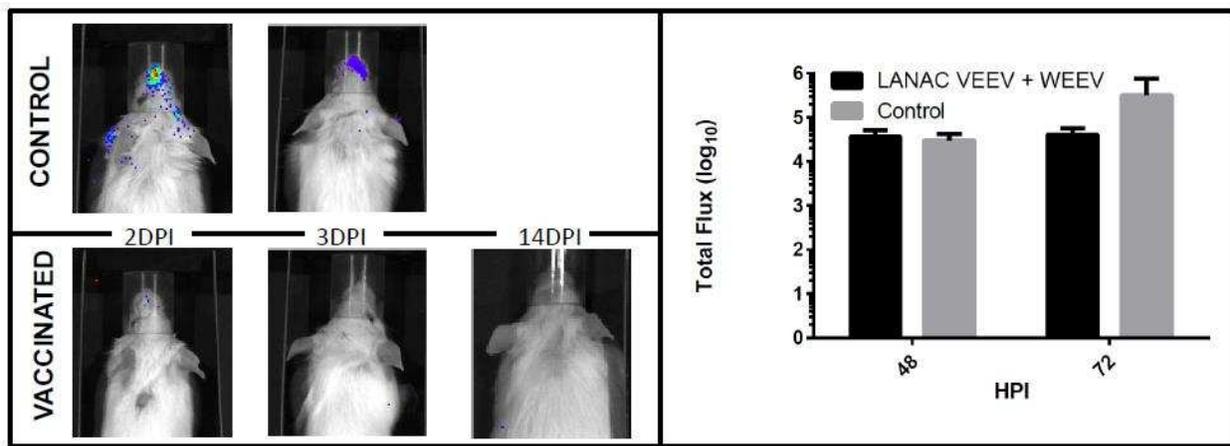


Figure 2. 10 *In vivo* bioluminescence imaging of EEEV-FL93-NLuc IN infected mice.

LANAC VEEV E1 + WEEV E1 immunized and unimmunized mice (n=7/group) were challenged by IN inoculation with 10⁴ PFU of EEEV-FL93-NLuc virus then imaged at 2, 3 and 14 DPI. Left- Bioluminescence. Right- Bioluminescence quantification. Each bar represents the average bioluminescence signal for each group.

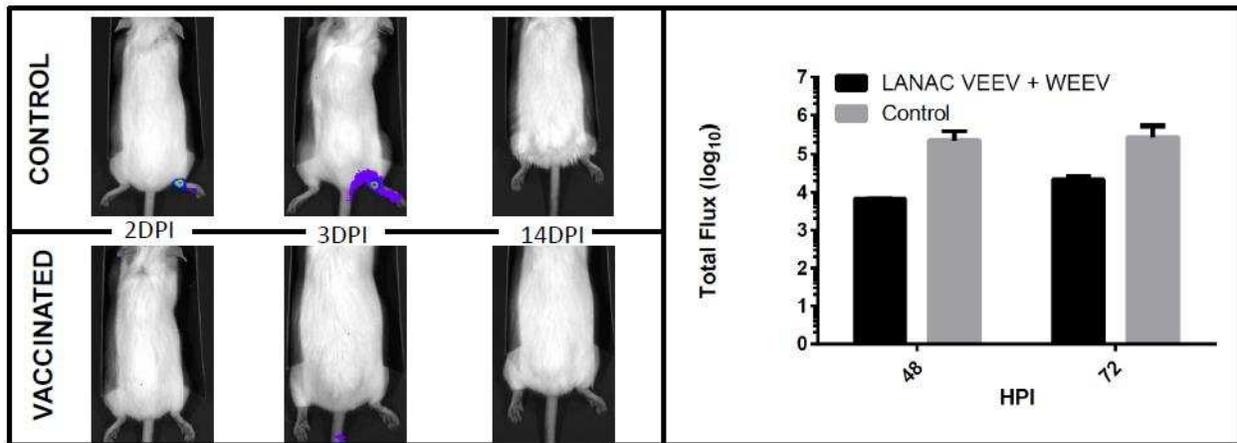


Figure 2.11 *In vivo* bioluminescence imaging of EEEV-FL93-NLuc SC infected mice.

LANAC VEEV E1 + WEEV E1 immunized and unimmunized mice (n=7/group) were challenged by SC inoculation with 10⁴ PFU of EEEV-FL93-NLuc virus then imaged at 2, 3 and 14 DPI. Left- Bioluminescence. Right- Bioluminescence quantification. Each bar represents the average bioluminescence signal for each group.

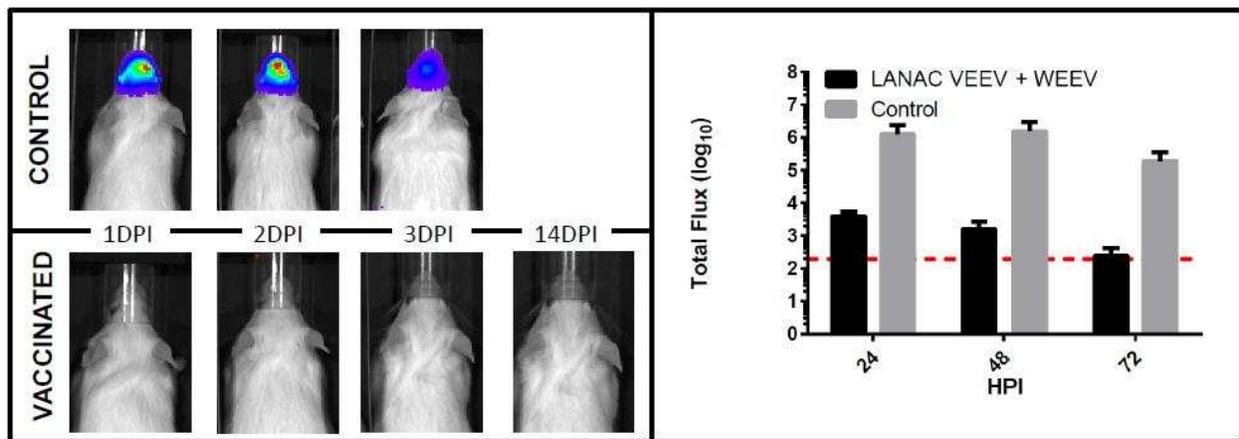


Figure 2. 12 *In vivo* bioluminescence imaging of VEEV-3908-FLuc IN infected mice.

LANAC VEEV E1 + WEEV E1 immunized and unimmunized mice (n=7/group) were challenged by IN inoculation with 10⁴ PFU of VEEV-3908-FLuc virus then imaged at 1, 2, 3 and 14 DPI. Left- Bioluminescence. Right- Bioluminescence quantification. Each bar represents the average bioluminescence signal for each group. The red line establishes background bioluminescence.

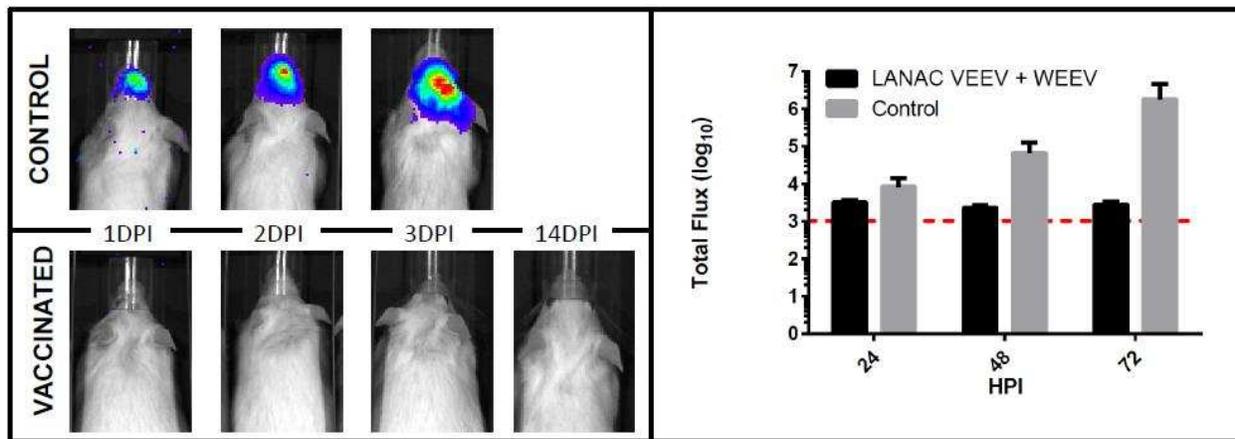


Figure 2. 13 *In vivo* bioluminescence imaging of WEEV-5'McM-FLuc IN infected mice.

LANAC VEEV E1 + WEEV E1 immunized and unimmunized mice (n=7/group) were challenged by IN inoculation with 10^4 PFU of WEEV-5'McM-FLuc virus then imaged at 1, 2, 3 and 14 DPI. Left- Bioluminescence. Right- Bioluminescence quantification. Each bar represents the average bioluminescence signal for each group. The red line establishes background bioluminescence.

Table 2. 1 Neutralizing titers of sera from VEEV E1 + WEEV E1 vaccinated mice following challenge.

PRNTs of sera from LANAC VEEV E1 + WEEV E1 vaccinated mice which had survived EEEV, VEEV or WEEV challenge.

Challenge Virus	PRNT⁸⁰ Before Challenge	PRNT⁸⁰ After Challenge	Sterilizing Immunity following Vaccination?
WEEV	<40	<40	Yes
VEEV	<40	<40	Yes
EEEV	<40	>200	No

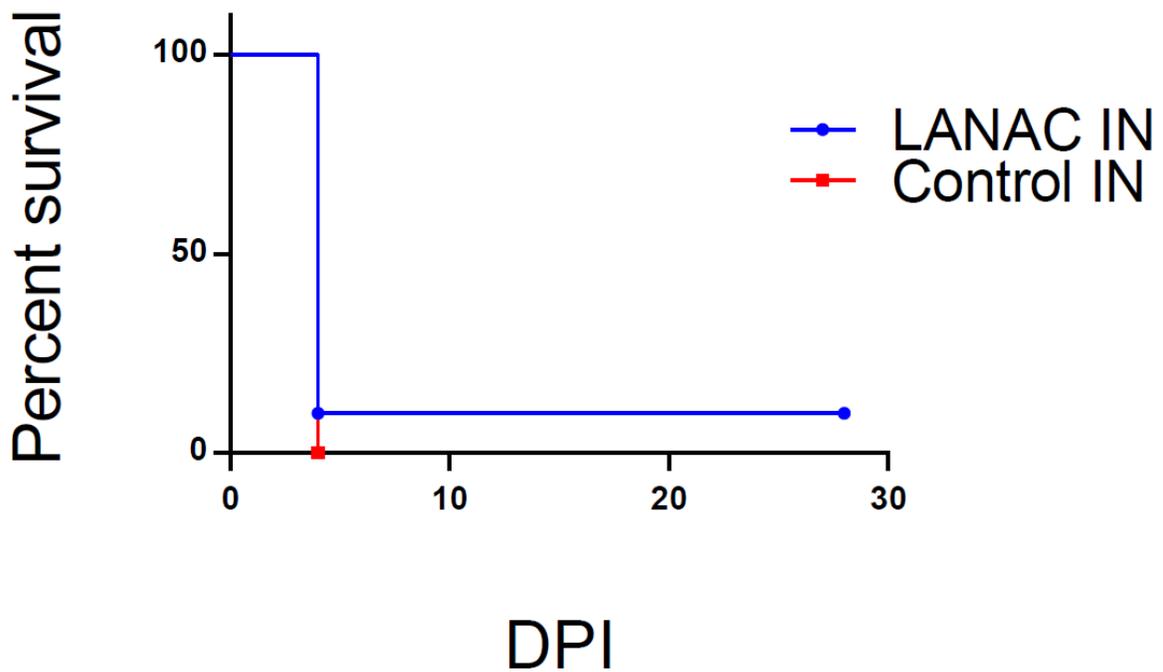


Figure 2. 14 LANAC WEEV E1 protection against IN challenge with WEEV.

Hamsters were prime-boost immunized with LANAC WEEV E1 and challenged with 10^4 PFU WEEV-5'McM-FLuc. The differences in survival among hamsters immunized with LANAC WEEV E1 or control hamsters were not statistically significant ($p=0.3173$).

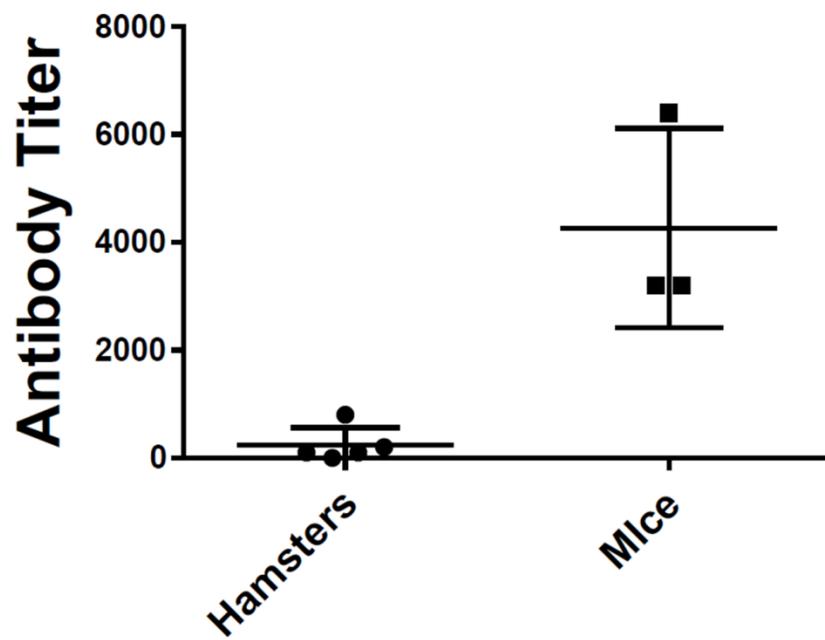


Figure 2. 15 Humoral immune response to LANAC WEEV E1 immunization.

Antigen specific E1 ELISA. Geometric mean titers of antibody from LANAC WEEV E1 hamster and mouse serum.

recombinant alphavirus E1. E1 is highly conserved among alphavirus species and antibodies towards E1 can be alphavirus group reactive (Hunt and Roehrig, 1985; Schmaljohn et al., 1982; Strauss and Strauss, 1994).

Our LANAC E1 platforms, described herein and in Phillips et al. (2014) have the potential to protect against all three major NWAs: EEEV, VEEV, and WEEV. Surprisingly, both VEEV and WEEV E1 provided partial protection towards EEEV suggesting that EEEV E1 antigen alone might confer protection against all three viruses.

Unexpectedly, hamsters were not protected from lethal WEEV challenge by LANAC WEEV E1 vaccination. Hamsters did not seroconvert following LANAC WEEV E1 vaccination. Previous studies utilizing hamsters as an alphavirus vaccination model have, notably, utilized attenuated viruses still capable of initiating infection (Paessler et al., 2006; Turell and Parker, 2008). However, Cole and McKinney (1971) used adjuvanted inactivated EEEV, VEEV, and WEEV vaccines with human serum albumin in hamsters and observed the development of protective immune responses. Hamsters are an underdeveloped animal model and lack the wealth of information available with more traditional animal models. It is possible, that hamsters are unable to respond to LANAC WEEV E1 vaccination in the context of dsRNA and CpG DNA adjuvants. Importantly, the CpG DNA used in this study was mouse specific and could potentially not cross react with hamster TLR-9. Future studies will be required to identify the components necessary for hamsters to respond to alphavirus E1 vaccination.

We have demonstrated the utility of LANAC alphavirus E1 vaccination against EEEV, VEEV, and WEEV. We remain optimistic that LANAC alphavirus E1 vaccination has potential as a broad-acting alphavirus vaccine. Studies to test LANAC E1 vaccination in additional animal models are needed to meet the FDA's animal efficacy rule.

CHAPTER THREE: MECHANISM OF PROTECTION OF E1 ANTIBODIES

Introduction

Alphaviruses are mosquito-borne RNA viruses that cause pathology in humans and other mammals. The alphavirus genome is approximately 11,700 nucleotides long with a 5' methylated cap and a 3' polyadenylated termini. The message sense alphavirus genome is translated into four non-structural proteins (nsP1-4) that form the replication complex. A subgenomic RNA is also generated which encodes the five structural proteins (C, E3, E2, 6K, and E1) functioning in the assembly of progeny virions (Strauss and Strauss, 1994).

The three major alphavirus structural proteins (nucleocapsid, E2, and E1) are each immunogenic and their antibodies have distinct activities *in vitro* (Schmaljohn et al., 1982). Antibodies towards E2 are predominantly neutralizing, whereas antibodies towards E1 and nucleocapsid traditionally do not interact with intact virions (Schmaljohn et al., 1982). Though, neutralizing and non-neutralizing antibodies have been described following alphavirus infection, the respective role each plays in mediating host recovery is unclear.

Due to the known importance of alphavirus antibody in recovery from alphavirus infection (Griffin et al., 1997; Levine et al., 1991; Schmaljohn et al., 1982), alphavirus antibody has long been used as a surrogate marker of immunity. Though the association between alphavirus immunity and neutralizing antibody is unclear, neutralizing antibody is generally considered the most important antibody for recovery and immunity to alphavirus infection. However, non-neutralizing antibodies can prevent lethal alphavirus disease, though the mechanism behind protection is unclear (Schmaljohn et al., 1982).

Repeated alphavirus vaccination has been associated with immunological interference, the inability to mount protective immune responses towards related viruses upon subsequent immunizations (Reisler et al., 2012). As the major antibody produced during alphavirus infection, it is believed that antibodies towards E2 are responsible. These antibodies partially cross react, preventing new antibodies

from being generated but not protecting from disease upon infection. Unlike E2, E1 is highly conserved among alphaviruses and is a promising pan-alphavirus vaccine and therapeutic candidate.

Recently, we have described a novel alphavirus vaccine platform that uses recombinant, baculovirus-expressed E1 as viral antigen (Phillips et al., 2014). Vaccination of CD-1 mice results in the production of high titer, class-switched E1 antibody. In the present study, sera derived from alphavirus E1 immunization were assessed for cross-protection and mechanism of protection *in vitro* and *in vivo*. We demonstrate that E1 antibody is non-neutralizing, inhibits virus replication at late stages of viral infection, and is sufficient for protection *in vivo*. Together these studies, support the use of E1 directed prophylactics and therapeutics to combat alphavirus infections.

Methods

Virus strains

Recombinant luciferase-expressing VEEV plasmid (VEEV-3908-FLuc) was obtained from Dr. Scott Weaver (University of Texas Medical Branch) and virus was generated as follows: MluI linearized VEEV-3908-FLuc plasmid was purified by QIAprep Spin MiniPrep Kit (Qiagen) and genomic RNA was *in vitro* transcribed using a T7 RNA Polymerase MAXIscript kit (Life Tech). Vero cells (4×10^6 cells in 400 μ L) (ATCC) were electroporated with genomic RNA using an ECM 630 electroporator (BTX Harvard Apparatus). Two pulses (450 V, 1200 Ω , and 150 μ F) were administered. Forty-eight hours post electroporation virus was recovered from supernatant. SINV-TE3'2J-FLuc, SINV-TE3'2J Replicon-Helper and SINV-TE3'2J Replicon-GFP plasmid were obtained from Dr. Brian Geiss (CSU) and virus was generated as follows: respective plasmid/s was transfected into BHK15 cells (ATCC) using Lipofectamine 2000 transfection reagent (Life Tech). Twenty-four hours post transfection virus was harvested from supernatant. CHIKV-ReUnion-NLuc and EEEV-FL93-NLuc plasmids were obtained from Dr. William Klimstra (The University of Pittsburgh) and rescued as described (Sun et al., 2014). pWEEV-5'McM-FLuc was generated by duplication of the subgenomic promoter sequence immediately downstream of nsP4. MfeI linearized pWEEV-5'McM-FLuc was purified by QIAprep Spin MiniPrep Kit (Qiagen) and infectious clone genomic RNA was *in vitro* transcribed using a T7 RNA Polymerase

MAXIscript kit (Life Tech). Vero cells (4×10^6 cells in 400 μ L) (ATCC) were electroporated with genomic RNA using an ECM 630 electroporator (BTX Harvard Apparatus). Two pulses (450 V, 1200 Ω , and 150 μ F) were administered. Forty-eight hours post electroporation virus was recovered from supernatant. All recombinant viruses were used without further passage after rescue. Viral stocks were titered by plaque assay on Vero or BHK15 cells as described previously (Mirchamsy and Rapp, 1968) and stored at -80C.

Mouse studies

The animal use in this study was approved by the Institutional Animal Care and Use Committee at CSU. Mice were handled in compliance with the PHS Policy and Guide for the Care and Use of Laboratory Animals. All experiments were conducted at biosafety level 3 in a CDC Select Agent-approved facility. Outbred, 4- to 6- week old female CD-1 mice (Charles River Laboratories) were allowed to acclimate to the facility for three to five days. Intranasal (IN) inoculations were performed by installing inocula into each nostril of lightly anesthetized mice. Mice were anesthetized by administration of isoflurane (Minrad Incorporated) through an XGI-8 anesthesia system (Caliper Life Sciences). Inocula were back-titered to confirm dosages. Mice were observed twice daily for signs of morbidity. Moribund mice were euthanized by CO₂ inhalation, and the day of euthanasia was taken as the day of death to calculate mean times to death (MTD).

Generation of alphavirus E1 serum

For preparation of E1 serum, mice or rabbits were vaccinated with VEEV, WEEV (Toth et al., 2011), or VEEV E1 + WEEV E1 antigen (10 μ g/antigen/dose) provided by Dr. Donald Jarvis (University of Wyoming). Antigens were expressed at Expression Systems LLC. in Davis, CA. Jarvis et al. characterized and purified baculovirus expressed antigens. This priming dose was followed by an identical boost vaccination two weeks later. Serum was collected nine weeks post boosting, heat inactivated at 56C for 30 minutes, and stored at -80C. Naïve serum was collected from control animals.

Immunofluorescence assay

Vero cells on coverslips were infected with VEEV-3908-FLuc or WEEV-5' McM-FLuc at RT for one hour at a MOI of 0.1 with rocking every 15 minutes. Following infection, cells were overlaid with fresh media and the infection was allowed to develop. Twenty-four HPI cells were acetone:methanol fixed overnight at 4C. Following 5-five minute PBS washes, cells were blocked with 5% bovine serum albumin (BSA) in PBS for 30 minutes at RT. Coverslips were incubated with 400 μ L 1:100 diluted homologous immune sera from immunized mice and rocked at RT for one hour then incubated with 400 μ L of a 1:200 dilution of goat anti-mouse IgG Alexa Fluor 488 antibody (Life Technologies) and a 1:1000 dilution of 5 μ g/mL DAPI. Coverslips were washed with PBS and mounted on glass slides with 3:1 glycerol: PBS. Immunostained cells were visualized with a fluorescent microscope. Control slides included uninfected cells stained with immunized mouse sera.

Western blot

Recombinant VEEV or WEEV E1 antigen (27.5 μ g) were resolved on a NuPAGE Novex 4-12% Bis-Tris Mini Gels (Invitrogen). Protein was blotted onto a nitrocellulose membrane. Blots were then blocked with 5% BSA in PBS and probed with homologous immunized mouse sera (1:5000). Bound primary antibodies were detected using horseradish peroxidase (HRP) labeled secondary antibody (1:20000) and SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific). Chemiluminescence was detected using an IVIS 200 system (PerkinElmer).

Plaque reduction neutralization titer

Vero cells were seeded in 6-well plates. Plaque reduction neutralization titers (PRNTs) were measured by incubating virus with serial dilutions of homologous immunized mice sera, inoculating samples onto seeded cells, and incubating the plates at 37C for 1 hr. After aspiration of inocula, cells were washed and overlaid with Traggacanth gum overlay. After incubation at 37C for 2 days, plaques were visualized by crystal violet staining. Controls consisting of virus not treated with serum and treated with known neutralizing sera were included with each assay. A 80% PRNT (PRNT80) was used as the

neutralization endpoint and was expressed as the reciprocal of the lowest dilution of test sera able to neutralize 80% of input virus.

