Host Responses Associated with the Pathogenesis of Venezuelan Equine Encephalitis Virus in Mice and Evaluation of A Novel Approach to Generate Inactivated Virus Vaccines

Thesis

Submitted in the partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

Paridhi Gupta

Under the supervision of

Prof. R.K. Maheshwari, PhD

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI

RAJASTHAN, INDIA

2013

Completed at Uniformed Services University of the Health Sciences, Bethesda, Maryland, USA

under INDO-USUHS Collaboration

CERTIFICATE

This is to certify that the thesis entitled **Host Responses Associated with the Pathogenesis of Venezuelan Equine Encephalitis Virus in Mice and Evaluation of A Novel Approach to Generate Inactivated Virus Vaccines** and submitted by **Paridhi Gupta** ID No **2005PH29095** for award of Ph. D. Degree of the institute, embodies original work done by her under my supervision.

Radha K Maheshwari, Ph.D.

Professor, Department of Pathology and

Director, Combat Casualty and Life Sustainment Research,

Uniformed Services University of the Health Sciences, Bethesda, MD, USA

Coordinator, Indo-US Activities and

Adjunct Professor, BITS- Pilani, India

Date:

Acknowledgements

I express a deep sense of gratitude towards my mentor Prof. Radha K Maheshwari. He has been a constant source of support, encouragement and guidance throughout the course of my thesis work. He always gives a lot of freedom and respect to all his lab members which is very important for having a good research environment in the lab. He also emphasizes not only to be a good scientist but also a good human being. He always says working hard and being patient are keys to success. I have learnt a lot of things from him both at professional and personal level. He has been like a guardian to me over the past years and I feel privileged to work under his guidance.

I would like to thank Dr. Robert M Friedman, Chair, department of Pathology, USUHS for his constant support for the BITS-USUHS program without which I would not have got this opportunity. I would also like to acknowledge the immense help and guidance provided by Dr. Anuj Sharma. He was the only senior when I joined the lab and has been like a second mentor to me during my PhD work. Thanks for helping me with my questions, mistakes and troubleshooting over the years.

I also thank Dr. K. M. Birla, Chancellor; Dr. Bijendra Nath Jain, Vice Chancellor; Dr. R.N. Saha, Deputy Director, Research and Education Development and Administration. I extend my gratitude towards Dr. S. K. Verma, who played an key role in my joining the BITS-USU exchange student program and for his help over the years.. I appreciate the guidance and suggestions received from my Doctoral Advisory Committee members, Drs Suman Kapoor and Vidya Rajesh. I also extend a special thanks to Dr. Dinesh Kumar and Ms. Monica Sharma for serving as a bridge between the off-campus students and BITS, Pilani and helping through the course of my PhD.

I am greatful towards our collaborators, Drs. Raj K Puri, Jing Han and Amy Yang for helping with my microarray experiments; our collaborators at National Cancer Institute, NIH Drs. Robert Blumenthal, Yossuf Raviv and Mathias Viard for helping with the INA studies; our collaborators at United States Army Medical Research Institute for Infectious Diseases, Fort Detrick for their help with the V3526 studies. This work would not have been complete without the help and critical input from all of them.

I would like to thank all my lab colleagues Raghav, Manoshi, Shelley, Dr. Sethu and Manish for their support and help over the years as well as for providing a cordial working environment in the lab. I have been lucky to have found some wonderful friends over the years. Saumyaa, Swagata, Debargh, Rajesh, Sangeetha, Swadhinya, Naveen and Dimple di; thank you guys for being there and helping me navigate through the difficult evenings when my experiments would not work and celebrating with me the little joys over these years. It means even more when you are so far away from your family and country. I cannot forget my oldest friend and roommate, Monika, who has listened to all my happy and sad tales on a day to day basis and taken care of me like a sister.

Then there is "Family". I am grateful to my parents and siblings for their support, encouragement and guidance throughout my life and for supporting my decision of pursuing PhD in US. I know it was especially tough for my Mom to let her eldest kid go so far away from her. But I am here today because of their trust and confidence in me.

I am also lucky to have wonderful family through my marriage who supported and respected my work. Specially my mother-in-law, who always encourages me to give equal importance to my career and wants me to be a self-dependent person.

Words are not sufficient to appreciate the efforts and support from my loving husband and my best friend ever, Animesh. The credit of my PhD goes to him in several ways. First of all, he was the one who inspired me to pursue PhD and come to US for studies. Being in a long distance relationship is very tough, but instead of complaining he supported and encouraged me to complete my PhD. He has been my constant wall of love, strength and support through thick and thin over the past several years. I feel very lucky to have him as my life partner.

Last but not the least; I thank the Almighty for keeping me surrounded by all these people who make my world a better happier place to live and perform well.

Abstract

Viruses are obligate microbes that have evolved plethora of mechanisms to interact with their host and exploit the host factors for replication. Vaccines are given to the host prior to infection to prevent disease development against such pathogens whereas therapeutic drugs are used to fight against the infection once the virus has already entered the host system. Newer strategies are often required to develop efficient drugs and vaccines to fight against these infections. The present study focuses on both these aspects by identifying novel host derived drug development targets and evaluating second generation inactivated vaccine candidate against Venezuelan equine encephalitis virus (VEEV) infection.

VEEV is a human pathogen which causes flu like disease and spreads through mosquito bite. The host-virus interactions are generally reflected as specific changes in the gene-expression pattern and signal transduction pathways in the host. However, the host–virus interactions which lead to the development of the VEEV disease are not well characterized. VEEV was first recognized in 1938 in Venezuela. Even after more than 70 years of its discovery, there is no safe, licensed vaccine or antiviral therapy against VEEV infection. A better understanding of virus-host interactions and host responses triggered upon VEEV infection is needed to develop effective antiviral drugs against VEEV. More specifically, since VEEV is a neurotropic virus, host factors that may play a critical role in the entry of VEEV to the host brain need to be identified. In this study, the global gene expression response of the host against neuroinvasive and non-neuroinvasive VEEV infections has been characterized. Several host signaling pathways specifically modulated against each type of VEEV infections were identified in blood, brain and spleen along with a VEEV specific gene signature which can be developed as VEEV specific biomarker. The effect of chemotherapeutic agents such as tunicamycin,

antimalarials and environmental pollutants on these virus-host interactions was also studied. Modulation of Interferon pathway was observed in presence of these chemotherapeutic agents during virus infections which may be responsible for worsening the disease.

Studies were conducted to evaluate a novel inactivation strategy to generate second generation inactivated VEEV vaccine. INA (1, 5 iodonapthyl azide), is a photo-reactive compound which has been shown to inactivate enveloped viruses. Since, there is no licensed vaccine against VEEV infection, an attenuated strain of VEEV, V3526, which failed during clinical trials due to severe immunoreactivity in the patients, was further inactivated using INA. Additionally, INA-inactivation was extended to Encephalomyocarditis virus (EMCV), a non-enveloped virus and the safety and protective efficacy of the inactivated vaccine was evaluated in the mouse model.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

BBB	Blood brain barrier
BSA	Bovine serum albumin
BSL-3	Biosafety level 3
Ccl	Chemokine (C-C motif) ligand
$CdCl_2$	Cadmium chloride
CHL	Chroloquine
CNS	Central nervous system
CPE	Cytopathic effect
CV	Crystal violet
Cxcl	Chemokine (C-X-C motif) ligand
Cxcr	Chemokine (C-C motif) receptor
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
EIi	INA (100µM) -treated and UV-irradiated EMCV
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
EMCV	Encephalomyocarditis virus
FDA	Food and drug administration, USA
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GOFFA	Gene ontology for functional analysis
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
ICAM1	Intercellular adhesion molecule-1
IFN	Interferon
IFNAR1	Interferon- alpha receptor-1
IL	Interleukin
INA	1-5 iodonapthyl azide
IRF	Interferon regulatory factors
mAdb	Microarray database