In vitro antibody inhibition assay

Venezuelan equine encephalitis virus E1, WEEV E1, and VEEV E1 + WEEV E1 sera were tested against CHIKV, EEEV, SINV, VEEV, and WEEV as described previously (Phillips et al., 2014). Briefly, Vero or BHK15 cells were infected for one hour at a MOI of 0.01 with CHIKV-ReUnion-NLuc, EEEV-FL93-NLuc, SINV-TE3'2J-FLuc, SINV-TE3'2J Replicon-GFP, VEEV-3908-FLuc, or WEEV-5'McM-FLuc. Following infection, cells were washed three times with ice cold PBS and sera was diluted in growth media and added to each well. IVIS/fluorescence images were acquired at 24 HPI. For homologous virus/sera combinations supernatants were, additionally, collected and used to quantify virus by plaque assay (data not shown).

Passive transfer of immune sera

Sera were obtained from rabbits following prime-boost immunization with VEEV E1, WEEV E1 or VEEV E1 + WEEV E1. Naïve mice received 0.1 mL of pooled immune or naïve serum (intraperitoneally) four hours prior to IN challenge with 1×10^4 PFU of EEEV-FL93-NLuc, WEEV-5'McM-FLuc, or VEEV-3908-FLuc in 20 μ L total volume. Mice were imaged daily to monitor infection.

Bioluminescent imaging

Starting 24 HPI, black flat well plates or mice infected with luciferase-expressing viruses were imaged using an IVIS 200 system (Perkin Elmer). Plate imaging was performed directly after application of 0.3mg/mL D-luciferin. Exposure time was kept to five seconds under standard settings. Mouse imaging was performed as described previously (Phillips et al., 2013; Sun et al., 2014). Living Image 3.0 software (Caliper Life Science) was used to analysis and process images. Total light emission from each well/mouse was determined using software provide grids and collecting light emission data.

Statistics

Kaplan-Meier (log rank test) analyses were applied to survival curves using Prism version 6.00 for Windows (Graphpad). Quantitative analysis of bioluminescence and fluorescence was conducted using Living Image 3.0 software (PerkinElmer). Quantitative analyses of bioluminescence and virus titers were performed by Mann-Whitney test.

Results

Alphavirus E1 antibodies bind to E1

Immunofluorescence assays demonstrated that VEEV E1 and WEEV E1 sera preferentially binds to infected cells (Figure 3.1). Western blotting results showed that VEEV E1 and WEEV E1 sera additionally binds to purified E1 (Figure 3.2), but not intact virions (data not shown).

Antiviral activities of sera derived from E1 immunization

Using standard PRNT assays, the viral neutralization titers of E1 sera were measured (Table 3.1). We then tested E1 polyclonal sera using our *in vitro* antibody inhibition assay (Phillips et al., 2014). Mouse WEEV E1 sera significantly inhibited CHIKV-ReUnion-NLuc, EEEV-FL93-NLuc, SINV-TE3'2J-FLuc, and WEEV-5'McM-FLuc (Figure 3.3). However, mouse WEEV E1 sera did not significantly inhibit the replication of SINV-TE3'2J Replicon-GFP (Figure 3.4). Rabbit VEEV E1 sera significantly inhibited CHIKV-ReUnion-NLuc and VEEV-3908-FLuc (Figure 3.5). Rabbit WEEV E1 sera significantly inhibited CHIKV-ReUnion-NLuc and WEEV-5'McM-FLuc (Figure 3.6). Lastly, rabbit VEEV E1 + WEEV E1 sera significantly inhibited CHIKV-ReUnion-NLuc, VEEV-3908-FLuc, and WEEV-5'McM-FLuc (Figure 3.7)

Passive transfer of E1 antibodies confers protection

Venezuelan equine encephalitis virus + WEEV E1 or non-immune sera were passively transferred to mice four hours prior to IN infection with EEEV, VEEV, or WEEV. Mice receiving non-immune sera showed progressive intensification of bioluminescence and neurological deficits until euthanasia (Figure 3.8-3.10). Mice receiving VEEV E1 + WEEV E1 sera and VEEV or WEEV challenge showed no

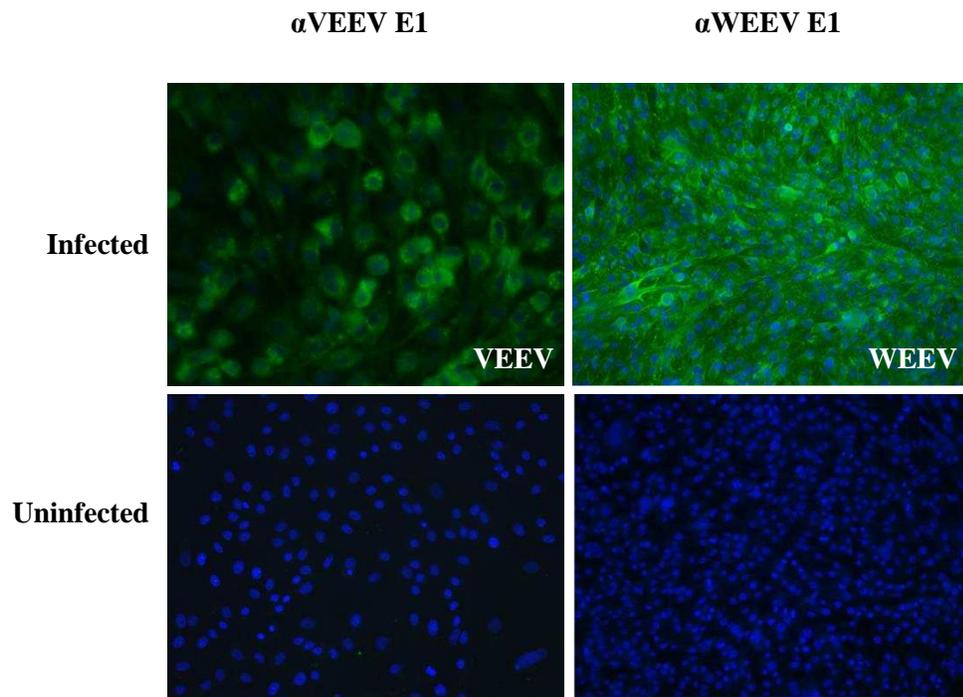


Figure 3. 1 Antibody binds infected cells.

Infected (VEEV or WEEV) or uninfected cells stained with mouse E1 sera.

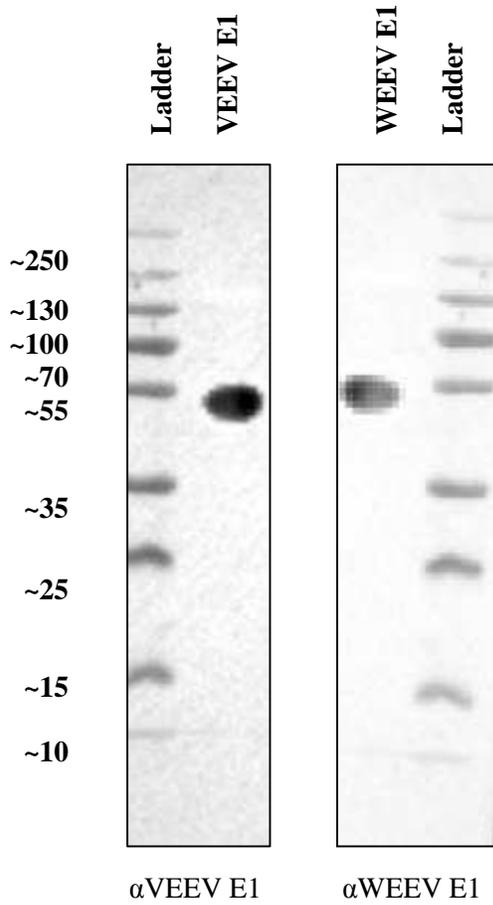


Figure 3. 2 Alphavirus E1 sera binds purified E1.

Western blotting demonstrating mouse alphavirus E1 sera binds to recombinant alphavirus E1. Left panel- VEEV E1 sera binds VEEV E1. Right panel- WEEV E1 sera binds WEEV E1.

Table 3. 1 Neutralizing titers of alphavirus polyclonal serum.

PRNTs of mouse E1 sera, naïve mouse sera, and sera from mice which had survived VEEV or WEEV challenge.

Sample	PRNT⁸⁰
WEEV E1 serum	<40
VEEV E1 serum	<40
WEEV Survivor serum	200
VEEV Survivor serum	200
Non-immune serum	<40

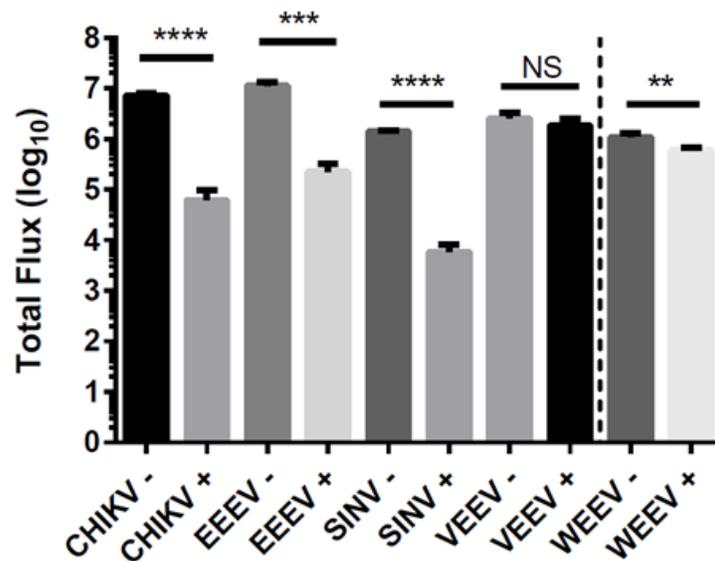
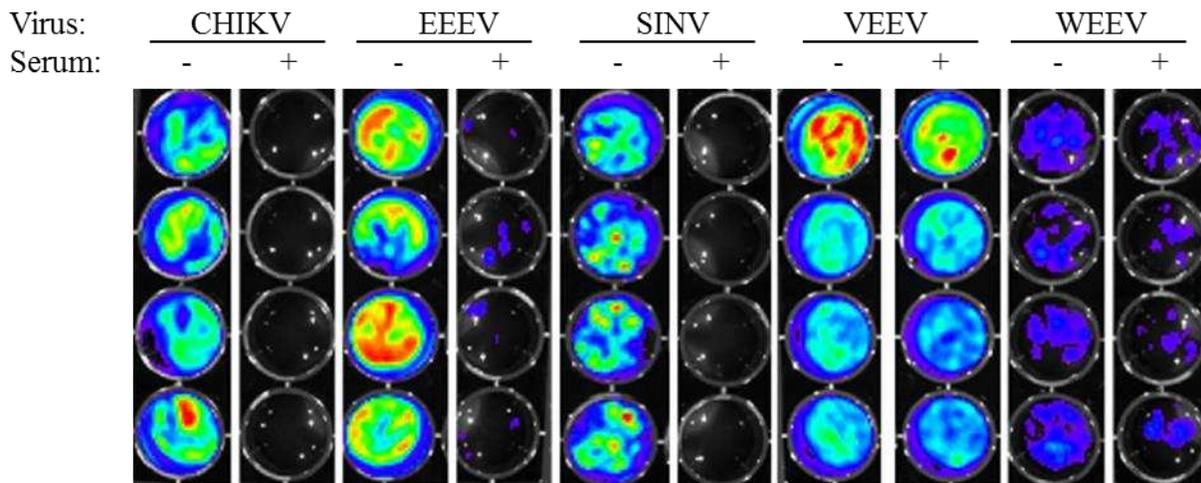


Figure 3. 3 *In vitro* antibody inhibition assay with mouse WEEV E1 sera.

Cells were infected and treated with mouse WEEV E1 sera. Top- Bioluminescence. Bottom- Quantification of bioluminescence. For homologous viruses sera was diluted 1:50. For heterologous viruses sera was diluted 1:10.

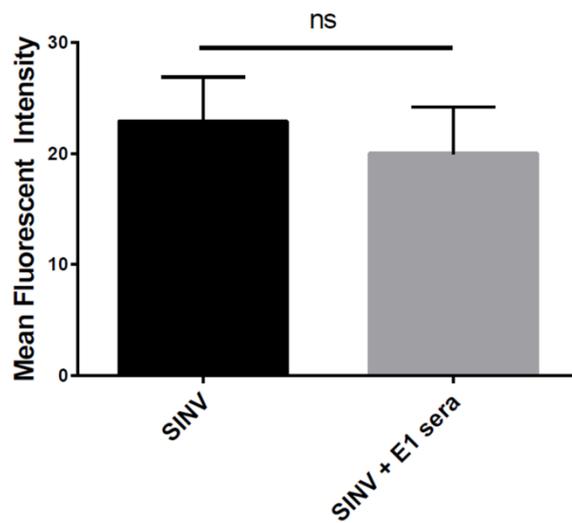
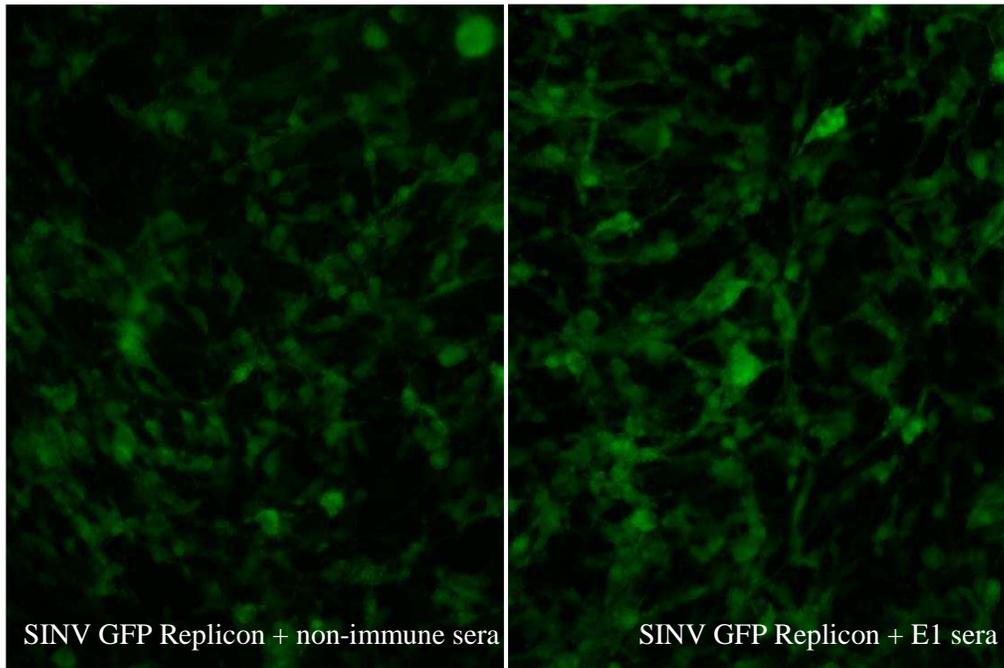
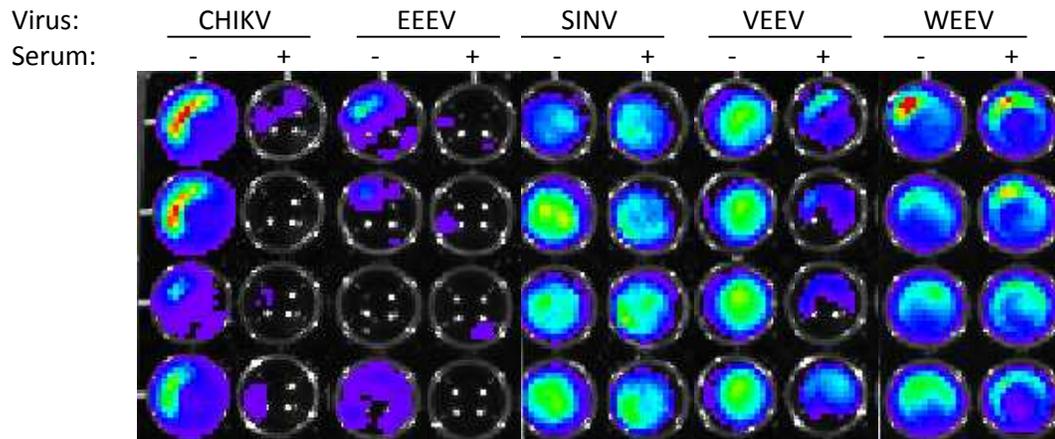


Figure 3. 4 WEEV E1 sera does not inhibit SINV replicon replication.

SINV-TE3'2J Replicon-GFP infected cells treated with mouse WEEV E1 sera (1:10).



aVEEV E1

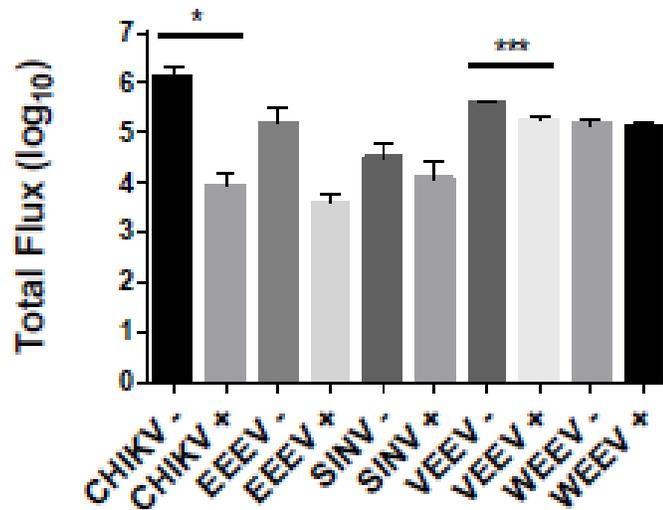
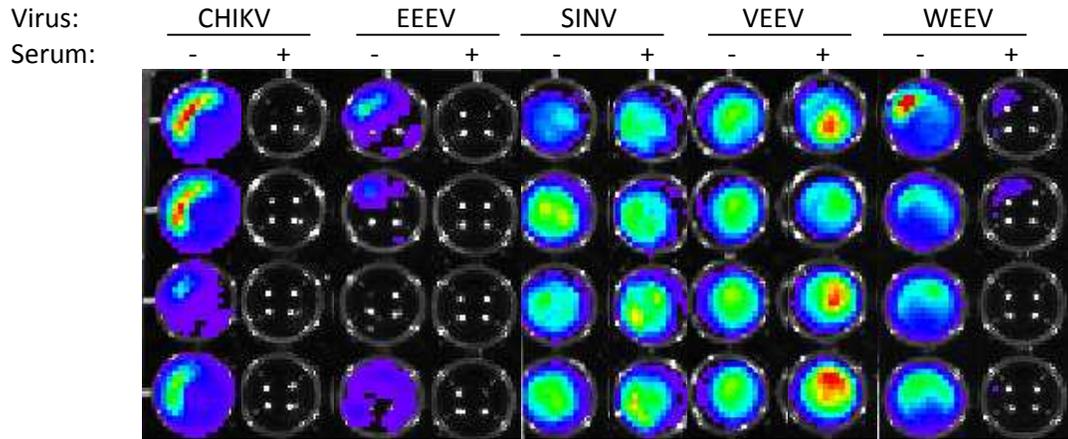


Figure 3. 5 *In vitro* antibody inhibition assay with rabbit VEEV E1 sera.

Cells were infected and treated with rabbit VEEV E1 sera. Top- Bioluminescence. Bottom- Quantification of bioluminescence. Sera was diluted 1:10.



aWEEV E1

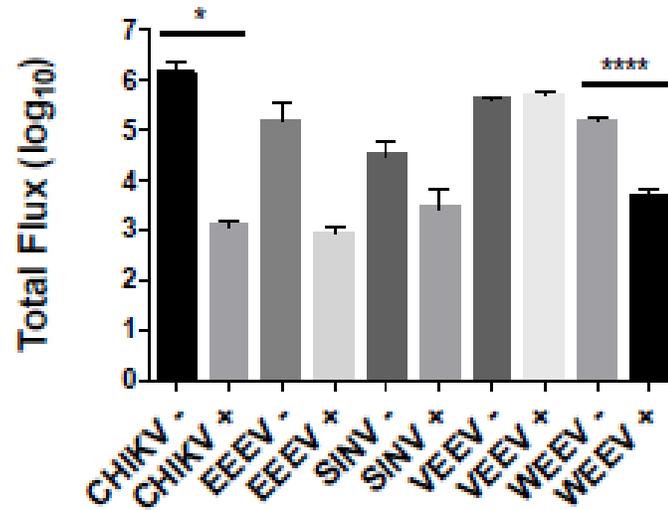
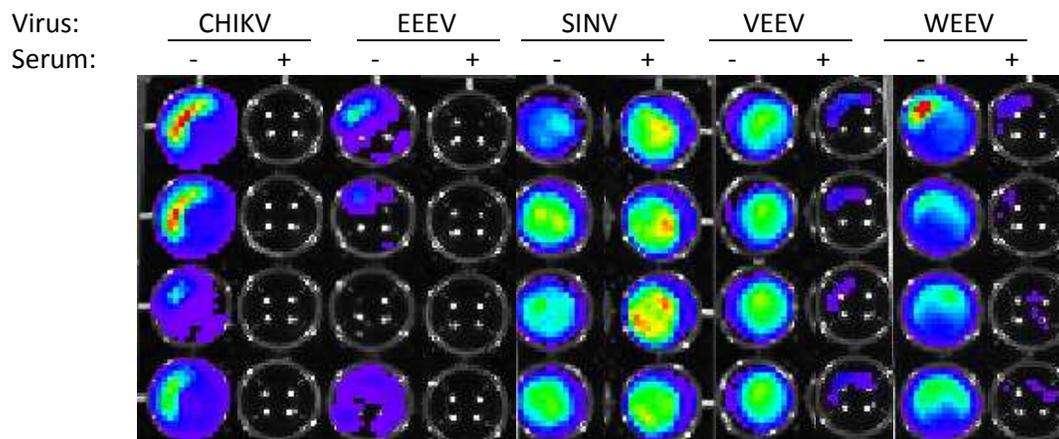


Figure 3. 6 *In vitro* antibody inhibition assay with rabbit WEEV E1 sera.

Cells were infected and treated with rabbit WEEV E1 sera. Top- Bioluminescence. Bottom- Quantification of bioluminescence. Sera was diluted 1:10.



aVEEV + WEEV E1

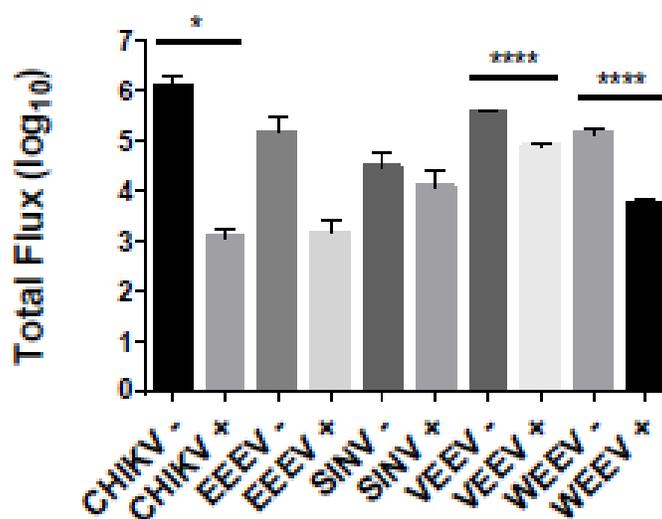


Figure 3. 7 *In vitro* antibody inhibition assay with rabbit VEEV E1 + WEEV E1 sera.

Cells were infected and treated with rabbit VEEV E1 + WEEV E1 sera. Top- Bioluminescence. Bottom- Quantification of bioluminescence. Sera was diluted 1:10.

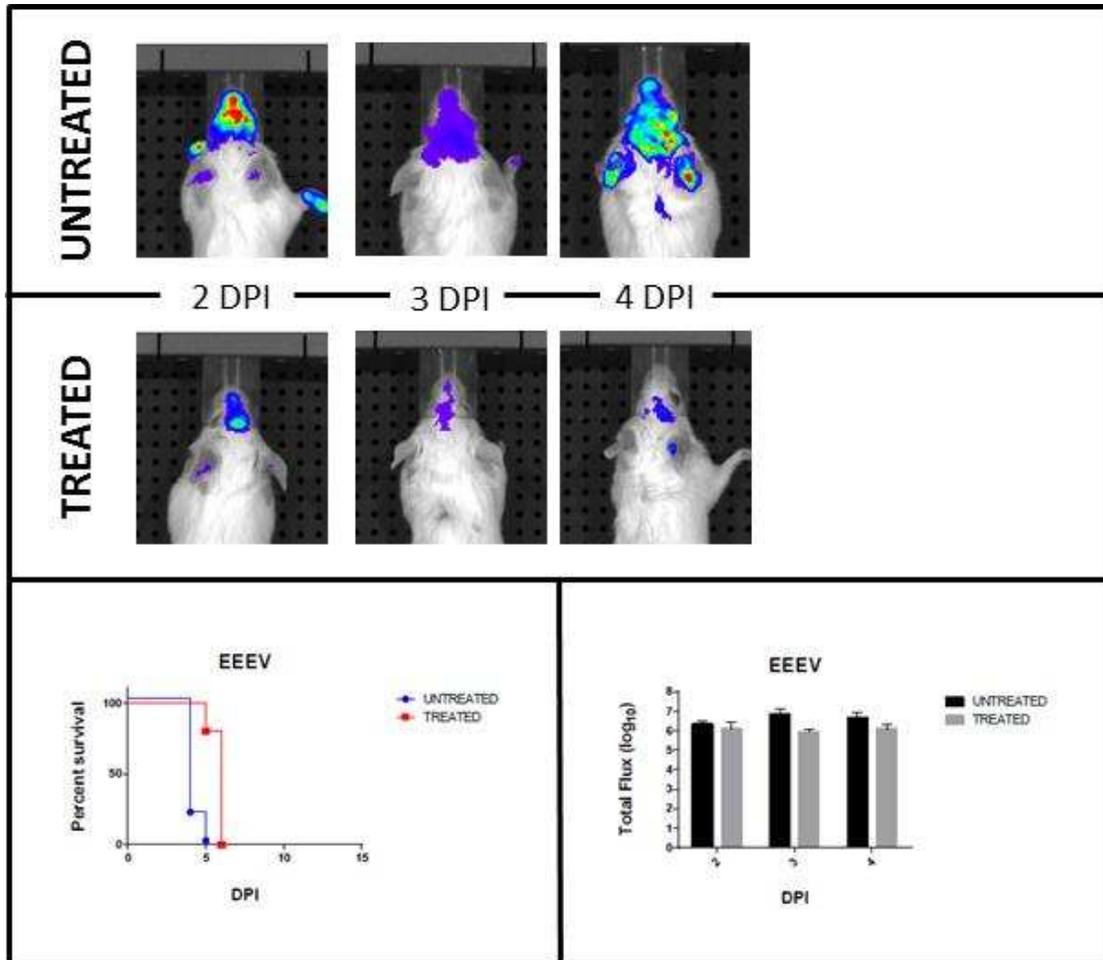


Figure 3. 8 Passive transfer of VEEV E1 + WEEV E1 sera to EEEV-FL93-NLuc IN infected mice.

Mice (n=5/group) were challenged by IN inoculation with 10^4 PFU of EEEV-FL93-NLuc virus then imaged at 2, 3 and 4 DPI. Treated mice received rabbit VEEV E1 + WEEV E1 4 hours before infection. Top - Bioluminescence. Bottom left- Survival. Bottom right- Bioluminescence quantification. Each bar represents the average bioluminescence signal for each group.

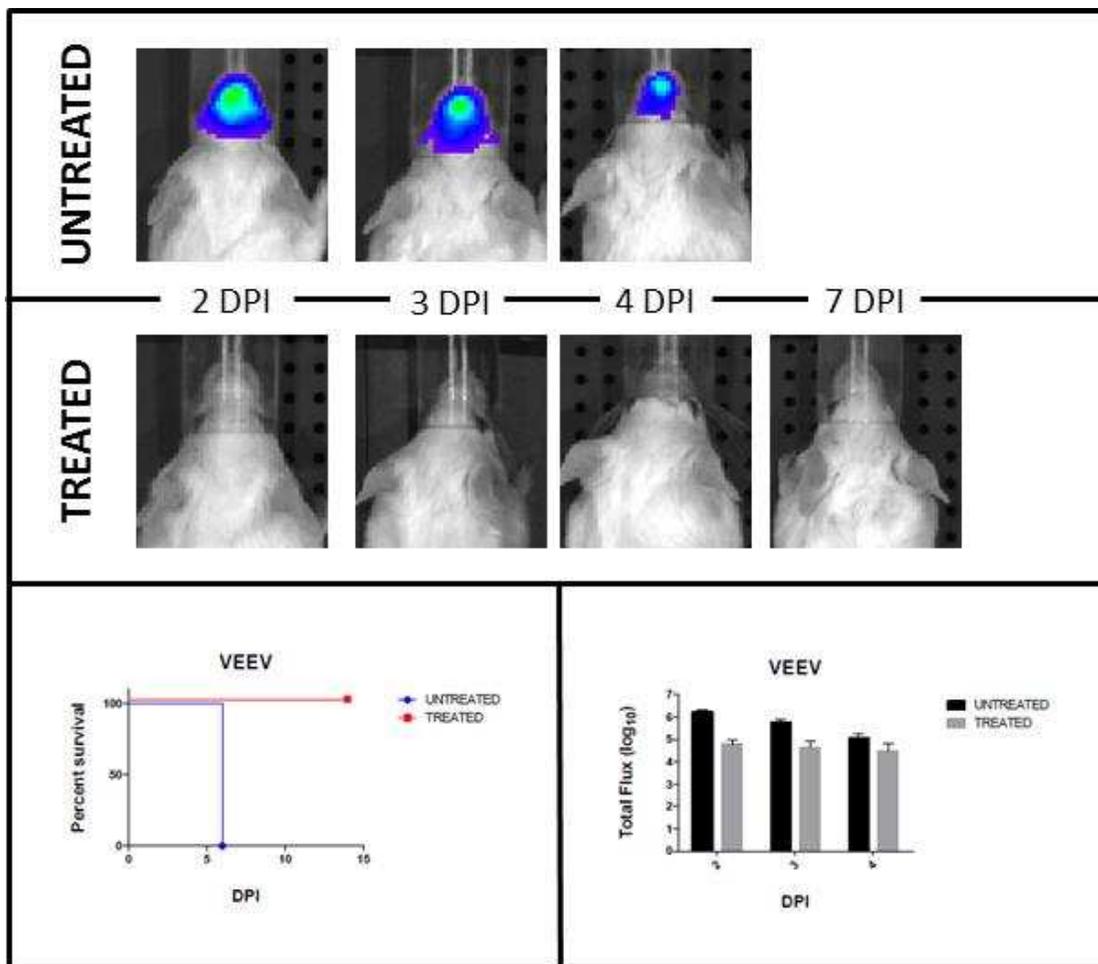


Figure 3. 9 Passive transfer of VEEV E1 + WEEV E1 sera to VEEV-3908-FLuc IN infected mice.

Mice (n=5/group) were challenged by IN inoculation with 10⁴ PFU of VEEV-3908-FLuc virus then imaged at 2, 3, 4 and 7 DPI. Top - Bioluminescence. Treated mice received rabbit VEEV E1 + WEEV E1 4 hours before infection. Bottom left- Survival. Bottom right- Bioluminescence quantification. Each bar represents the average bioluminescence signal for each group.

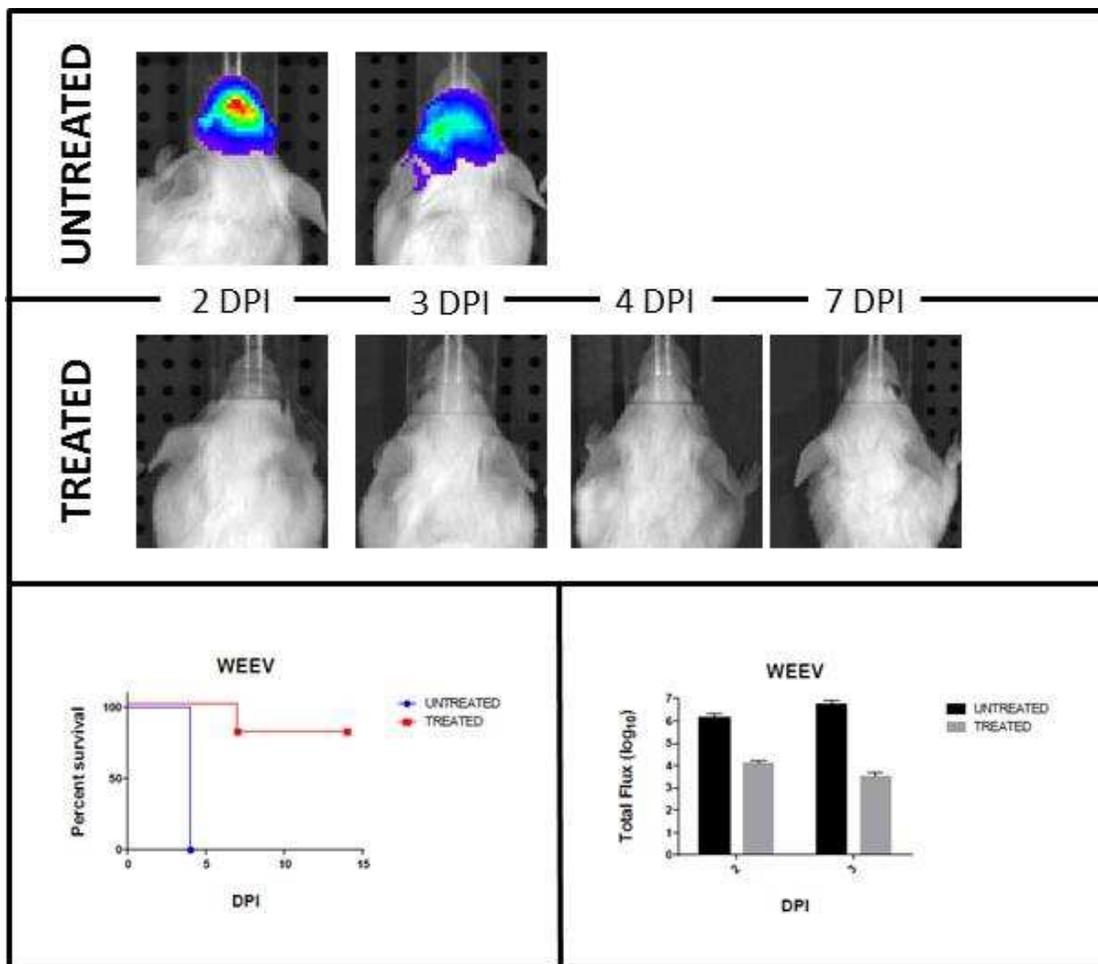


Figure 3. 10 Passive transfer of VEEV E1 + WEEV E1 sera to WEEV-5'McM-FLuc IN infected mice.

Mice (n=5/group) were challenged by IN inoculation with 10^4 PFU of WEEV-5'McM-FLuc virus then imaged at 2, 3, 4 and 7 DPI. Treated mice received rabbit VEEV E1 + WEEV E1 4 hours before infection. Top - Bioluminescence. Bottom left- Survival. Bottom right- Bioluminescence quantification. Each bar represents the average bioluminescence signal for each group.

bioluminescence signal compared to mice receiving non-immune sera. Mice receiving VEEV E1 + WEEV E1 sera and EEEV challenge had progress intensification of bioluminescence signal until euthanasia. Mice receiving VEEV E1 + WEEV E1 sera showed no observable neurological deficits despite virus replication within the CNS (Figure 3.11). Of mice receiving VEEV E1 + WEEV E1 sera, 100% of VEEV infected mice were alive at 14 DPI ($p=0.0027$), 80% of WEEV infected mice were alive at 14 DPI ($p=0.0027$), and 0% of EEEV infected mice were alive at 14 DPI (2 day MTD extension ($p=0.0047$)). All mice receiving non-immune sera were euthanized due to neurological deficits.

Discussion

In this report, we describe the characterization of alphavirus E1 antibodies. We demonstrate that alphavirus E1 polyclonal antibodies bind to infected cells and purified E1, but not intact virions. E1 polyclonal antibodies are, additionally, non-neutralizing yet capable of inhibiting virus replication using our *in vitro* antibody inhibition assay. These findings are not unexpected as similar binding and neutralization properties have been previously reported for alphavirus E1 monoclonal antibodies (Hunt and Roehrig, 1985; Wust et al., 1989). Of particular significance, are the data derived from our *in vitro* antibody inhibition assays.

Using our *in vitro* antibody inhibition assay, we were able to assess the efficacy of non-neutralizing antibodies as alphavirus therapeutics *in vitro*. Traditionally, the therapeutic evaluation of non-neutralizing alphavirus antibodies has been restricted to passive transfer experiments. With this assay, we report a convenient system with which to pre-screen alphavirus E1 antibodies before *in vivo* studies are carried out.

Using our *in vitro* antibody inhibition assay (Phillips et al., 2014), we additionally demonstrate the highly cross-protective nature of E1 antibody. Significantly, previous studies demonstrating E1 cross-reactivity have relied on ELISA based assays. To our knowledge, this is among the first studies to show cross-protection in the context of E1 alone. Though E1 is highly conserved among alphaviruses and E1 antibodies are more cross-reactive than antibodies to E2, we did not expect the high level of cross-



Figure 3. 11 E1 antibody probably crosses the BBB to inhibit virus replication.

WEEV-5'McM-FLuc infected CD-1 mouse passively transferred rabbit E1 antibody. Mouse recovered from virus infection without intensification of neurological symptoms. Note the high level of viral replication within the CNS. Clearance of virus infection from CNS without intensification of neurological symptoms indicates that passively transferred antibody cleared virus from the CNS in a non-lytic manner.

protection observed. We found that mouse WEEV E1 antibody was cross-protective across three alphavirus complexes. Significantly, we report differences between the efficacy of mouse and rabbit sera *in vitro*. Currently, it is unclear whether these *in vitro* differences are due to a difference in titer of the antibodies or whether the differences are related to the antibody mechanism of action. Lastly, we show that E1 polyclonal sera is protective *in vivo*. Significantly, we show that E1 polyclonal sera protects against VEEV and WEEV and cross-protects (extension in MTD) against EEEV. These results extend E1 cross-reactivity data (Hunt and Roehrig, 1985; Wust et al., 1989) to the context of *in vitro* and *in vivo* cross-protection. Importantly, we have observed differences in *in vivo* protection resulting from passive transfer of mouse versus rabbit antibody (data not shown). In this case, it was clear that the survival differences were not related to antibody titer, but likely resulted from the inability of the Fc fragment of rabbit antibody to react with mouse immune cells. Previous studies have demonstrated that cytotoxicity is an unlikely mechanism for clearance of infection from neurons (Levine et al., 1991); however, Fc interactions could be utilized in transfer of antibody across the blood brain barrier (Hirsch et al., 1979) or to stimulate effector cells in a non-cytotoxic manner (DiLillo and Ravetch, 2015). Further studies will be required to identify the role of the Fc fragment in *in vivo* alphavirus protection.

We have made considerable progress towards identifying the mechanism of protection through which protective, non-neutralizing E1 antibodies act. Importantly, we show that antibody is acting at a late stage of virus replication as alphavirus replicons are uninhibited by E1 antibody. Based on this information and cross protection data, we suspect that a limited number of E1 epitopes are involved in protection. We have identified several E1 amino acid differences between alphavirus E1 glycoproteins that could account for the cross-protection profile observed; including amino acid 76, 136, 244, 308, and 392. Importantly, one such amino acid change (136) corresponds to the E1 fusion domain and is, additionally, glycosylated, a factor that has been previously shown to be important for protection (Das et al., 2004). Further studies will be required to examine the number and location of the E1 epitopes targeted by our polyclonal sera. Lastly, we demonstrate that E1 polyclonal sera is capable of protecting mice from lethal infection after the establishment of CNS infection. Antibody is likely crossing the blood-brain

barrier and acting in a non-lytic manner to do so as virus is cleared from the CNS without any increase in neurological symptoms (Figure 3.10). Importantly, at the time of recovery from CNS infection the BBB is still intact (Schafer et al., 2011) and does not represent a possible route of antibody transport into the brain. Further experiments involving Evans blue administration, to monitor BBB integrity, and whole brain staining for E1 antibody will be required to confirm antibody presence within the CNS.

This report describes the characterization of non-neutralizing alphavirus E1 antibodies. We report the use of a convenient system for the screening of non-neutralizing alphavirus E1 antibodies. Using this screen, we have provided data demonstrating the cross-protective efficacy of alphavirus E1 antibodies and have made progress towards the characterization of their mode of action. Importantly, the humanization of the E1 antibodies described here and elsewhere (Hunt and Roehrig, 1985; Wust et al., 1989) could greatly expand current alphavirus therapeutic options.

CHAPTER FOUR: ANTIVIRALS TO TREAT ALPHAVIRUS INFECTIONS

Introduction

Alphaviruses are mosquito-borne viruses that cycle between mosquitoes and vertebrates, and cause disease in humans. The public health threat posed by alphaviruses is a result of two factors: natural transmission and biosafety. Since 2014, over one million cases of alphavirus infection have occurred due to natural transmission with numerous deaths. In addition to naturally occurring infections, alphaviruses pose a threat to public health because of their potential use as weapons of bioterrorism (Pamela, 2011). In 1942, the United States began weaponizing VEEV as part of its offensive biological weapons program. Though VEEV was readily weaponized, efforts to develop defensive therapeutic countermeasures were unsuccessful. Today, there are no licensed antivirals for the treatment of alphavirus infection, and treatment options are limited to supportive care. New antivirals for the treatment of alphavirus infection are required to oppose both natural and bioterror related alphavirus infections.

Alphaviruses are small, enveloped, positive-sense RNA viruses. The alphavirus genome is approximately 11,700 nucleotides long and is capped and polyadenylated (Strauss and Strauss, 1994). Alphaviruses possess two open reading frames (ORFs) that encode for nine viral proteins: four non-structural proteins and five structural proteins. Each of these proteins is essential and a potential therapeutic target (Reichert et al., 2009; Strauss and Strauss, 1994). Host-derived proteins and neuroprotective agents are, additional, therapeutic targets (Reichert et al., 2009).

In this report, we identify and characterize novel inhibitors of alphavirus replication. Following a high-throughput ELISA-based screen (Spurgers et al., 2013) conducted by our collaborators, 35 compounds were identified that inhibit CHIKV, EEEV, VEEV, and WEEV replication. Using traditional and bioluminescence-based *in vitro* and *in vivo* assays, we demonstrate that several lead compounds identified by cell-based ELISA screen are effective alphavirus inhibitors. We, additionally, define a working mechanism of action for several compounds. Together, these studies support the use of cell-

based ELISAs and bioluminescence-based screens for the identification of alphavirus antivirals and definition of compound mechanisms of action.

Methods

Virus strains

Chikungunya virus 15561 strain, EEEV FL93-939 strain, VEEV Trinidad strain, and WEEV CBA-87 strain were obtained from USAMRIID archives. Recombinant luciferase-expressing VEEV plasmid (VEEV-3908-FLuc) was obtained from Dr. Scott Weaver (University of Texas Medical Branch) and virus was generated as follows: MluI linearized VEEV-3908-FLuc plasmid was purified by QIAprep Spin MiniPrep Kit (Qiagen) and genomic RNA was *in vitro* transcribed using a T7 RNA Polymerase MAXIscript kit (Life Tech). Vero cells (4×10^6 cells in 400 μ L) (ATCC) were electroporated with genomic RNA using an ECM 630 electroporator (BTX Harvard Apparatus). Two pulses (450 V, 1200 Ω , and 150 μ F) were administered. Forty-eight hours post electroporation virus was recovered from supernatant. SINV-TE3'2J-FLuc plasmid was obtained from Dr. Brian Geiss (CSU) and virus was generated as follows: plasmid was transfected into BHK cells using Lipofectamine 2000 transfection reagent (Life Tech) according to the manufacturer's instructions. Twenty-four hours post transfection virus was harvested from supernatant. CHIKV-ReUnion-NLuc and EEEV-FL93-NLuc plasmids were obtained from Dr. William Klimstra (The University of Pittsburgh) and rescued as described (Sun et al., 2014). pWEEV-5'McM-FLuc was generated by duplication of the subgenomic promoter sequence immediately downstream of nsP4. MfeI linearized pWEEV-5'McM-FLuc was purified by QIAprep Spin MiniPrep Kit (Qiagen) and infectious clone genomic RNA was *in vitro* transcribed using a T7 RNA Polymerase MAXIscript kit (Life Tech). Vero cells (4×10^6 cells in 400 μ L) (ATCC) were electroporated with genomic RNA using an ECM 630 electroporator (BTX Harvard Apparatus). Two pulses (450 V, 1200 Ω , and 150 μ F) were administered. Forty-eight hours post electroporation virus was recovered from supernatant. All recombinant viruses were used without further passage after rescue. Viruses were titered by plaque assay on Vero or BHK cells as described previously (Mirchamsy and Rapp, 1968) and stored at -80C.

Compound screening by ELISA

The library of approximately 2600 previously existing compounds was comprised of FDA-approved and ex-US approved drugs, targeted molecular probes, and nutraceuticals. ELISA based drug screens were performed blinded by our collaborators at USAMRIID with the compound identities unknown to the researchers. Compounds were diluted (0.0-40 μ M depending on compound) and mixed with CHIKV (15561) (0.1 MOI) , EEEV (FL93-939) (0.01 MOI), VEEV (Trinidad) (0.01 MOI), or WEEV (CBA-87) (0.01 MOI) virions. Confluent Vero cell monolayers were then infected with compound/virus mixture at an MOI of 0.01 (EEEV, VEEV, and WEEV) or 0.1 (CHIKV). At 24 HPI, cells were fixed in 3.7% formaldehyde in PBS at 4C for 18-24 hours. Cells were washed in PBS and blocked with 3% BSA in PBS for one hour at RT. Anti-CHIKV, EEEV, VEEV, or WEEV antibody was diluted in PBS + 3% BSA and added to cells for two hours at RT. After washing with PBS, cells were incubated with HRP conjugated secondary antibody diluted in PBS + 3% BSA for one hour at RT. Color was developed and read at an absorbance of 425 nm.

Cell viability

Cell viability assays were completed by our collaborators at USAMRIID. Compounds were diluted to the indicated concentrations in complete media and added to Vero cells. Cells were incubated for 48 hours at 37C and then moved to RT for 30 minutes. Cell Titer Blue reagent (Promega) was added to cell culture supernatant in each well, and incubated for 3 hours at 37C. Fluorescence was then measured in each well.

Yield reduction and time of addition assays

Yield reduction and time of addition assays were done at both USAMRIID and CSU. Vero cells were infected with VEEV Trinidad or 3908 at a MOI of 0.1 for one hour at RT with rocking every 15 minutes. After infection, virus inocula were removed, cells were washed 3X with PBS, and standard media was added. Complete media containing diluted compound was added to the cells at -1, 0, 0.5, 1, 2, 4, 6, or 8 HPI. Twenty-four HPI, plates were checked for CPE and either imaged for luciferase expression or supernatant was removed for titration by plaque assay.

Virucidal assay

Virucidal assays were done at CSU. Venezuelan equine encephalitis virus 3908 was diluted to 10^7 PFU in diluted compound and incubated for one hour at 37C. Following incubation, samples were immediately diluted 1000X and titered on Vero cells. Positive control samples incubated in 10% bleach were included in each assay.

Pretreatment assay

Pretreatment assays were done at CSU. Cells were seeded the day before infection. On infection day, cells were pretreated with diluted compound for three hours immediately prior to infection. Cells were then washed 3X with PBS and infected with VEEV-3908-FLuc at a MOI of 0.1 for one hour at RT with rocking every 15 minutes. After infection, cells were washed 3X with PBS and overlaid with standard media. Twenty-four HPI plates were imaged for luciferase expression.

Attachment assay

Attachment assays were done at CSU. Cells were seeded the day before infection. On infection day, cells were infected with VEEV-3908-FLuc at a MOI of 0.1 in infection media containing diluted compound for one hour at RT with rocking every 15 minutes. After infection, cells were washed 3X with PBS and incubated in standard media. Twenty-four HPI plates were imaged for luciferase expression.

Mouse studies

The animal use in this study was approved by the Institutional Animal Care and Use Committee at CSU. Mice were handled in compliance with the PHS Policy and Guide for the Care and Use of Laboratory Animals. All experiments were conducted in a biosafety level 3, and in a CDC Select Agent-approved facility. Outbred, 4- to 6- week old female CD-1 mice (Charles River Laboratories) were allowed to acclimate to the facility for one day. Mice were administered 2.5 or 5 mg/kg P-75802 (compound) via the intraperitoneal route. All mice were treated one hour post-challenge. Mice receiving 2.5 mg/kg were treated daily for 7 days and mice receiving 5 mg/kg were treated daily for 5 days. Mice were infected with 10^4 PFU VEEV-3908-FLuc by IN route. Intranasal inoculations were performed by installing inocula into each nostril of lightly anesthetized mice (20 μ L total volume). Inocula were back-

titered to confirm dosages. Mice were observed twice daily for signs of morbidity, and imaged for bioluminescence once daily on days 0-7 and 14. Moribund mice were euthanized by CO₂ inhalation, and the day of euthanasia was taken as the day of death to calculate MTD. Survivorship was followed for 14 days.

Bioluminescence imaging

At 24 HPI, cells infected with luciferase-expressing viruses were imaged using an IVIS 200 system (Perkin Elmer). Plate imaging was performed directly after application of 0.3mg/mL D-luciferin. Exposure time was kept to five seconds under standard settings. Mouse imaging was performed daily as described previously (Phillips et al., 2013). Living Image 3.0 software (Caliper Life Science) was used to analysis and process images. Total light emission from each well/mouse was determined using software provide grids and collecting light emission data.

Statistics

Time of addition studies, yield reduction assays, virucidal, pretreatment, and attachment assays statistical comparisons were done using Kruskal-Wallis non-parametric ANOVA (Graphpad). Clinical scores and weight loss were compared using linear regression followed by multiple comparisons tests. Kaplan-Meier (log rank test) analyzes were applied to survival curves using Prism version 6.00 for Windows (Graphpad).

Results

Identification of lead anti-alphavirus compounds by ELISA

Our collaborators assessed the ability of a panel of compounds to inhibit CHIKV, EEEV, VEEV, and WEEV *in vitro* by ELISA. Cut off criteria was greater than 50% virus inhibition and less than 30% cytotoxicity. Of 2316 tested compounds: 35 inhibited all four alphaviruses; two inhibited CHIKV, EEEV, and WEEV only; one inhibited CHIKV and WEEV only; three inhibited CHIKV and EEEV only; and eight compounds selectively inhibited CHIKV yielding an overall hit rate of 2.1% (Table 4.1 and Figure 4.1). Table 4.2 summarizes compound inhibitory concentrations and cytotoxicity concentration.

Table 4. 1 Alphavirus *in vitro* therapeutic screen hit rates.

Three dosage compound screen (3-pt), eight dosage compound screen (8-pt).

Virus	Screen	# of Compounds Tested	#/% of Active Compounds
VEEV	3-pt Vero	2316	48/2.1%
VEEV	8-pt Vero	69	35/51%
EEEV	8-pt Vero	69	40/58%
WEEV	8-pt Vero	69	38/55%
CHIKV	8-pt Vero	69	49/71%

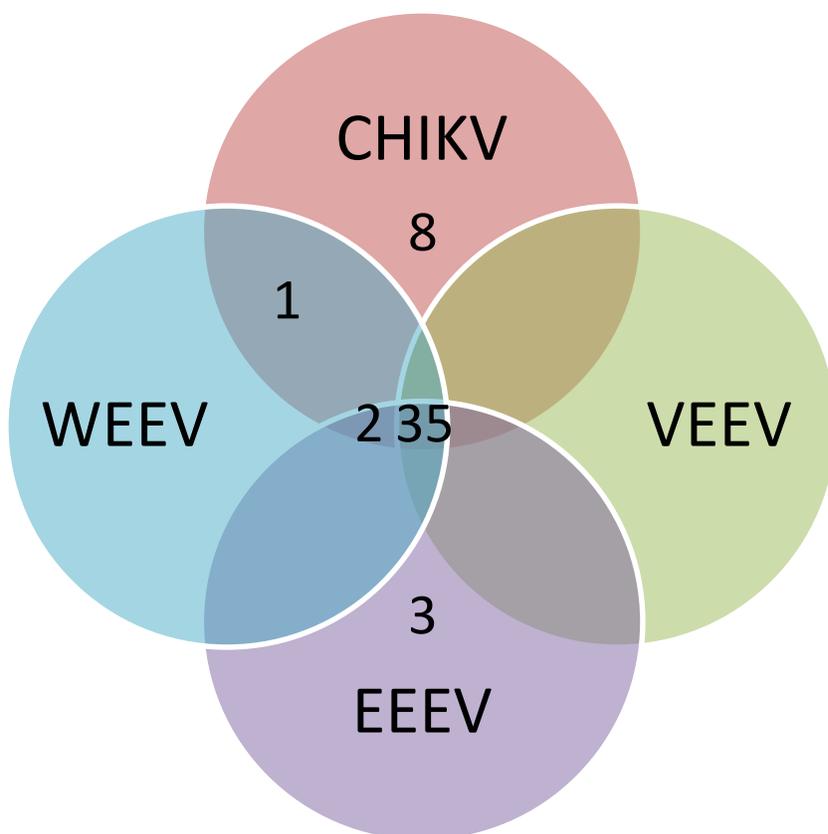


Figure 4. 1 Virus specific compound hit distributions.

Table 4. 2 Alphavirus therapeutic candidates from *in vitro* screens.

50% inhibitory concentration (IC⁵⁰) for CHIKV, EEEV, WEEV, and VEEV; 50% cytotoxicity concentration (CC⁵⁰); maximum concentration tested (Max []); minimum concentration tested (Min []); less than minimum concentration tested (<MCT). Concentrations in μM.

Drug	VEEV-IC ⁵⁰	EEEV-IC ⁵⁰	WEEV-IC ⁵⁰	CHIKV-IC ⁵⁰	CC ⁵⁰	Max []	Min []
	29.3	25.6	24.2	18.6	37.1	39.9	0.6
	0.04	0.05	0.05	0.04	ND	3.9	0.06
	1.5	1.8	1.6	0.6	6.0	7.9	0.06
	19.2	6.8	4.5	2.7	ND	39.9	0.6
	1.0	4.8	4.2	3.4	ND	12	0.1
	<MCT	<MCT	<MCT	<MCT	ND	7.9	0.0
	15.3	13.0	8.4	5.0	17.2	39.9	0.6
	0.005	0.003	0.002	<MCT	ND	3.9	0.0
	<MCT	<MCT	<MCT	<MCT	ND	3.9	0.0
	<MCT	<MCT	<MCT	<MCT	ND	7.9	0.1
	0.7	0.6	0.4	0.2	ND	7.9	0.1
	0.2	0.3	0.1	0.2	ND	3.9	0.0
	0.4	.04	.02	.01	ND	3.9	0.0
	5.6	4.4	4.0	1.2	ND	19.9	0.3
	14.3	13.0	8.2	5.4	ND	39.9	0.6
P-75802	0.1	0.09	0.09	<MCT	ND	3.9	0.0
	2.9	2.9	1.3	1.4	ND	27.9	0.4
P-75811	0.1	0.08	0.1	0.07	ND	3.9	0.0
	0.4	0.2	0.3	0.3	ND	14	0.2
	24.0	11.6	8.2	7.1	ND	39.9	0.6
	1.8	1.6	1.8	1.3	ND	19.9	0.3
	9.4	7.2	7.5	4.7	13.1	19.9	0.3
	0.2	0.1	0.1	0.08	ND	3.9	0.0
	15.1	11.8	11.8	7.4	ND	19.9	0.3
	21.7	23.7	29.1	12.9	ND	39.9	0.6
	<MCT	<MCT	<MCT	<MCT	ND	3.9	0.0
	41.3	30.3	44.9	20.8	68.4	119	0.9
P-75805	<MCT	<MCT	<MCT	<MCT	ND	3.9	0.0
	1.6	1.3	1.4	1.2	ND	39.9	0.6
	2.4	1.2	1.8	0.2	ND	3.9	0.0
	7.8	5.1	6.1	3.1	ND	19.9	0.3
	12.9	13.8	12.1	7.2	ND	19.9	0.3
	26.2	17.1	25.6	9.5	28.6	39.9	0.6
P-75803	<MCT	<MCT	<MCT	<MCT	ND	3.9	0.0
	32.8	23.8	26.9	10.4	ND	39.9	0.6

Compound identity was kept blinded throughout the study. Several compounds (11) were near the cut off criteria in the three dosage compound screen and were included in the eight dosage compound screen.

Verification of lead anti-alphavirus compounds by VEEV yield reduction assay

Following lead compound identification, four compounds were tested for VEEV inhibition by yield reduction assay (Figure 4.2). Yield reduction assay results closely resembled those of our ELISA screen. P-75802, P-75803, and P-75805 each inhibited VEEV replication by greater than four logs. P-75811 inhibited VEEV replication by three logs.

Characterization of the mechanism of action for lead anti-alphavirus compounds

Compounds verified for anti-VEEV activity by both ELISA and yield reduction assay were subjected to mechanism of action experiments. Using time of addition assays, we identified the time point in the virus life cycle each compound acted. All compounds significantly inhibited VEEV replication compared to controls with no differences observed with time of compound addition (Figure 4.3 and 4.4). These results indicated that all four compounds acted at early time points in virus replication.

Compounds were tested by virucidal, pretreatment, and attachment assays to identify specific mechanisms of action. Using virucidal assay, no compound significantly inhibited VEEV infectivity (Figure 4.5). P-75802 and P-75803 significantly inhibited VEEV replication following pretreatment of cells with compound ($p < 0.001$ and $p < 0.05$ respectively). When compound was added at the time of virus attachment, no compound significantly inhibited VEEV replication.

In vivo efficacy

P-75802 was tested for antiviral efficacy *in vivo*. Mice were treated with either 2.5 mg/kg or 5 mg/kg P-75802 daily for seven or five days, respectively. Treatment with 2.5 mg/kg P-75802 extended the mean time to death of mice from 6 DPI to 8 DPI ($p < 0.01$) (Figure 4.6 and 4.7). Mean time to death of mice in the 5 mg/kg group was unchanged (Figure 4.6 and 4.7). P-75802 was slightly toxic on its own at the high dose (data not shown) and could explain the lack of protection seen at the high dose. Despite the extension in mean time to death, mice in the 2.5 mg/kg treatment group had similar clinical scoring and

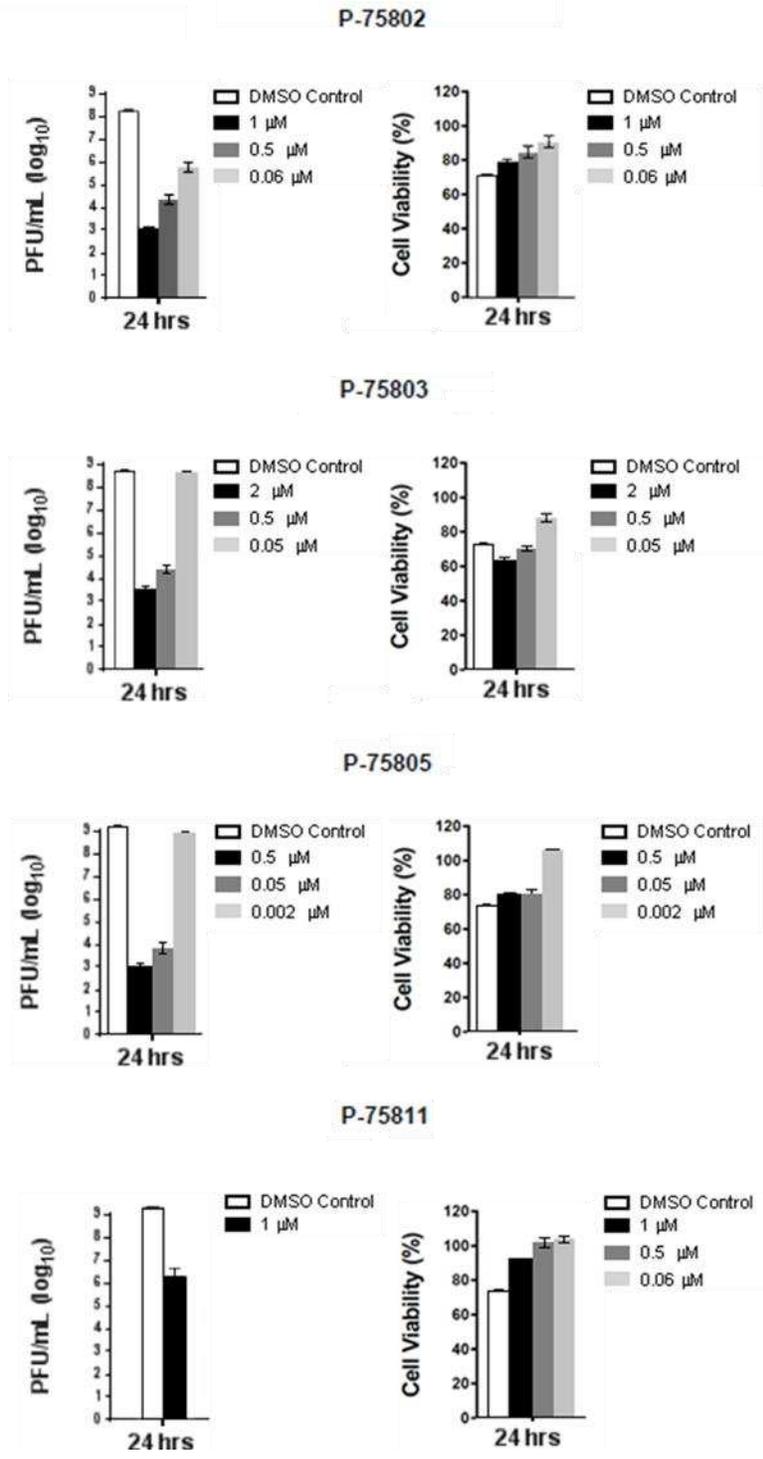


Figure 4. 2 Compounds inhibit VEEV replication by yield reduction assay.

PFU/mL media in supernatants infected with VEEV 3908 0.1 MOI at 24 HPI. Compound was added at 0 HPI.

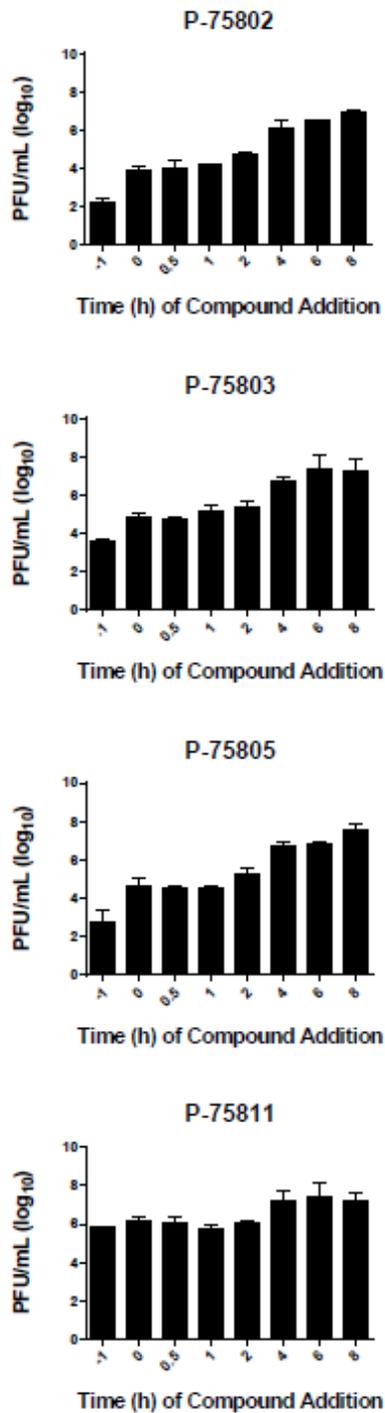


Figure 4. 3 Compounds inhibit early stages of virus replication (PFU/mL).

Time of addition assays. PFU/mL media in supernatants infected with VEEV 3908 0.1 MOI at 24 HPI. Compound was added at either -1, 0, 0.5, 1, 2, 4, 6, or 8 HPI. Differences in time of compound addition were not significant.

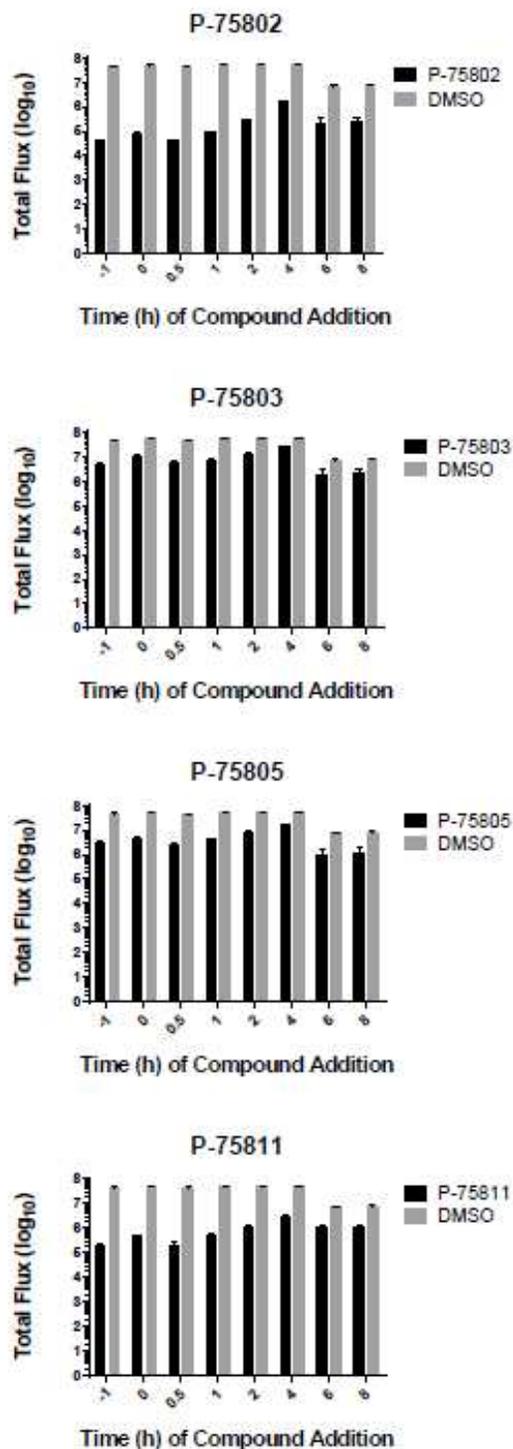


Figure 4. 4 Compounds inhibit early stages of virus replication (Bioluminescence).

Time of addition assays. *In vitro* bioluminescence of cells infected with VEEV-3908-FLuc 0.1 MOI at 24 HPI. Compound was added at either -1, 0, 0.5, 1, 2, 4, 6, or 8 HPI. Differences in time of compound addition were not significant.

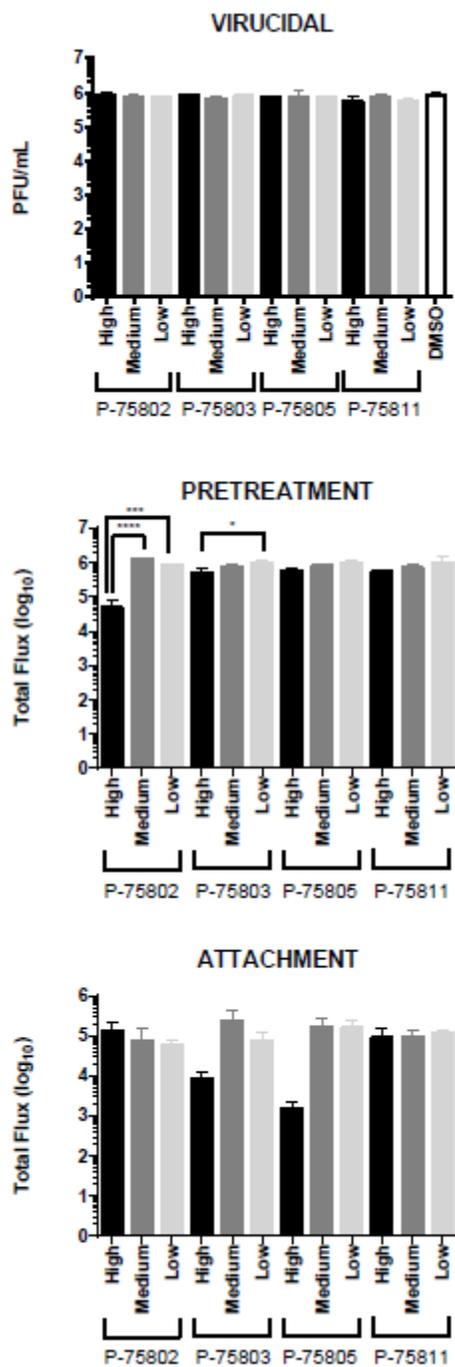


Figure 4. 5 Compound mechanism of action. .

In vitro bioluminescence or PFU/mL of VEEV-3908-FLuc infected Veros. Compounds were tested in virucidal, pretreatment, and attachment assays. No compound was virucidal, P-75802 inhibited VEEV replication upon pretreatment ($p < 0.001$), and no compound affected virus attachment.

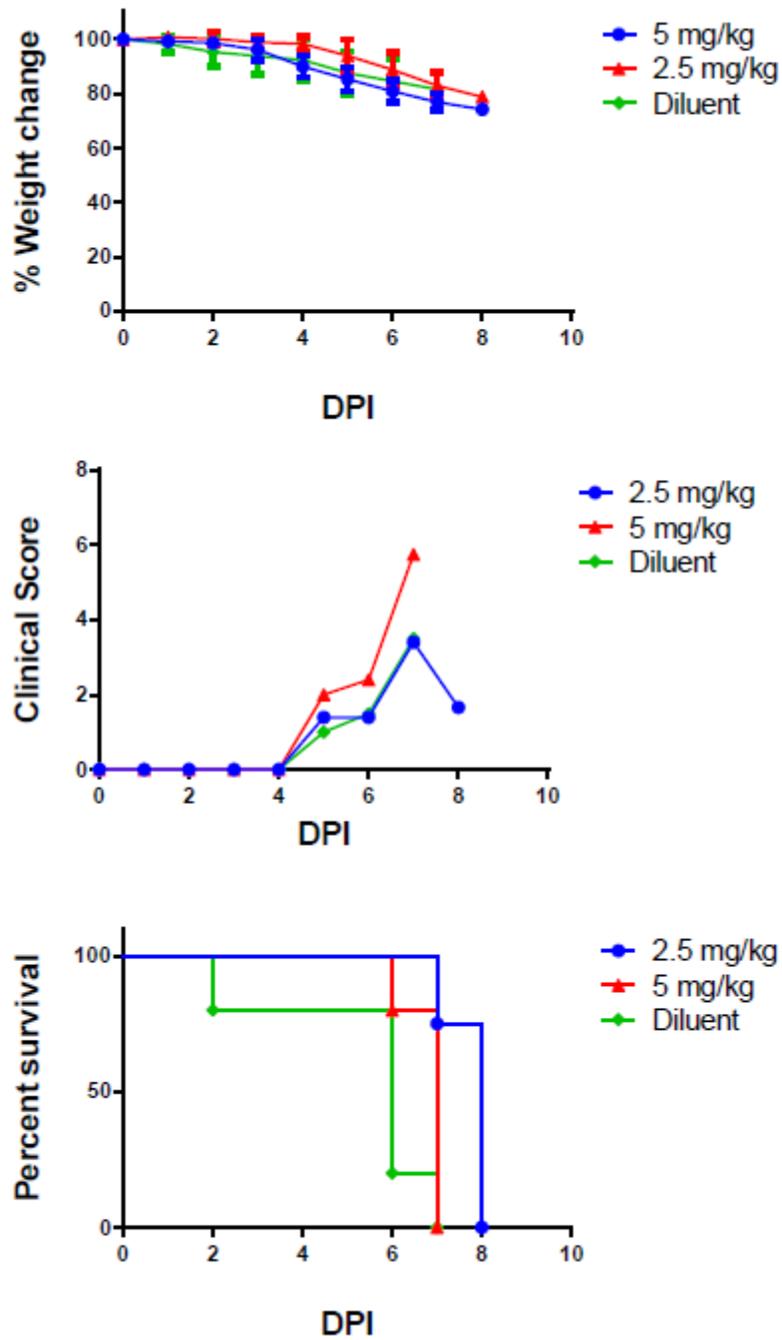


Figure 4. 6 *In vivo* efficacy of P-75802.

VEEV-3908-FLuc infected mice treated with P-75802. Mice in the 5 mg/kg group lost more weight than controls ($p < 0.0001$), no significant difference in clinical scores were observed, and mice treated with 2.5 mg/kg P-75802 had an extended mean time to death ($p < 0.01$).

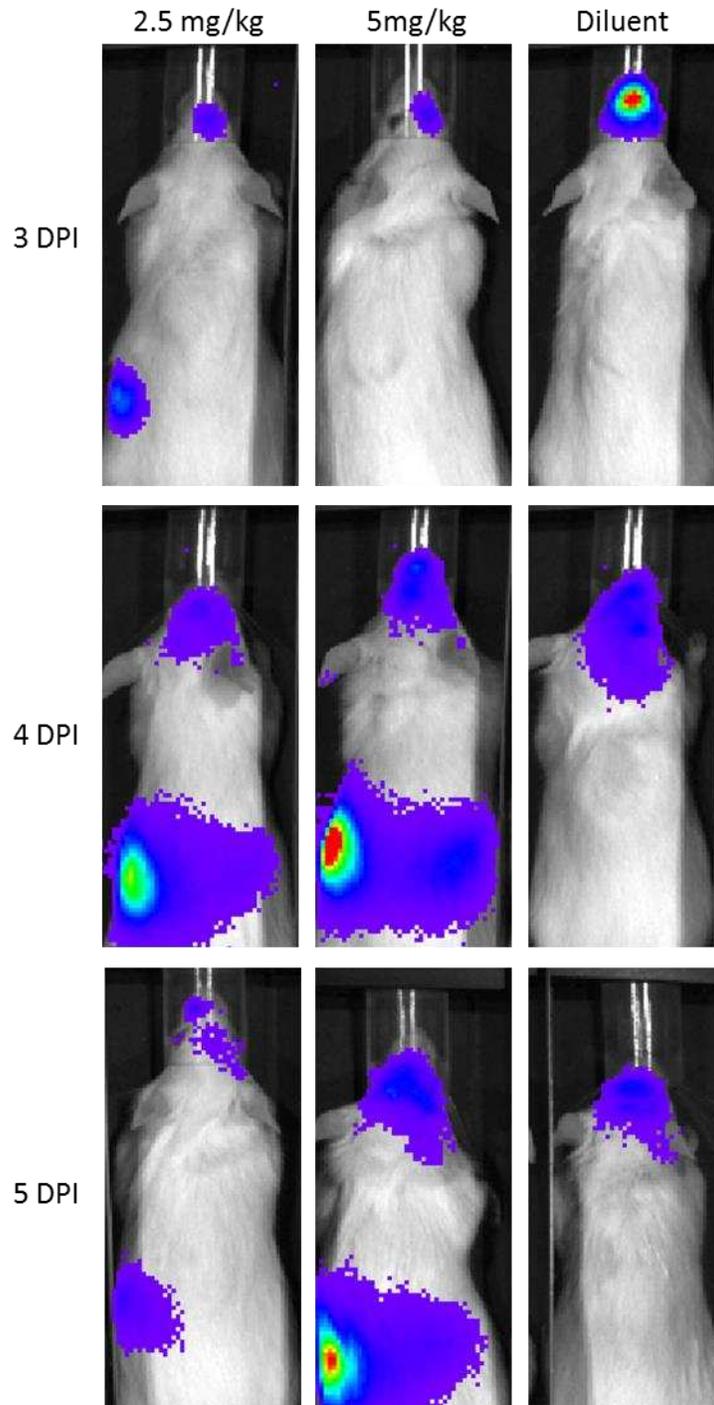


Figure 4. 7 *In vivo* bioluminescence imaging of VEEV-3908-FLuc infected mice.

Mice (n=5/group) were challenged by IN inoculation with 10^4 PFU of VEEV-3908-FLuc virus then treated with 2.5 or 5 mg/kg P-75802 and imaged daily.

weight loss as control animals (Figure 4.6). Mice in the 5 mg/kg treatment group had similar clinical scoring and higher weight loss ($p < 0.0001$) compared to controls (Figure 4.6). No significant differences in bioluminescence were observed between groups; however, mice treated with P-75802 displayed an altered distribution of virus replication as indicated by bioluminescence outside of CNS (Figure 4.7).

Other alphaviruses

P-75802, P-75803, P-75805, and P-75811 were tested for antiviral efficacy against CHIKV, EEEV, SINV, and WEEV by yield reduction assay (Figure 4.8). P-75802 was the most effective compound inhibiting CHIKV ($p < 0.001$), EEEV ($p < 0.001$), SINV ($p < 0.05$), and WEEV ($p < 0.001$). Chikungunya virus was additionally inhibited by P-75803 and P-75805 ($p < 0.05$), and EEEV was inhibited by P-75803 ($p < 0.05$). Lastly, WEEV was inhibited by P-75811 ($p < 0.01$).

Discussion

Screening assays to identify compounds with antiviral activity vary in spectrum from catch all screens that identify any virus inhibitor regardless of mechanism of action to narrow screens that identify a few inhibitors each with a specific mechanism of action. Each type of screen has its inherent advantages and disadvantageous. This study reports the use of cell-based ELISA screens in which the target of any inhibitor is unknown with mechanism of action screens to identify novel alphavirus inhibitors for which the mechanism of action is defined.

We present the results of a screen of 2316 compounds for anti-alphavirus activity. We identified both pan-alphavirus hits and virus-specific hits. Forty-nine of 2316 compounds tested by our collaborators displayed activity against at least one alphavirus with greater than 50% inhibition and less than 30% cytotoxicity. In general, CHIKV had lower IC^{50} concentrations than EEEV, VEEV, and WEEV.

Following confirmation of compound anti-alphavirus activity, compounds were subjected to descriptive mechanism of action experiments. Time of addition experiments were used to separate mechanisms of action into early and late time points within the virus replication cycle. All lead compounds affected early stages. No compound had direct virucidal activity. P-75802 and P-75803 were effective at inhibiting VEEV replication upon compound pretreatment suggesting that P-75802 and P-

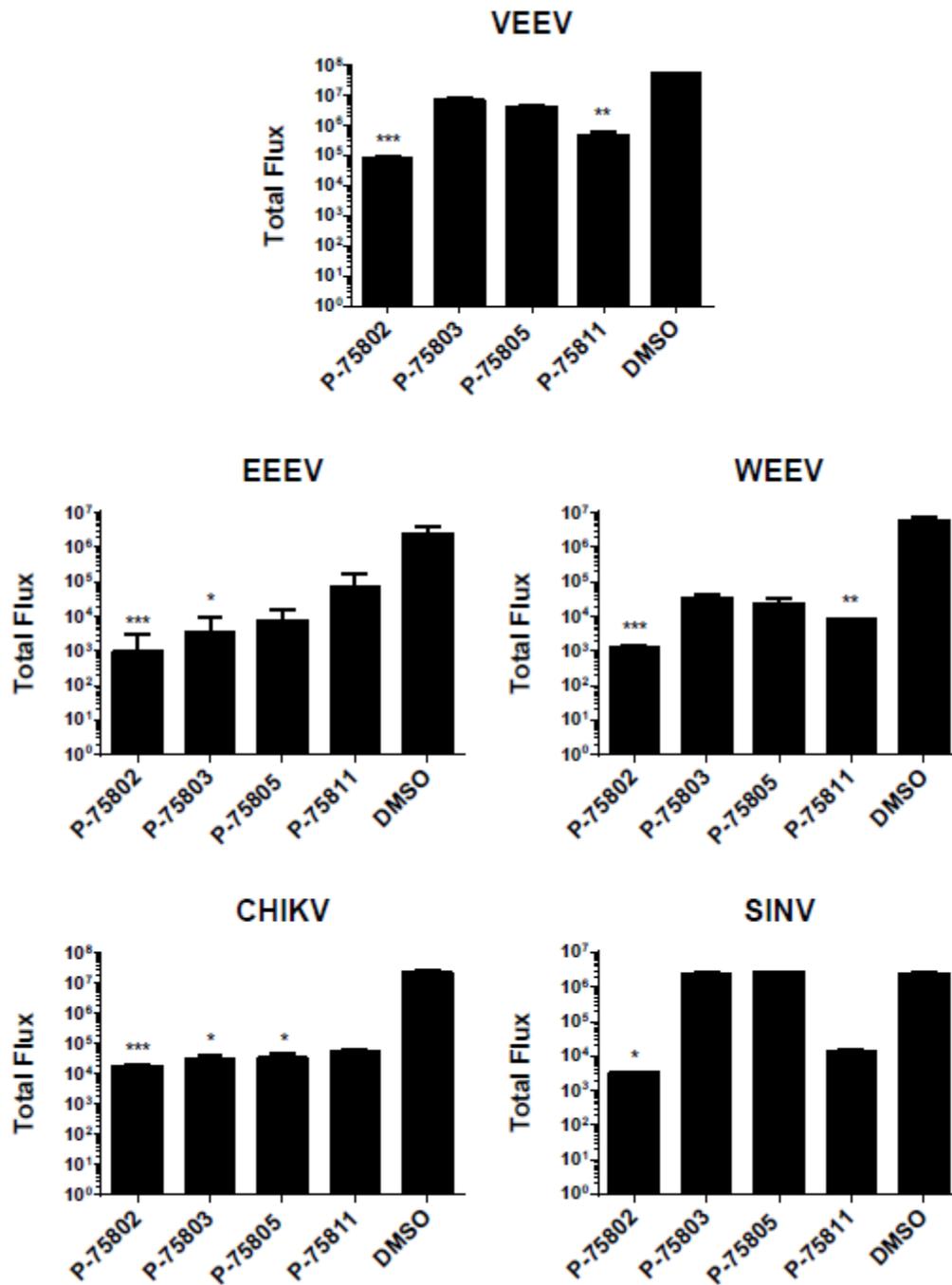


Figure 4. 8 Compounds inhibit the replication of non-VEEV alphaviruses by yield reduction assay.

In vitro bioluminescence of CHIKV-ReUnion-NLuc, EEEV-FL93-NLuc, SINV-TE3'2J-FLuc, VEEV-3908-FLuc, and WEEV-McM-FLuc infected cells (0.1 MOI) treated with P-75802, P-75803, P-75805, or P-75811.

75803 inhibition may involve cellular host factors. Additional investigation suggested that P-75803 and P-75805 may inhibit virus entry at higher concentrations. We were unable to further define the mechanism of action of P-75811. Though we were able to narrow down the mechanism of action of several compounds, we still are unable to fully define the mechanism of action of the lead compounds identified herein. Further experimentation and assay development is needed.

We tested P-75802 for *in vivo* efficacy against IN VEEV-3908-FLuc challenge. P-75802 had a significant but limited effect on MTD and no effect on survival, weight loss, clinical score, or virus replication (as measured by bioluminescence). Despite these results, we remain optimistic that P-75802 has *in vivo* anti-alphavirus efficacy. Importantly, P-75802 treated animals were euthanized due to weight loss of greater than 20% starting weight (n=5); whereas, untreated animals were euthanized due to clinical score (n=4). At the time of euthanasia, P-75802 treated animals were recovering from virus infection as demonstrated by improving clinical score. We believe that with less restricted, weight loss euthanasia criteria these animals would have survived infection and recovered fully.

Due to the blinded nature of our compound screens, we are notably lacking information regarding the pharmacokinetics of P-75802. P-75802 treated animals had a distinctly altered virus distribution with higher levels of virus replication occurring outside of the CNS. Without pharmacokinetics data, the importance behind this observation is unclear. Altered virus distribution upon P-75802 treatment could be indicative of the mechanism of action of P-75802, could be due to altered replication kinetics or extension of MTD resulting from P-75802 treatment, or could be related to P-75802 metabolism.

We assessed lead compounds for antiviral activity against CHIKV, EEEV, SINV, and WEEV by yield reduction assay. Similar to the data collected from our cell-based ELISA, CHIKV was the most responsive to antiviral treatment; followed by EEEV, VEEV, and WEEV; and SINV was the least inhibited. No clear trends regarding virus species relationship and compound efficacy were noted.

The present study described the development and validation of a cell-based ELISA paired with mechanism of action descriptions. We show that a catch all screen identifying inhibitors of alphavirus replication paired with mechanistic studies allows for the selection of anti-alphavirus compounds with

known activities. The development of additional mechanistic assays will allow for a clearer definition of compound mechanism of action. The identification of anti-alphavirus compounds using cell-based ELISAs and the description of their mechanism of actions using bioluminescence-based mechanistic studies will facilitate the development of antivirals for the treatment of alphavirus infections.

CHAPTER FIVE: ALPHAVIRUS NEUROINVASION

Introduction

Eastern equine encephalitis virus, VEEV and WEEV are mosquito-borne viruses found in the Americas and cause CNS disease in humans and equids (Dembek, 2007). Eastern equine encephalitis virus, VEEV, and WEEV are maintained in nature via transmission cycles between vertebrate hosts and specific mosquito species (Weaver and Barrett, 2004). Historically, these alphaviruses have caused sporadic epizootics in horses and spillover epidemics in humans (Dembek, 2007). Outbreaks have led to significant rates of morbidity and mortality. Survivors can suffer from debilitating and sometimes progressive neurological sequelae (Mulder et al., 1951). Many aspects of alphavirus-induced CNS disease remain to be fully characterized including the sites of alphavirus CNS entry.

We have previously shown that the WEEV McM strain, like other neurovirulent alphavirus strains (Steele and Twenhafel, 2010), readily causes encephalitis in outbred CD-1 mice after aerosol, IN, or SC inoculation (Logue et al., 2009). Several reports have described VEEV and WEEV infection in the CNS of laboratory animals following IN exposure of alphaviruses (Dembek, 2007). We have reported that IN inoculation of mice with WEEV-3'McM'FLuc led to virus entry of the CNS through the long axonal projections of olfactory sensory neurons (OSNs) (Phillips et al., 2013). This finding is in agreement with other studies with EEEV and VEEV (Honnold et al., 2015; Ryzhikov et al., 1995). Virus entry into the host CNS via OSNs is a common feature shared by numerous neurovirulent viruses of medical importance (Koyuncu et al., 2013). Alphaviruses then disseminate into the rest of the brain along the neuronal axis, with infection of the olfactory bulb glomerular layer and lateral olfactory tract being prominent features of IN inoculation.

Peripheral infection of neuroinvasive alphavirus species can be modeled by SC inoculation of the mouse footpad. Charles et al. (1995) reported that SC injection of VEEV leads to CNS entry through olfactory or peripheral nerves. Conversely, EEEV has been reported to enter the brain directly from the

blood stream (Vogel et al., 2005). The precise entry points of EEEV and WEEV into the brain remain to be determined.

Here, we used *in vivo* bioluminescence imaging and confocal fluorescence microscopy to track experimental alphavirus infection following footpad injection and determined the site(s) of alphavirus entry into the CNS. We used recombinant, double-subgenomic alphaviruses based on WEEV strain McM and VEEV strain 3908 expressing FLuc or DsRed. We compared our *in vivo* and *ex vivo* BLM imaging studies with histological analyses of tissues to determine CNS entry points and route of dissemination of neuroinvasive alphaviruses throughout the brain. A non-recombinant wild-type EEEV (FL93) was also used to assess sites of entry by traditional histochemical approaches. Peripheral infection with each virus demonstrated a consistent spatiotemporal distribution of virus. We conclude that CNS entry of neuroinvasive alphaviruses occurs in areas of the CNS where the BBB is naturally absent. These areas include the hypothalamus, anteroventral third ventricle (AV3V) region, area postrema, and the pineal body. Virus subsequently disseminated via centripetal spread along neural pathways to other areas of the brain.

These observations are consistent with a model of hematogenous seeding of virus from sites of peripheral infection and highlight previously unreported areas within the CNS which we hypothesize possess specific vulnerability to infection during viremia. These findings are important to understand the pathogenesis of alphavirus encephalitides. Our studies should lead to a better understanding of the reported neurological sequelae among survivors of alphavirus-induced CNS disease and may contribute to the development of alphavirus therapeutics.

Methods

Viruses

A full-length infectious clone of WEEV strain McM was derived from a virus isolate obtained from the Arbovirus Reference Collection at the Center for Disease Control and Prevention in Fort Collins, CO, USA and has been previously described (Logue et al., 2009). Descriptions of the molecular cloning

methods used to engineer recombinant Fluc or DsRed expressing McM viruses are provided or have been previously published (Phillips et al., 2013). In brief, transgene expression in recombinant WEEV was achieved by duplication of the subgenomic promoter sequence immediately downstream of the last nucleotide of E1 or immediately downstream of nsP4 creating 3' and 5' variants, respectively. Recombinant luciferase-expressing VEEV plasmid (VEEV-3908-FLuc) was obtained from Dr. Scott Weaver (University of Texas Medical Branch). Linearized VEEV (Mlu1) and WEEV (Mfe1) constructs were purified by QIAprep Spin MiniPrep Kit (Qiagen) and genomic RNA was *in vitro* transcribed using a T7 RNA Polymerase MAXIscript kit (Life Tech). Vero cells (4×10^6 cells in 400 μ L) (ATCC) were electroporated with genomic RNA using an ECM 630 electroporator (BTX Harvard Apparatus). Two pulses (450 V, 1200 Ω , and 150 μ F) were administered. Forty-eight hours post electroporation media was taken from electroporated cells and passaged once in BHK cells to make stock virus. Supernatant was collected at 48 HPI and stored at -80C. EEEV strain FL93-939 was obtained from Dr. Richard Bowen (CSU). EEEV FL93-939 stocks were produced by infecting Vero cells at a MOI of 0.1 PFU/cell. Cell culture supernatants were collected 24 HPI. Virus stocks were quantified using plaque titration in Vero cells prior to experimental use. Virus titrations were performed in duplicate and plaque assays were performed as described by Liu et al. (1970) .

Mouse infections and imaging

All animal protocols used in the study were reviewed and approved by the Animal Care and Use Committee at CSU. Mice were handled in compliance with the PHS Policy and Guide for the Care and Use of Laboratory Animals. All experiments were conducted in a biosafety level 3, and in a CDC Select Agent-approved facility. Female 4–5 week old CD-1 mice (Charles River Laboratory) were used in this study. Footpad inoculation was conducted at a dose of 1×10^4 PFU in a volume of 20 μ L. Intranasal inoculations were performed at a dose of 1×10^4 by installing inocula into each nostril of lightly anesthetized animals. Imaging was performed after 150 mg/kg of luciferin substrate (30 mg/mL stock) was injected (SC) dorsal to the cervical spine of each animal. Each animal was imaged 10–15 minutes after injection of luciferin. Uninfected mice were used as an imaging control to adjust for background

signal. Mice were anesthetized by administration of isoflurane (Minrad Incorporated) through an XGI-8 anesthesia system (Caliper Life Sciences) connected to the IVIS 200 (Perkin Elmer) imaging camera. Exposure time was three minutes under standard settings for the camera. Living Image 3.0 software (Caliper Life Science) was used to analyze and process images taken using the IVIS 200 camera. A threshold for significant BLM was established using negative imaging controls at 5×10^3 p/s/cm²/sr. Total light emission from each mouse was accomplished by creating a region of interest of standard size for each mouse and collecting light emission data using the software.

For imaging of bisected heads, mice received 150 mg/kg of luciferin (30 mg/mL stock diluted in PBS). After 10 minutes, mice were injected with a second dose of luciferin, and promptly euthanized via inhalation of a lethal dose of isoflurane. Animals were decapitated and whole heads bisected along the medial sagittal plane. Resulting sections were briefly rinsed with PBS and promptly imaged.

Immunohistochemistry

Paraffin -embedded formalin fixed tissue was rehydrated, treated with Tris-EDTA pH 9.0 at 90C for 15 minutes, and blocked with SuperBlock T20 (Thermo Scientific). Biotinylated polyclonal rabbit anti-FLuc antibody (Abcam) was used at 1:1000 dilution and incubated overnight at 4C. Primary antibody was washed three times with Tris-buffered saline (TBS) containing 0.03% Tween 20 (TBST). Secondary antibody was streptavidin-HRP (Rockland) and was used at a 1:6000 dilution and incubated for 30 minutes at RT. Slides were again washed three times with TBST. 3, 3'-diaminobenzidine (DAB) was added to the slides and allowed to develop stain for five minutes. Hematoxylin was used to counterstain. Hyperimmune horse serum generated against WEEV Fleming strain (CDC, Fort Collins, CO) was used for anti-WEEV IHC (1:600 dilution). Secondary antibody was HRP-conjugated rabbit polyclonal antibody to horse IgG (Abcam) used at a 1:3500 dilution. For anti-VEEV and anti-EEEV immunostaining, antigen retrieval method used was to incubate tissue sections in Proteinase K (20 µg/mL) diluted in Tris-EDTA CaCl₂ buffer at pH 8.0 for 20 minutes at 37C. Primary antibodies were diluted and used as follows: 1:300 anti-VEEV (ATCC) and 1:100 mouse anti-EEEV NA with 1:100 mouse anti-EEEV SA (CDC, Fort Collins, CO). Secondary antibody was goat anti-mouse conjugated to

HRP (Abcam) used at 1:1000 dilution. All other conditions remained unchanged. Negative controls consisting of staining done without the addition of primary antibody were conducted with each batch. When possible staining was done with both virus and reporter directed antibody to confirm findings.

Imaging coronal sections of mouse brain

Formalin-fixed whole-brains were cryo-sectioned into 400 μm sections using a Microm HM 450 (Thermo Scientific). Resulting coronal sections were submerged for 15 minutes in TBS containing 0.01 mg/mL of Hoechst 33342 dye (Molecular Probes). Sections were rinsed in TBS and imaged using a BX51 microscope (Olympus), ORCA-ER camera (Hamamatsu), ProScan III stage controller (Prior), and CellSens Dimension v1.12 imaging software (Olympus). Fluorescence was detected for DsRed (WEEV-3' McM-Red virus) and Hoechst dye (nuclear counter-stain) using a 2X air objective. Resulting images were assembled into an individual montage image for each coronal section using CellSens software.

For enhanced fluorescence imaging, whole-brains were first embedded in polyacrylamide hydrogel as previously reported (Chung and Deisseroth, 2013). Embedded brains were cryo-sectioned into 180 μm sections and each section was placed into individual wells of 12-well tissue-culture plate, with each well containing 2 mL of clearing solution (4% sodium dodecyl sulfate and 200 mM boric acid in deionized water, pH8.5). Sections were cleared at room temperature for four days with clearing solution replaced after two days. Sections were then washed twice with 2 mL of TBS (24 hours per wash) at RT. Washed sections were moved to TBS containing 0.01 mg/mL of Hoechst 33342 dye (Molecular Probes). Sections were washed once in TBS for five minutes at RT. Sections were placed onto positively charged glass slides and coverslipped using fluorescence imaging mounting media. Coverslips were sealed along the outer edge with clear fingernail polish. Images were acquired using a BX51 microscope (Olympus), ORCA-ER camera (Hamamatsu), ProScan III stage controller (Prior), and CellSens Dimension v1.12 imaging software (Olympus). Fluorescence was detected for DsRed (WEEV-3' McM-Red virus) and Hoechst dye (nuclear counter-stain) using a 10X air objective. Additional images were acquired using a FluoView 1200 scanning-laser confocal microscope (Olympus).

Results

BLM imaging and histological examination of CNS invasion following IN inoculation

Neuroinvasion and CNS dissemination were monitored in CD-1 mice intranasally inoculated with VEEV-3908-FLuc. Mice (n=10) were euthanized at various time points post IN inoculation, decapitated, and whole heads were imaged. Representative images are presented (Figure 5.1). BLM signal was first observed in the nasal turbinates and olfactory bulb before infection proceeded along the lateral olfactory tract and broadly disseminated. Histological examination supported a route of neuroinvasion occurring via the olfactory tract and associated tissues (Figure 5.2).

Distribution of DsRed within coronal slices of IN or SC infected mice

Following our preliminary characterization of the recombinant reporter virus constructs (Figure 5.3 and 5.4); we monitored infection of the CNS following inoculation of the footpad. We compared the distribution of DsRed within coronal sections of brains obtained from CD-1 mice receiving either IN or SC inoculation. Mice were euthanized upon showing neurological signs of disease. All mice (n=10) receiving IN inoculation required euthanization at 3.5 DPI. Approximately 30% of SC-inoculated mice (n=10) required euthanization. Time to euthanasia increased in mice receiving footpad inoculations, compared to IN inoculations, and ranged from 4-7 DPI. Mouse brains were harvested and prepared for low-resolution montage imaging of whole coronal sections. Examination of DsRed distribution among the imaged sections revealed that SC-inoculated animals exhibited a distinct virus distribution pattern compared to IN-inoculated animals (Figure 5.5).

BLM imaging and histological examination of CNS invasion following SC inoculation

The distinct patterns of DsRed expression indicated that WEEV enters the CNS at different locations dependent on the route of inoculation. Therefore, we examined early entry events associated with CNS entry of alphaviruses following SC inoculation. We monitored infection of the CNS following SC inoculation of the footpad with WEEV-5' McM-FLuc or VEEV-3908-FLuc. Whole animals were

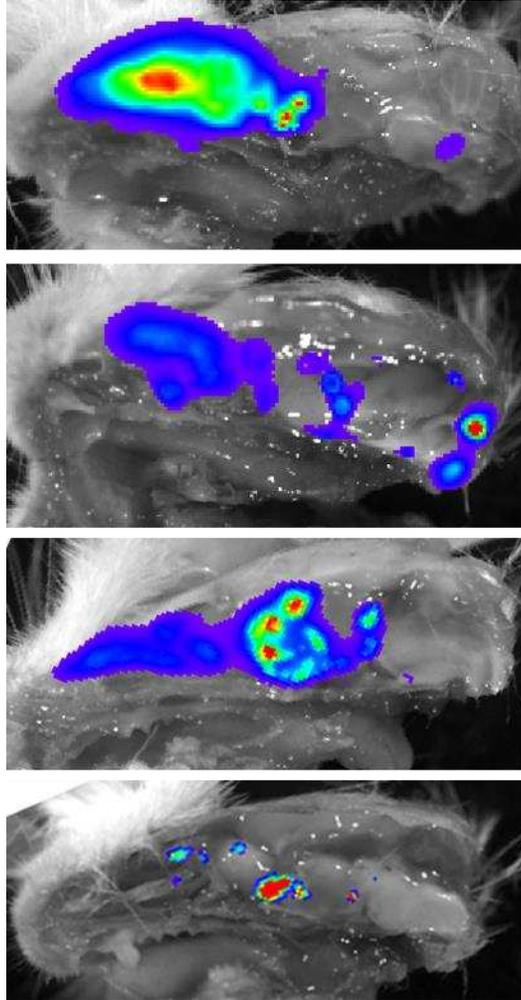


Figure 5. 1 *Ex vivo* imaging showing CNS invasion with VEEV-3908-FLuc following IN inoculation

Schematic representation of the anatomical locations of the olfactory tract and associated tissues. Whole brains were bisected along the sagittal midline and imaged at 4, 5, 5, and 6DPI (top to bottom).

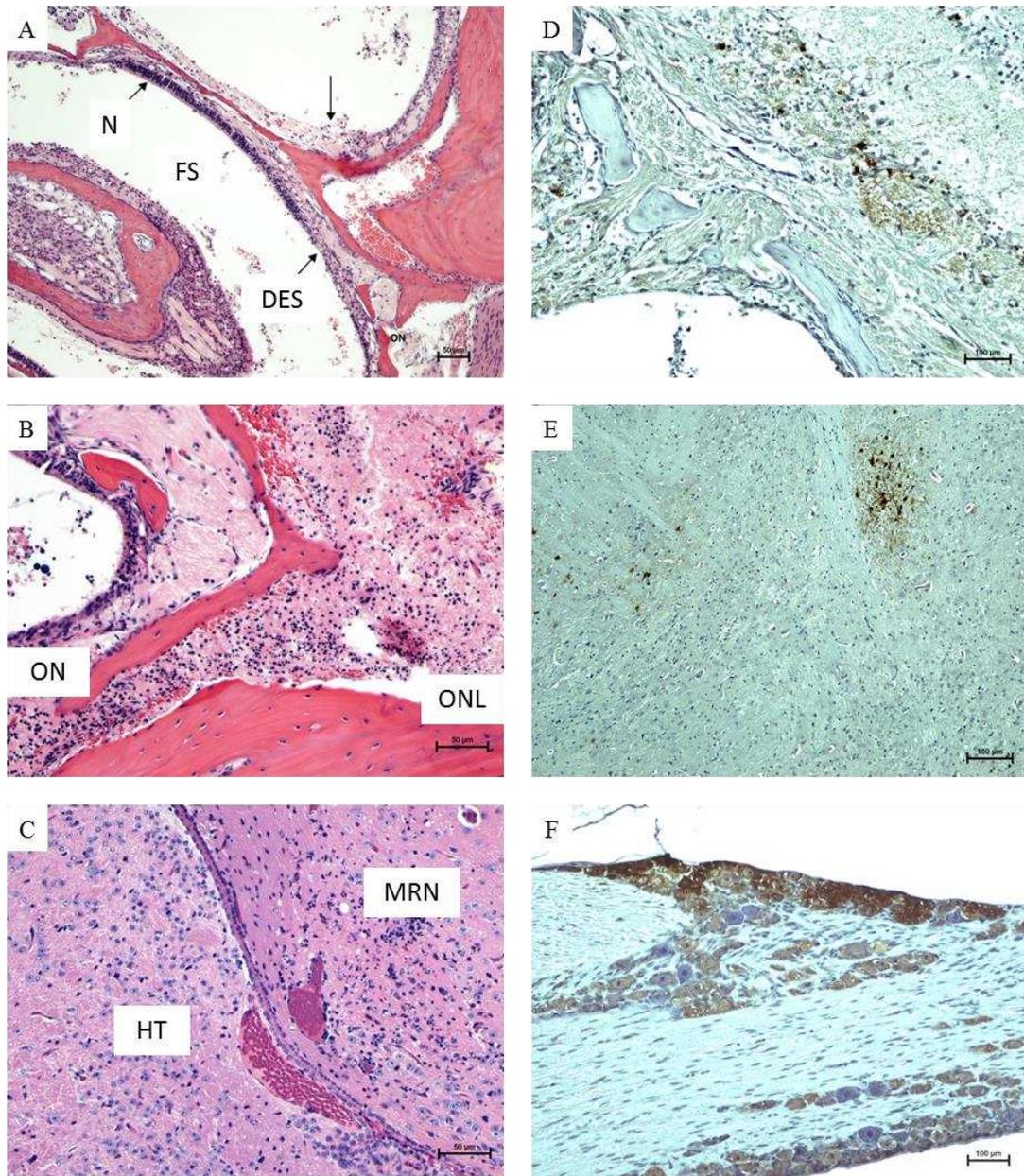


Figure 5. 2 Histological analysis of CD-1 mouse heads following IN VEEV-3908-FLuc infection.

A- C.) Hemotoxylin and eosin staining. D- F.) IHC staining. A.) Frontal sinus (FS) and olfactory nerve (ON). B.) ON and olfactory nerve layer (ONL). C.) Hypothalamus (HT) and midbrain. D.) Anti-luciferase IHC staining (brown) of main olfactory lobe. E.) Anti-luciferase IHC staining (brown) of thalamus. F.) Anti-luciferase IHC staining (brown) of trigeminal ganglia. normal (N), desquamation (DES), superior central nucleus raphe (MRN).

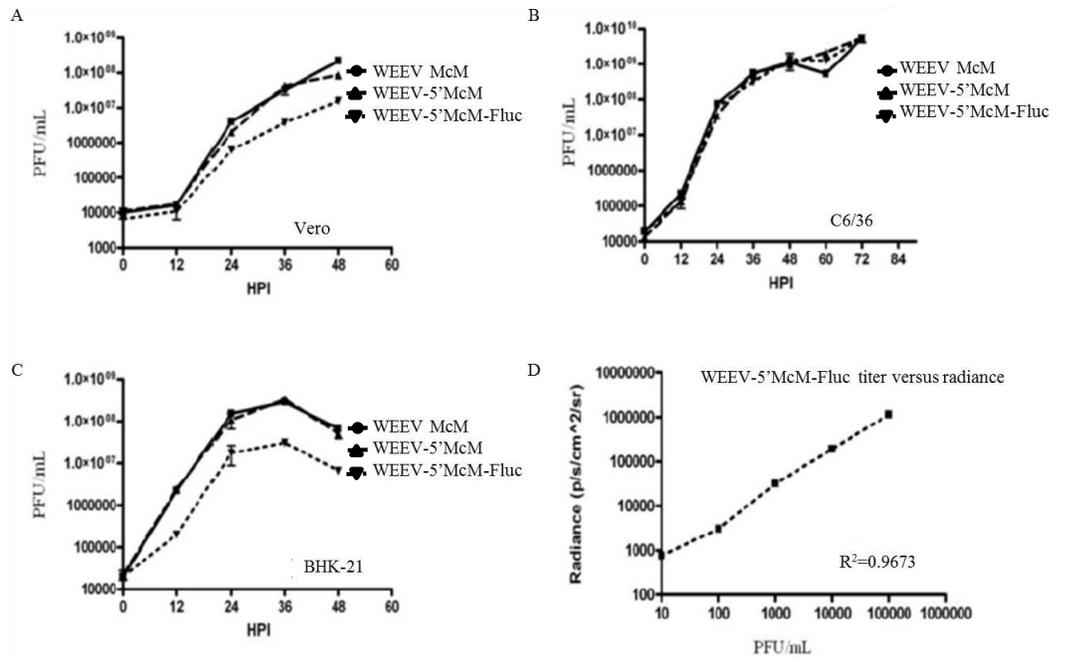


Figure 5. 3 Growth curves for WEEV McM constructs.

PFU/mL in supernatants at indicated time points following infection at 0.01 MOI. Vero (A), C6/36 (B), and BHK cells (C). The relationship between virus titer of WEEV-5'McM-FLuc added (PFU/mL) and amount of light emitted from expressed FLuc in BHK cells was examined by correlating WEEV-5'McM-FLuc titer (10^5 , 10^4 , 10^3 , 10^2 , and 10^1 PFU/mL) with bioluminescence at 8 hours post infection as measured by radiance (p/s/cm²/sr).

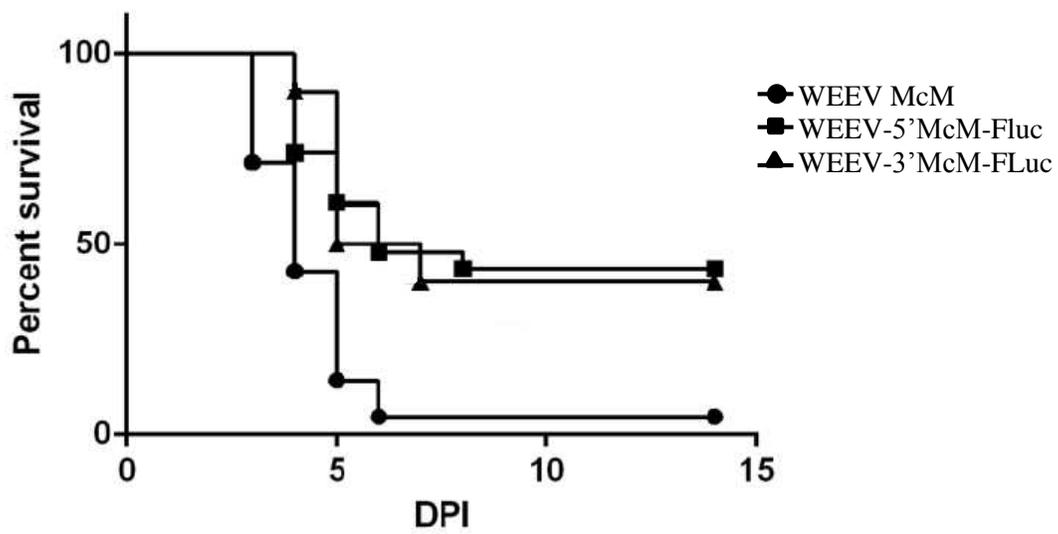


Figure 5. 4 Survival analysis of mice infected with parental wild-type or recombinant WEEV McM.

Survival percentages are given for each virus following footpad inoculation of CD-1 mice with 10^4 PFU.

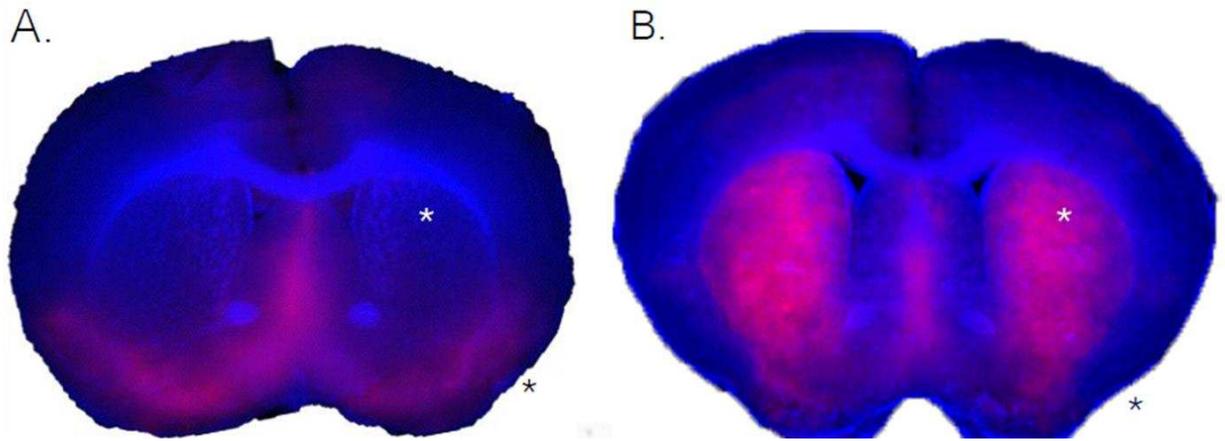


Figure 5. 5 Virus distribution in coronal slices of brain from CD-1 mice inoculated via IN or SC route.

Following euthanasia of affected mice at 7 DPI for SC mice and 3.5DPI for IN mice, brains were sectioned into 400 μm coronal sections and imaged for DsRed expression using fluorescence microscopy. Distinctive patterns of virus distribution were observed among brains receiving virus IN versus SC. Brain sections were imaged at 20X total magnification for WEEV-3'McM-Red (red) and nuclear counter-stain (blue). Representative images show two comparable coronal sections of whole brain from animals receiving either IN (A) or SC (B) inoculation. Distinct patterns of virus distribution are marked within the caudoputamen (indicated with white asterisks) and lateral olfactory tract (indicated with black asterisks)

imaged daily by BLM detection. For both WEEV and VEEV, increased BLM was detected as early as 12 HPI in the mouse footpad and 72 HPI in the CNS (Figure 5.6). Mice exhibiting FLuc activity within the head region of $>10,000$ p/sec/cm²/sr, went on to display neurological signs of disease. Mice exhibiting $<10,000$ p/sec/cm²/sr did not show neurological signs of disease and did not succumb to infection at any point during the four week holding period.

We performed a sagittal bisection of the whole head at the earliest sign of increasing luciferase activity, constituting a measured luciferase activity in the head region of $>10,000$ p/sec/cm²/sr. Early detection of CNS infection by *in vivo* imaging typically occurred at 3-5 DPI. *Ex vivo* imaging on bisected heads showed that specific areas of the brain were consistently associated with FLuc activity, identifying likely sites of entry into the CNS. In contrast, BLM was consistently absent in other areas of the brain, most notably the cerebellum and the olfactory bulbs. The brain regions showing the earliest signs of BLM were the area postrema (Figure 5.7E), pineal body (Figure 5.7D and 5.7A), and hypothalamus with associated organum vasculosum lamina terminalis (OVLT) and subfornical organ (SFO) (Figure 5.7A, 5.7C, and 5.7F). Notably, the OVLT and the SFO are interconnected with the hypothalamus, and together, comprise the anteroventral third ventricle (AV3V) region of the brain. Therefore, it is not surprising that BLM activity occasionally included multiple regions associated with the AV3V (Figure 5.7C).

We extended these studies by performing histological examinations of the *ex vivo* imaged tissues to further describe CNS infection within those animals. Virus, WEEV-3'McM-FLuc, invaded the brain by 72hpi, inducing moderate to severe meningoencephalitis. Meninges and corresponding parenchyma showed moderate vascular congestion and infiltration of pleocellular exudate. Mononuclear cells (immunostained for WEEV antigen) seeded the perivascular areas in the connective tissue surrounding circumventricular organs (CVOs) (Figures 5.8, 5.9 and 5.10). Apoptosis and neuropil edema became evident in the parenchyma that surrounds the CVOs by 72-96 hours post neuroinvasion. The lesions and virus distribution of brains infected with VEEV-3908-FLuc were similar to WEEV-3'McM-FLuc, indicating that VEEV-3908-FLu

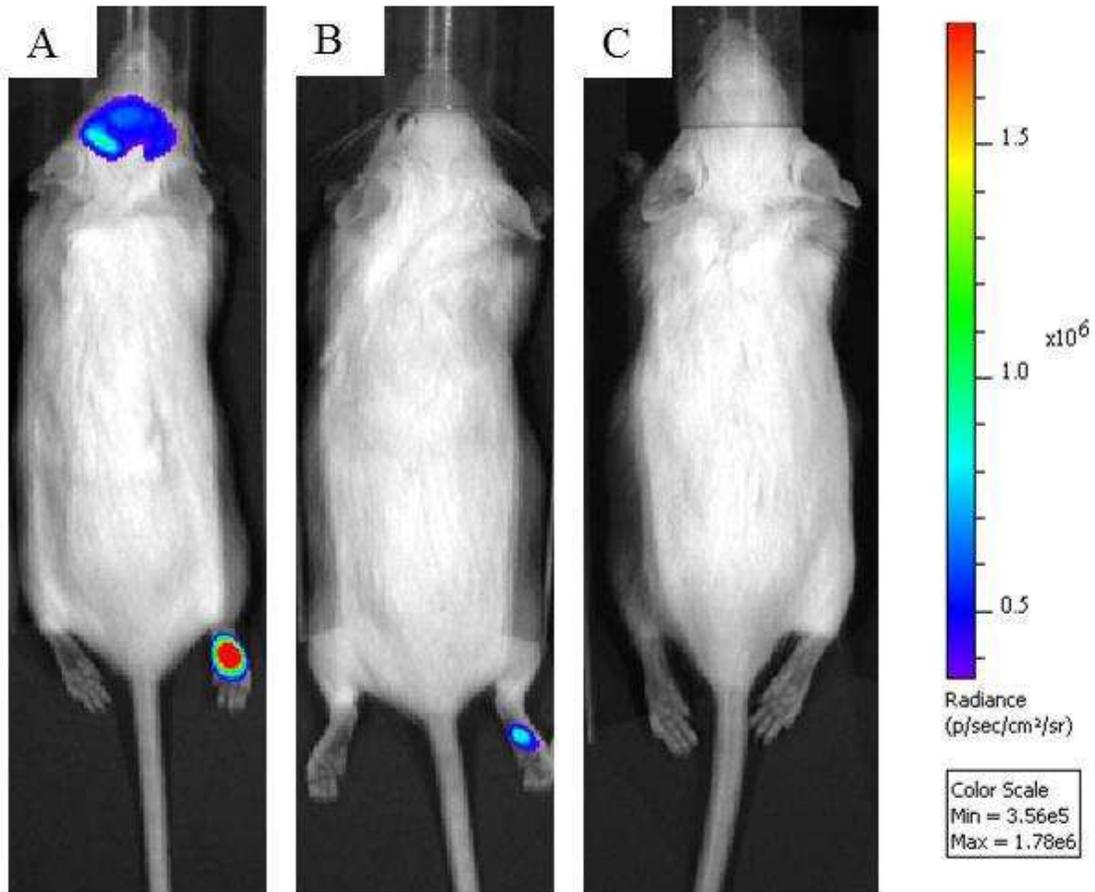


Figure 5. 6 Whole animal imaging showing CNS infection following footpad inoculation.

CD-1 mice were challenged with WEEV-5'McM-FLuc and were imaged daily for BLM activity. A.) Representative image shows CD-1 mouse with neurological signs of disease and CNS and inoculation site bioluminescence. B.) Mouse with no neurological signs of disease or luciferase activity within the brain region and inoculation site. C.) Uninfected control mouse.

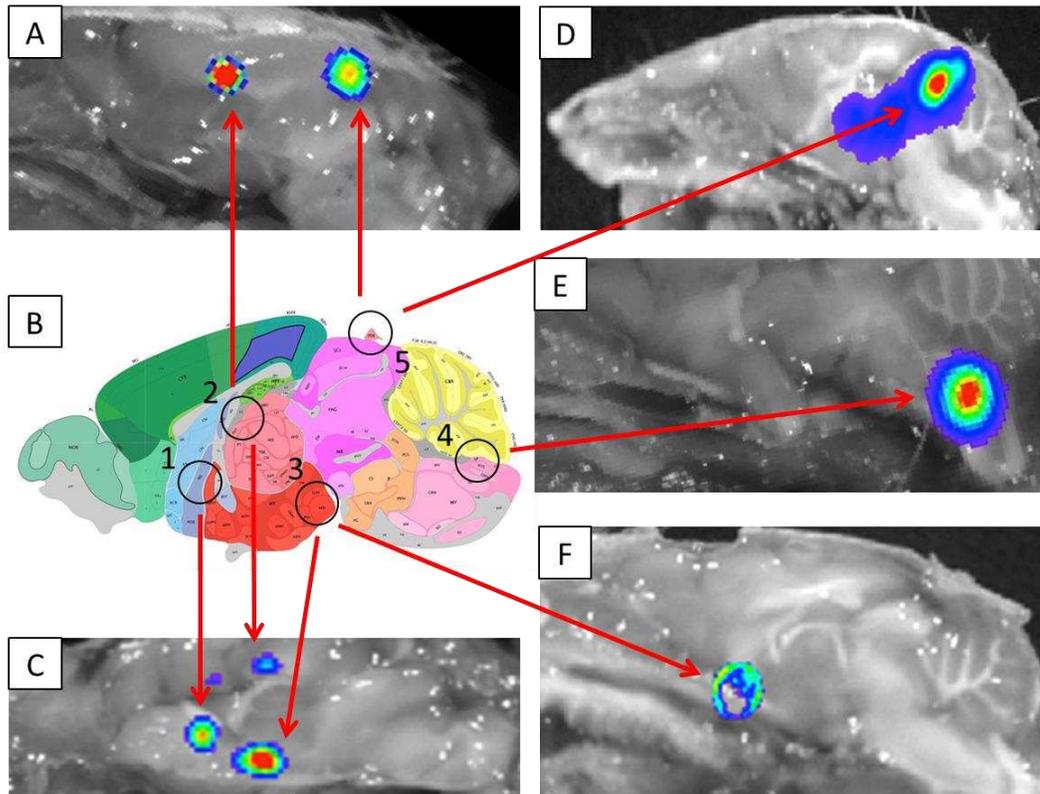


Figure 5.7 Ex vivo imaging showing early stages of CNS infection following footpad inoculation.

CD-1 mice were challenged with WEEV-5'McM-FLuc and were imaged daily for BLM activity. Upon detection of increased BLM activity within the head region, mice processed for *ex vivo* imaging. Representative images show CD-1 mice exhibiting each of the BLM patterns observed during early stages of CNS infection (3-5 DPI). A., C.,E.) VEEV-3908-FLuc., D., F.) WEEV-5'McM-FLuc. B) Schematic showing the anatomical locations of the CVOs along the sagittal axis. Numbered circles refer to the following anatomic locations: 1) vascular organ of the lamina terminalis. 2) subformal organ. 3) posterior hypothalamus. 4) area postrema. 5) pineal body.

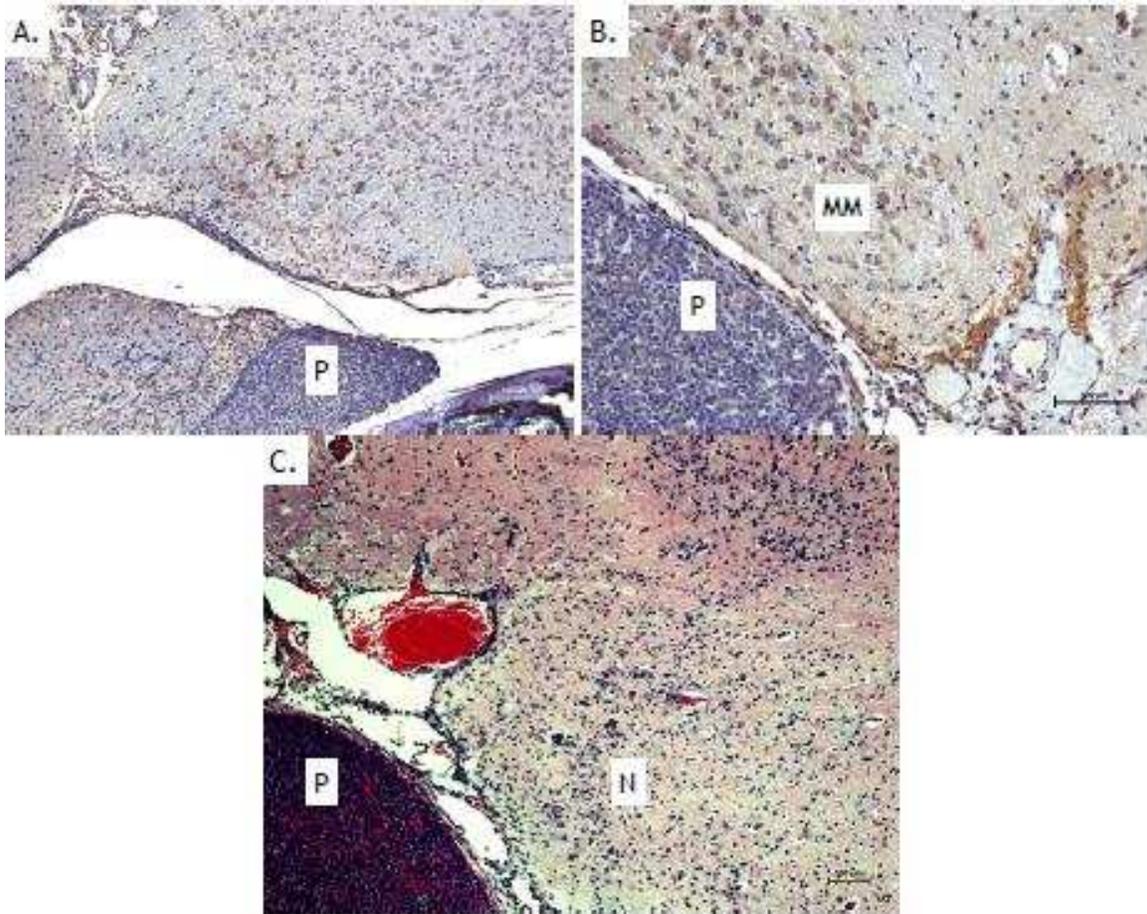


Figure 5. 8 Hypothalamic route of CNS entry for WEEV-5'McM-FLuc.

A.) Anti-WEEV antigen IHC staining (brown) of hypothalamic region with nearby pituitary (P) gland. B.) Anti-WEEV antigen IHC staining (brown) of medial mammillary nucleus (MM) region. C.) Hemotoxylin and eosin staining of median eminence region. necrosis (N).

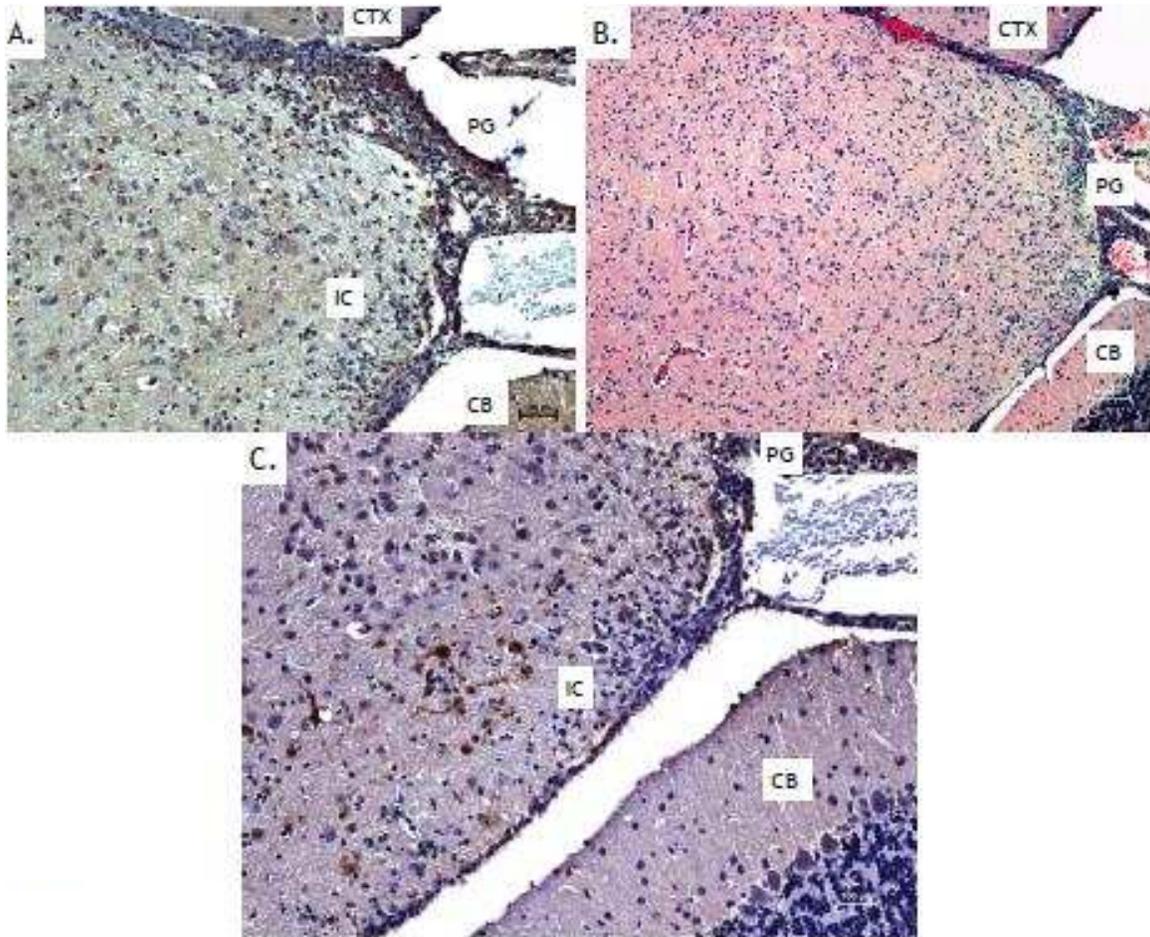


Figure 5. 9 Pineal gland route of CNS entry for WEEV-5'McM-FLuc.

A.) Anti-WEEV antigen IHC staining (brown) of inferior colliculus (IC) and pineal gland (PG) region. B.) Hemotoxylin and eosin staining of same region. C.) Anti-WEEV antigen IHC staining (brown) of another section of the inferior colliculus region. cerebellum (CB). cortex (CTX).

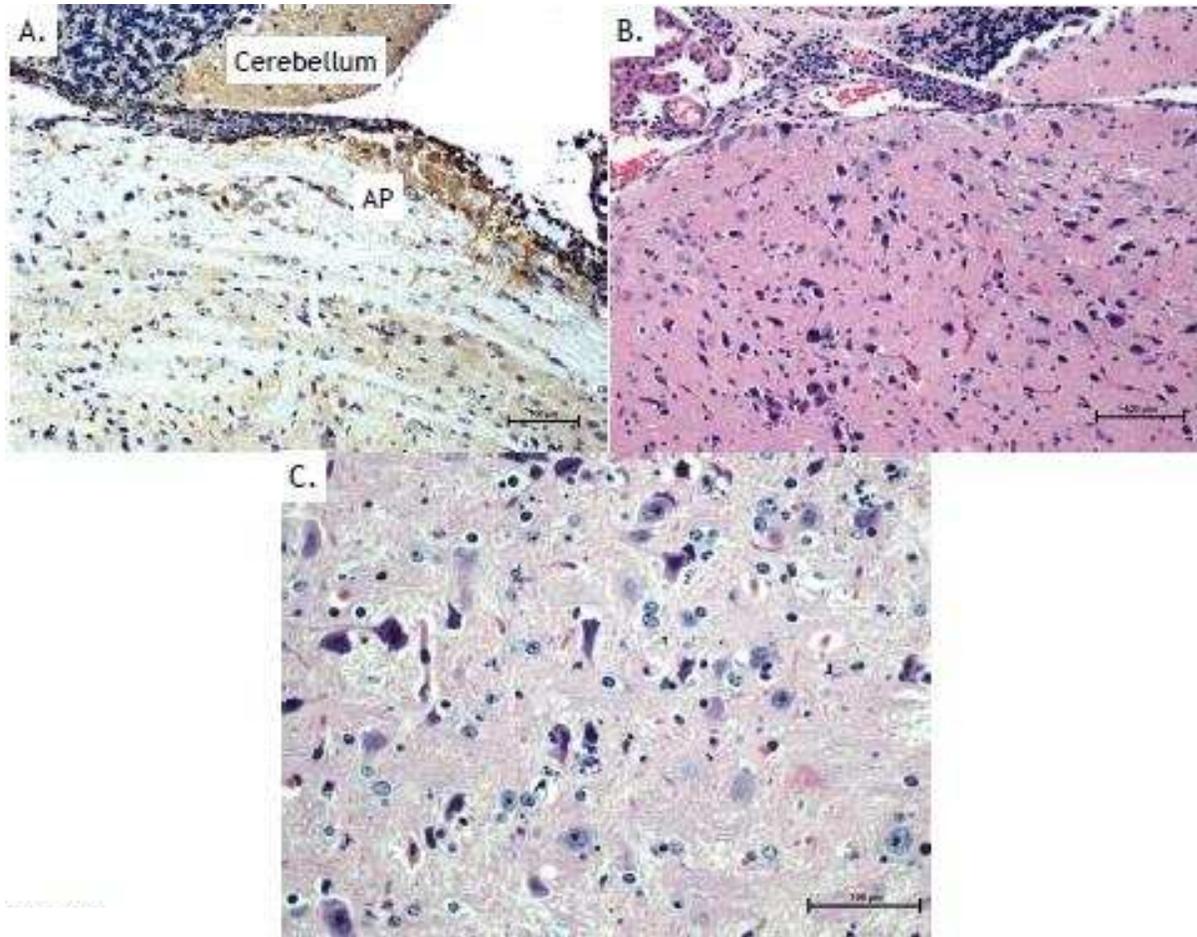


Figure 5. 10 Area postrema route of CNS entry for WEEV-5'McM-FLuc.

A.) Anti-WEEV antigen IHC staining (brown) of area postrema (AP) region. B.) Hemotoxylin and eosin staining of same region. C.) Hemotoxylin and eosin staining of hindbrain near area postrema region.

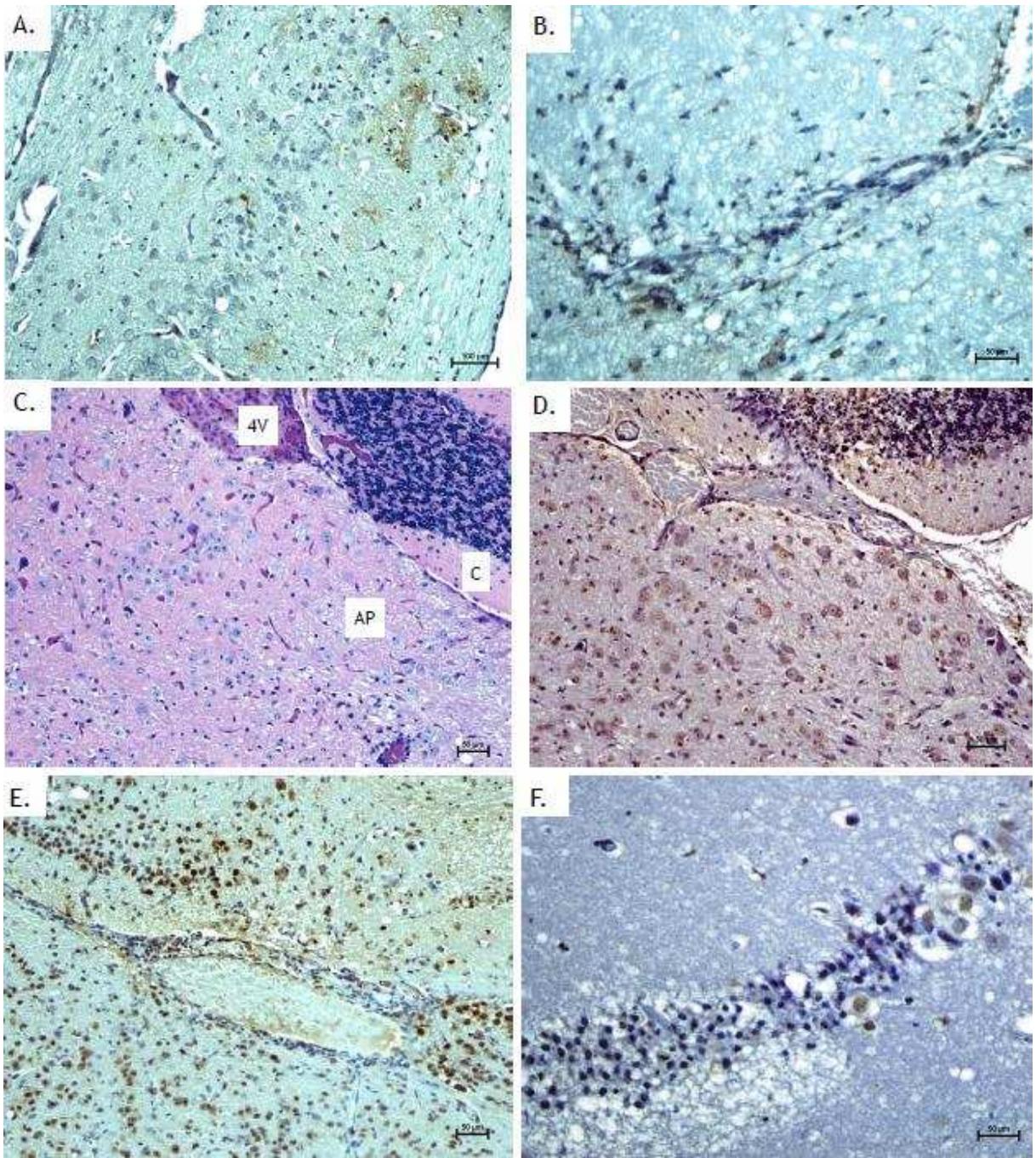


Figure 5.11 Histological analysis of CD-1 mouse tissue following footpad inoculation with VEEV.

A and B.) Anti-luciferase IHC staining (brown) of hypothalamus region. C.) Hemotoxylin and eosin staining of area postrema (AP) region. D.) Anti-VEEV IHC staining (brown) of a similar section. E.) Anti-VEEV staining (brown) of the superior colliculus. F.) Anti-VEEV staining (brown) of the hippocampus, fourth ventricle (4V), cerebellum (C).

Infection typically progressed along a bilateral symmetry with neurons as key targets of infection, especially in the caudate/putamen, superior and inferior colliculi, substantia nigra, hypothalamus, midbrain-tegmental region and hind-brain (Figure 5.12). Many neurons were apoptotic and occasional vessels in the most affected areas were cuffed by small numbers of mixed inflammatory cells including macrophages, lymphocytes and fewer neutrophils. Glial cells also appeared to be infected, but to a lesser extent than neurons. Both astrocytes and oligodendroglial cells showed moderate WEEV antigen immunoreactivity in the midbrain. Strong WEEV antigen immunoreactivity was observed in the hindbrain by 7 DPI. Apart from the brain, retinal ganglion neuronal cell bodies showed slight immunoreactivity along with scattered immunoreactivity of the retinal ganglion axons during this late-stage of disease. Cranial nerves also showed strong immunoreactivity especially cochlear, trigeminal and optic nerves. OSNs remained uninfected by immunochemical staining procedures.

As further evidence of the central role CVOs have in alphavirus entry of the CNS, we also inoculated CD-1 mouse footpads with wild-type EEEV (FL93). Once mice displayed neurological signs of disease, they were euthanized and brains were harvested and processed for immunohistochemistry staining. Sagittal brain sections staining positive for EEEV antigen were used to locate EEEV specific sites within the mouse brain. (Figure 5.13). Staining indicated the presence of EEEV antigen in regions consistent with the infection of the CVOs.

Enhanced fluorescence imaging of CLARITY-processed coronal sections

To more precisely track replicating virus, we inoculated the footpads of CD-1 mice (n=10) with WEEV-5' McM-Red. Mice were euthanized at 4 DPI, brains were treated to generate clarified coronal sections, and clarified sections were imaged for DsRed fluorescence. We found that WEEV-3' McM-Red entry into the CNS did not involve the olfactory tract (Figure 5.14A), although virus can spread rapidly along the neuronal axis into the olfactory bulb by 5 DPI (Figure 5.14C). Viral expression of DsRed in the olfactory bulb was limited to more internal layers, such as the subependymal zone, and was not detected in the olfactory bulb glomerular layer; an area shown to be the primary site of entry following IN inoculation (Figure 5.14B and 5.14A). A

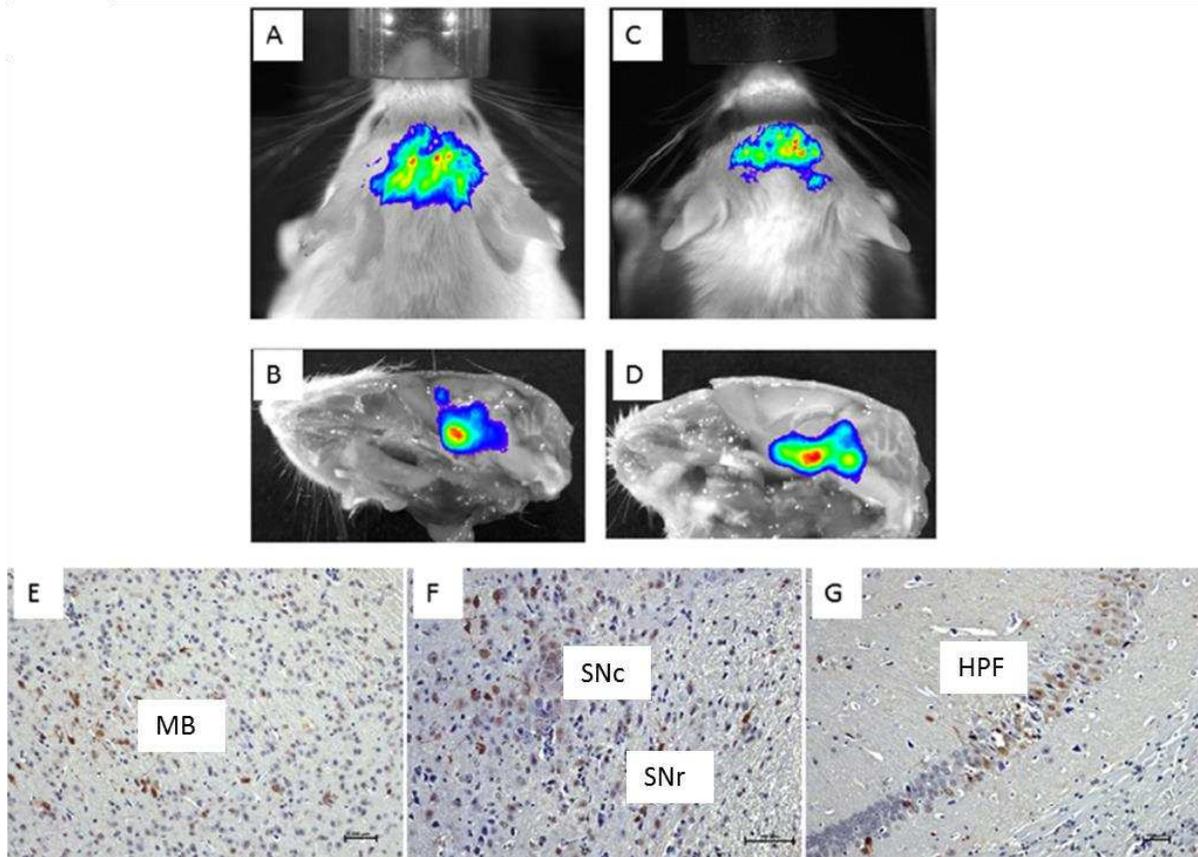


Figure 5. 12 Later stages of CNS infection following footpad inoculation with WEEV-5'McM-FLuc.

A-D.) Two separate CD-1 mice are shown which represent the later stages of CNS infection (5-7 DPI). Images are shown from the intact animal and the subsequent *ex vivo* imaging of the head of that animal. Anti-WEEV antigen IHC staining (brown) of E.) midbrain (MB), F.) substantia nigra pars compacta (SNc) and pars reticulata (SNr), and G.) hippocampal formation (HPF) showing extensive viral antigen immunoreactivity.

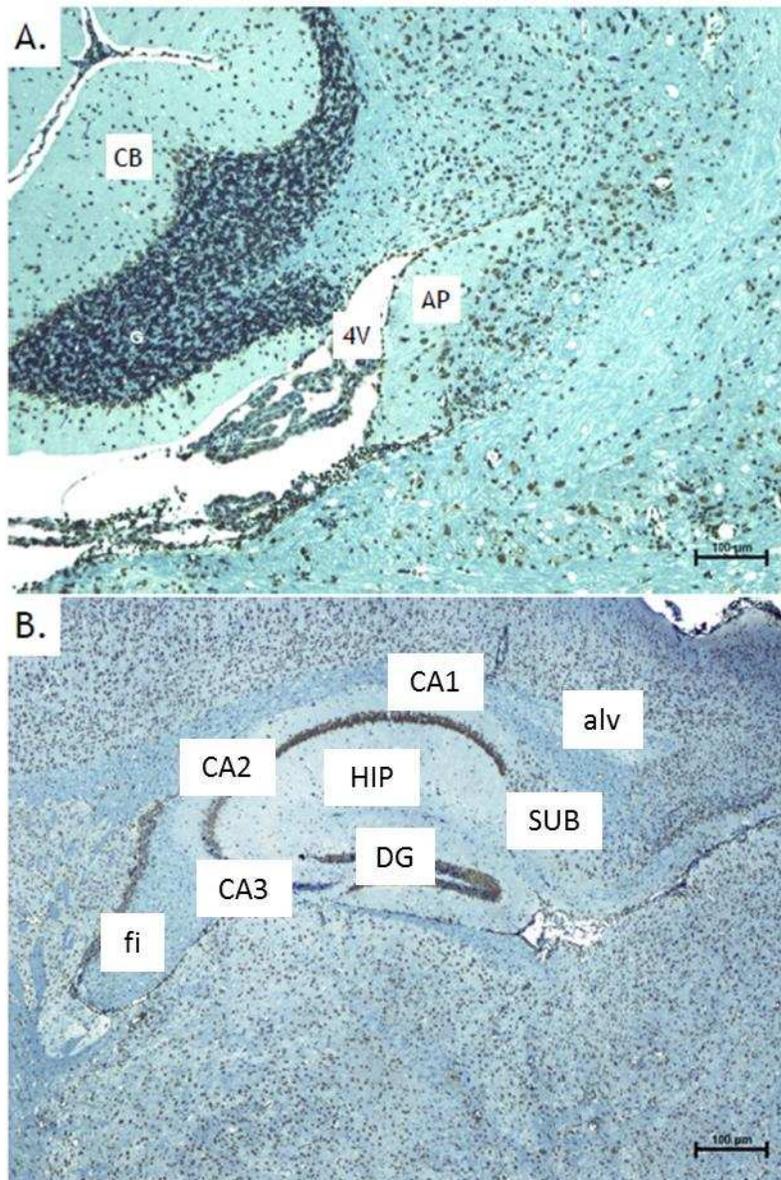


Figure 5. 13 Histological analysis of CD-1 mouse tissue following footpad inoculation with EEEV.

A.) Anti-EEEV IHC staining (brown) of area postrema region. B.) Anti-EEEV IHC staining (brown) of hippocampal region. area postrema (AP). fourth ventricle (4V). cerebellum (CB). hippocampus (HIP). *cornu ammonis* (CA). subiculum layer (SUB). fimbria (FI). alveus (Alv). dentri gyrus granular layer (DG).

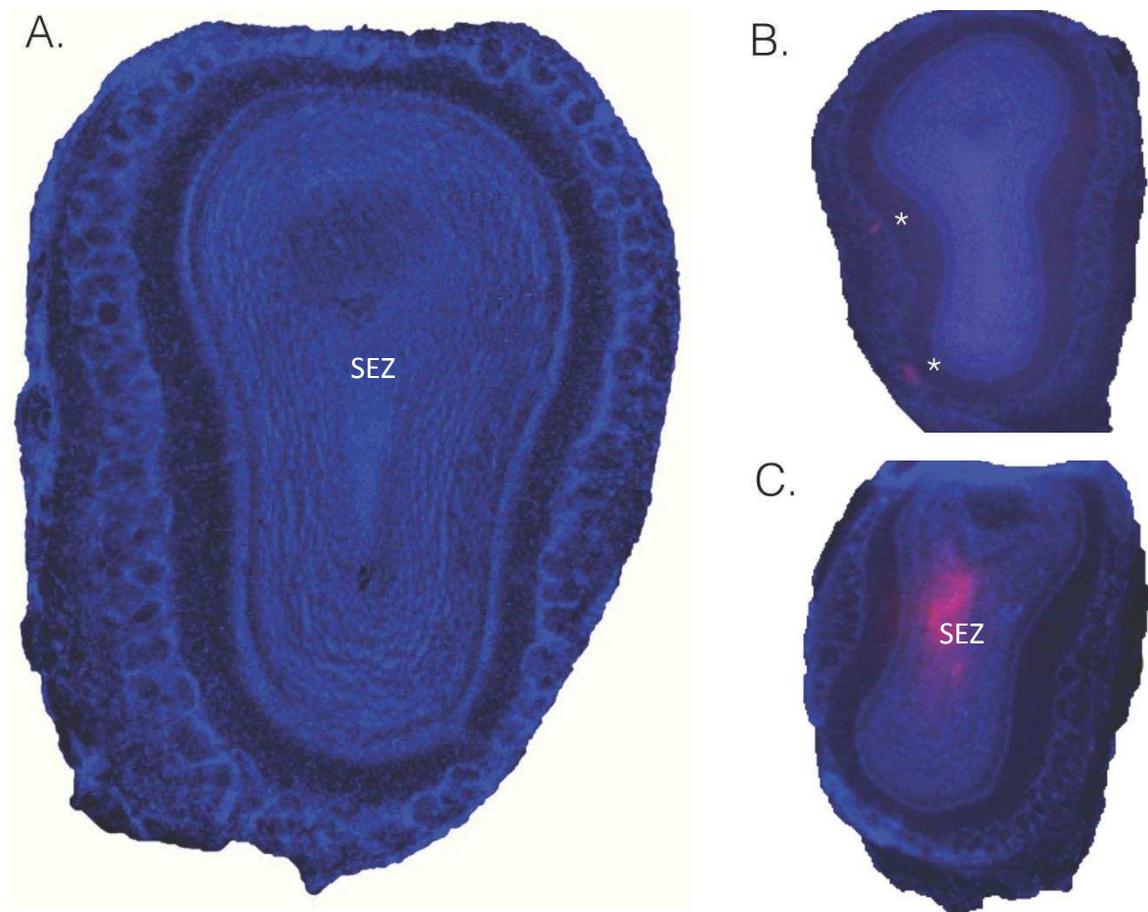


Figure 5. 14 Imaging of olfactory bulb sections at early time points post-inoculation.

CD-1 mice were inoculated in the footpad with WEEV-3'McM-Red virus and euthanized at 4 DPI. Whole brains were sectioned, clarified, and imaged at 100X total magnification. A.) Montage image shows an olfactory bulb from mouse with severe CNS infection - same animal as shown in Figures 5.15 and 5.16. B.) Representative image for comparison showing WEEV-3'McM-Red virus in the glomerular layer (asterisks) of the olfactory bulb following IN inoculation, 3 DPI. C.) Olfactory bulb from SC inoculated animal, 5 DPI, shows WEEV-3'McM-Red entering the olfactory bulb but not yet infecting the olfactory bulb glomerular layer.

comparison of olfactory bulbs from IN-inoculated animals versus SC-inoculated animals demonstrates this point (Figures 5.14B and 5.14C).

Among the CVOs, the hypothalamus and AV3V region appeared to be the most frequent route of virus entry. Closer examination of this region demonstrated that neurons associated with the hypophyseal portal system of the posterior hypothalamus/pituitary, principal mammillary tract, and mammillary bodies were key targets of infection (Figures 5.15 and 5.16). Robust expression of DsRed was visualized throughout the principal mammillary tracts. Additionally, robust expression was present in nuclei of the substantia nigra (Figure 5.16). Cortical regions showing more faint and limited expression of DsRed were typically motor-related areas prompting examination of retrograde spread from spinal cord neurons via the corticospinal tract. Examination of sections more caudally located provided evidence that the pyramids are void of virus expression of DsRed and do not lend support for the corticospinal tract route hypothesis (Figure 5.17).

Discussion

In this report, we IN inoculated CD-1 mice with VEEV-3908-FLuc and SC inoculated CD-1 mice with WEEV-5' McM-FLuc, WEEV-3' McM-Red, and VEEV-3908-FLuc to stimulate IN and peripheral infection and detect sites of virus replication in the CNS. We used immunohistochemical examinations and enhanced confocal fluorescence imaging (CLARITY technique) to further examine the virus entry points suggested by BLM and fluorescence imaging. We add further support to an olfactory route of CNS invasion for IN inoculated VEEV and find that EEEV, VEEV, and WEEV gain entry into the CNS at specific areas where the blood-brain barrier is naturally absent following peripheral inoculation. Importantly, we did not detect early BLM in CNS/ependymal tissues unassociated with CVOs nor did we detect initial BLM signal in the olfactory bulb or associated neuroepithelium at early time points following SC inoculation. This finding suggests CVOs as a previously undescribed site of neurotropic-virus entry into the CNS in a mouse peripheral infection model.

Our findings that VEEV-3908-FLuc enters the CNS via an olfactory route following IN exposure adds further support to previously published studies (Steele and Twenhafel, 2010). The *in vivo* and *ex vivo*

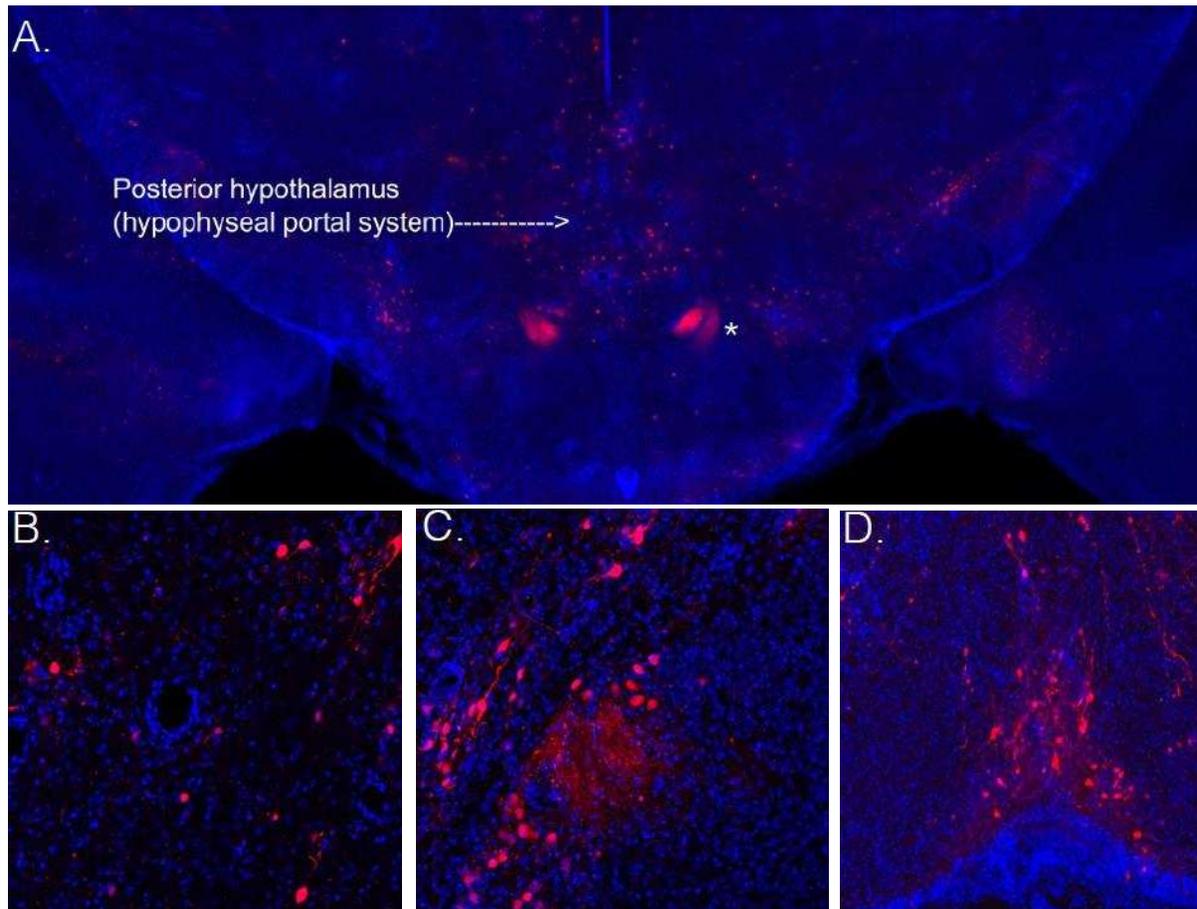


Figure 5. 15 Imaging of hypothalamus and AV3V at early time points post-inoculation.

CD-1 mice were inoculated in the footpad with WEEV-3'McM-Red virus and euthanized at 4 DPI. Whole brains were sectioned, clarified, and imaged at 100X total magnification. A.) Montage image shows a coronal section and includes the hypophyseal portal system. Asterisk indicates an area consistent with neuronal track leading to posterior pituitary. Area of interest (B-D) were imaged at 200X total magnification showing infected neurons surrounding portal blood vessels in the hypophyseal region (B), hypothalamic nuclei with fiber tracts leading to posterior pituitary (C), and OVLT region (D).

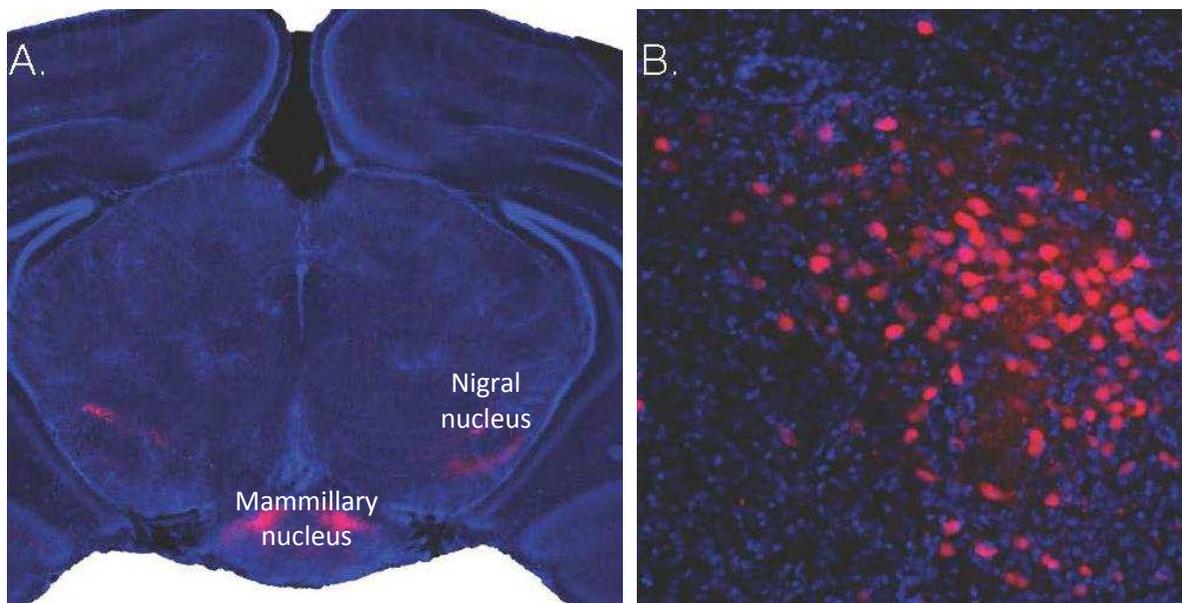


Figure 5. 16 Imaging of posterior hypothalamus and midbrain at early time points post-inoculation.

CD-1 mice were inoculated in the footpad with WEEV-3'McM-Red virus and euthanized at 4 DPI. Whole brains were sectioned, clarified, and imaged at 100X total magnification. A.) Montage imaging of coronal section, to include the posterior hypothalamus and midbrain nuclei, shows robust viral expression within nigral and mammillary tract nuclei. B.) Mammillary nuclei were viewed at 200X total magnification and show extensive infection.

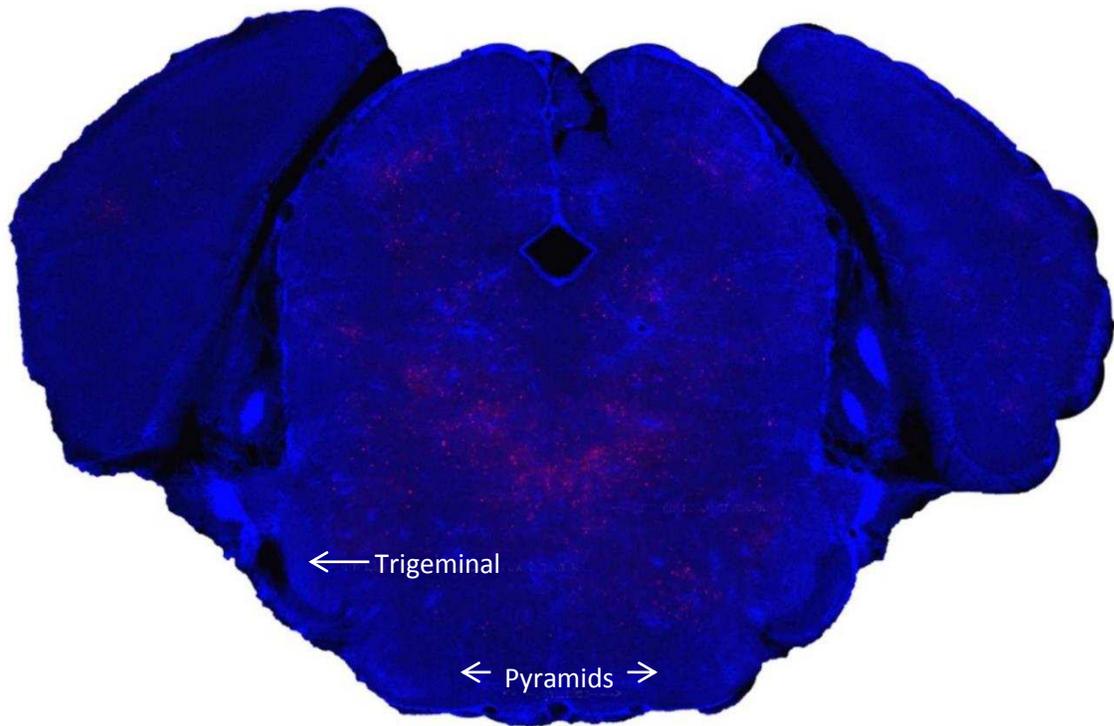


Figure 5. 17 Imaging of brainstem at early time points post-inoculation with WEEV-3'McM-Red.

CD-1 mice were inoculated in the footpad with WEEV-3'McM-Red virus and euthanized at 4 DPI. Whole brains were sectioned, clarified, and imaged at 100X total magnification. Montaged imaging of coronal section, to include the medullary pyramids, shows no viral expression areas consistent with the corticospinal tract (pyramids) or trigeminal.

imaging presented here, enhances our current model for alphavirus neuroinvasion following IN inoculation. We found that compared to WEEV-3'McM-FLuc (Phillips et al., 2013), VEEV-3908-FLuc had an identical yet delayed pattern of virus dissemination. These results highlight a common route for alphavirus neuroinvasion following IN inoculation.

Our findings, regarding neuroinvasion following SC WEEV-5'McM-FLuc, WEEV-3'McM-Red, and VEEV-3908-FLuc infection, differ from earlier studies reporting that VEEV enters the mouse CNS after SC inoculation by replicating in peripheral olfactory and dental tissues with retrograde movement of VEEV along the olfactory and trigeminal nerves (Charles et al., 1995). OSN and trigeminal nerve sites for EEEV, VEEV, and WEEV neuroinvasion were not supported in our studies. We found that the olfactory bulb can be infected later during virus dissemination in the CNS by spreading along the neuronal axis from CVO-associated entry points. The olfactory bulb and trigeminal nuclei have extensive neuronal connectivity with many other areas of the brain. This association may facilitate rapid movement of virus throughout the brain complicating interpretations of early sites of neuroinvasion.

In addition to the data presented here, evidence for non-olfactory routes of neuroinvasion following peripheral EEEV infection has been published (Vogel et al., 2005). Vogel et al. reported that following footpad inoculation, EEEV enters the CNS via a vascular route sparing the olfactory epithelium. On appearance in the CNS, EEEV exhibited a multifocal and random distribution suggesting that EEEV crosses the BBB by passive transfer or within infected leukocytes. Our findings are partially congruent with these observations. We likewise report a vascular route for EEEV, VEEV, and WEEV CNS entry; however, we found that specific areas of CNS are consistently targeted. These regions are collectively known as the CVOs and include the hypothalamus, AV3V, area postrema, and pineal body. Importantly, Vogel et al. reported EEEV infected neurons at the base of the cerebellum as an early entry site, this region is consistent with the area postrema and is congruent with the evidence we present.

The hypothalamus was the most frequently invaded CNS region by all viruses tested. Hypothalamic neurons are responsible for secreting neuroendocrine hormones into the hypophyseal portal

system and the anterior pituitary gland. Importantly, these neurons are in direct contact with circulating blood.

The AV3V region is composed of the OVLT and the SFO. The OVLT is important in blood pressure regulation with neurons of the OVLT projecting into the hypothalamus and regulating the activity of vasopressin-secreting neurons. The SFO is important in osmoregulation, as neurons in this region have receptors for many osmoregulatory hormones such as angiotensin. Like the OVLT, the SFO projects into the hypothalamus. The pattern of alphavirus dissemination seems to follow the route of the hypothalamus into the AV3V region. Thus it can be concluded that infection of the AV3V region is equivocal to hypothalamic infection.

The area postrema is important in the monitoring of toxins in the blood. This highly vascularized area is responsible for stimulating the emetic response when noxious stimuli are detected within the blood. Once again, in this region neurons are in direct contact with circulating blood.

Lastly, the pineal body is important in regulating the circadian cycle. A neuroendocrine gland itself, the pineal body was the least frequently used route for neuroinvasion. This may be a consequence of the decreased numbers of neurons when compared to those CVOs mentioned previously. Less is known about the specific innervations of the pineal body. More work is needed to further understand how important this route is to the overall CNS disease.

These CVOs are important in monitoring of blood contents and in neuroendocrine secretion functions aimed at modulating physiological and behavioral measures. Appropriately, these structures lack a normal blood-brain barrier. It is here that virus enters neuronal processes, which are in direct contact with viremic blood. Moreover, these structures are highly vascularized increasing the potential for encountering infectious viral particles in the circulating viremic blood. Future studies should investigate whether other neuroinvasive viruses gain entry to the CNS through similar means.

The studies presented here benefited from *in vivo* and *ex vivo* imaging and CLARITY immunostaining. Using these techniques, we were able to track virus dissemination in real-time and with accuracy. We confirmed that VEEV (VEEV-3908-FLuc) enters the CNS via the olfactory tract following

IN inoculation and we report a previously uncharacterized route for EEEV, VEEV, and WEEV entry into the CNS following SC inoculation. Our results have contributed to a better understanding of the pathogenesis of alphavirus encephalitides, and should lead to better targeted alphavirus therapeutics.

CHAPTER SIX: SUMMARY AND FUTURE DIRECTIONS

Summary

Alphavirus infections cause worldwide disease and death in humans and animals. Currently, alphavirus vaccines are limited and no therapeutics are available. These arboviruses are emerging/re-emerging into regions with naïve populations. As these alphaviruses spread, the need for vaccines and therapeutics has become more pronounced. The work presented in this dissertation provides a better understanding of the pathogenesis of alphaviruses and what constitutes a protective response to infection. These studies have: 1) shown the utility of alphavirus E1 vaccination in generating broad protection to alphaviruses, 2) characterized the mechanisms by which E1 antibodies provide protection to infection, 3) established alphavirus antiviral screens and identified a panel of alphavirus antivirals, and 4) resulted in the characterization of the routes of neuroinvasion for VEEV following IN inoculation and EEEV, VEEV, and WEEV following SC inoculation.

Future directions

Broad alphavirus vaccines

We have developed LANAC alphavirus E1 vaccines that provide complete protection from lethal EEEV, VEEV, and WEEV challenge in mice. We were unable to provide similar protection to hamsters following our vaccination scheme. Future studies should be aimed at identifying adjuvants that allow for the generation of antibody responses to alphavirus E1 in other animal models. Ideally, FDA-approved adjuvants should be used in the generation of alphavirus E1 antibody responses.

It would be worthwhile to identify an optimized cocktail of alphavirus E1 glycoproteins that allows for the broadest protection with the fewest number of E1 antigens. We found that LANAC VEEV E1 and LANAC WEEV E1 vaccination each provided partial cross-protection to EEEV challenge, these findings suggest that LANAC EEEV E1 vaccination alone might protect against EEEV, VEEV, and WEEV while requiring only one alphavirus E1 glycoprotein. In the studies presented here we focused on NWA

vaccination; however, OWAs pose an equal threat to human health and studies to identify LANAC alphavirus E1 vaccines that protect against OWAs should be conducted.

Mechanism of protection of E1 antibody

In the studies presented here, polyclonal E1 sera was used to characterize the mechanisms by which E1 antibodies provide protection to disease. These studies resulted in the finding that E1 antibody inhibits virus replication at late stages (i.e. budding; maturation). However, polyclonal E1 sera is limited in that multiple antibodies are present of which any number could be responsible for protection. Future studies characterizing E1 antibody mechanism should focus on monoclonal antibodies (Hunt and Roehrig, 1985).

Our studies using polyclonal E1 sera also suggested that E1 antibody gains access to the CNS. Studies aimed at demonstrating the presence of passively transferred antibody with the CNS are needed.

Antivirals to treat alphavirus infections

We have developed alphavirus antiviral drug screens that are able to identify potential alphavirus antivirals and their general MOAs. Importantly, we did not specifically identify the MOA of any compound. Characterization of the specific MOA of compounds would allow for the rational design of secondary compounds.

We tested one of our potential alphavirus antivirals, P-75802, for efficacy *in vivo*. With our study design we were unable to identify significant differences between P-75802 treated and control mice. Notably, we found that P-75802 treated mice were euthanized due to weight loss after their clinical scores were dropping; whereas, control mice were euthanized for clinical score. Future studies should be conducted that allow for 25-30% body weight reduction to determine whether P-75802 treated mice can survive infection.

Alphavirus neuroinvasion

Using *in vivo/ex vivo* imaging and fluorescence microscopy, we identified a previously uncharacterized route of hematogenous CNS invasion. We found that following peripheral challenge EEEV, VEEV, and WEEV enter the CNS via CVOs, regions that naturally lack a BBB. While this route

of CNS invasion is intuitive and has been previously proposed, to our knowledge this is the first time it has been demonstrated. Importantly, previous studies characterizing NWA neuroinvasion have reported CVOs as early entry points for alphavirus neuroinvasion. Unfortunately in these studies, the CVO route of neuroinvasion was masked by secondary sites of replication. Our utilization of *in vivo/ ex vivo* imaging and fluorescence microscopy, allowed for sample collection to occur at the time of neuroinvasion and resulting in a cleaner picture of the process of neuroinvasion. With these tools available, it will be interesting to discover if other neuroinvasive viruses are found that enter the CNS via CVOs.

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