MEM	Minimum essential medium
MOI	Multiplicity of infection
MST	Mean survival time
MTT	3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide
Nfkb	Nuclear factor kappa beta
NIH	National Institutes of Health
NSP	Non-structural protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming unit
PI	Post infection
RT	Reverse transcription
SFV	Semliki forest virus
$TCID_{50}$	50% tissue culture infective dose
TM	Tunicamycin
VEEV	Venezuelan equine encephalitis virus
VIi	INA (100µM) -treated and UV-irradiated V3526
V-mice	VEEV infected mice
VRP	Virus replicon particle
VT-mice	VEEV infected and tunicamycin treated mice
WNV	West nile virus

PART-1

Host Responses Associated with Neuroinvasive and Neurovirulent Venezuelan Equine Encephalitis Virus Infection and the Effect of Chemotherapeutic Agents on these Host Responses

Chapter-1.1: Introduction

Venezuelan equine encephalitis virus (VEEV) is a mosquito-borne virus and infects a wide range of hosts e.g. mice, rats, hamsters, guinea pigs, rabbits, monkeys, horses and humans. Due to frequent outbreaks at every 5-10 years intervals throughout the tropical Americas, VEEV has been categorized as an emerging infectious disease. VEEV is a highly infectious virus which can easily spread through aerosol. It has been bio-weaponized by Russia and USA in past and is considered a bio-warfare agent. Currently, no licensed vaccine or therapeutic drug is available against VEEV. Therefore, it is important to understand the pathogenesis of VEEV in order to identify new avenues for developing prophylactic or therapeutic measures.

A. Taxonomy:

Viruses transmitted through arthropods e.g. mosquitoes, are referred to as arboviruses. Initially, all the arboviruses with a morphological structure resembling to a Roman cloak (Latin: *toga*) were classified under one family, *Togaviridae* (Porterfield JS, ed. Plenum press, 1986). Togaviruses were classified as Group A (Alphaviruses), Group B (Flaviviruses) and later Group C (Rubella virus). Alphaviruses, the largest of the three genera, is further divided in seven antigenically related complexes (Powers et al 2001) (Table-1). Alphaviruses have a worldwide distribution and thus are also classified based on their geographic distribution as Old World viruses (that cause rash and arthritis) or New World viruses (that cause encephalitis). VEEV is a member of group A (New World viruses) along with eastern and western equine encephalitis virus (EEEV and WEEV respectively).

VEEV complex viruses have six subtypes based on hemagglutination inhibition and neutralization test called subtype I-VI (Calisher et al 1985). Subtype-I (VEE) is further divided into IA, IB, ID, IE, IF, II, IIIA, IIIB, IIIC, IIID`, IV, V and VI. These subtypes can be classified as epizootic (that cause epidemics) and enzootic (that do not cause epidemics) strains (Young and Jhonson 1969). Epizootic strains are IAB and IC and enzootic are ID, IE, IF, II, IIIA, IIIB, IIIC, IIID, IV, V and VI (reviewed in Weaver et al 2004 a, b).

B. History, outbreaks and epidemiology:

Several reports suggest that alphavirus epidemic outbreaks date back up to 18th and 19th centuries. The first alphavirus to be isolated and cultured was Western equine encephalitis virus in 1930 followed by Eastern equine encephalitis virus in 1933, however, first widely recognized VEEV outbreak appeared in the central river valleys of Colombia during 1935 where hundreds of thousands of horses died due to encephalitis. The epidemic then spread in Guajira region of northern Columbia and Venezuela. From 1936 to 1938, the VEEV outbreak spread across northern Venezuela and in 1943 appeared on the Island of Trinidad but was identified as a human pathogen only during 1943 in laboratory personnel (Beck and Wyckoff 1938, Kubes and Rios 1939, Casals et al 1943). Since 1938, VEEV outbreaks occurred periodically until 1973 after almost every 10 years involving tens of thousands of equines and people (Sanmartin-Barberi et al 1954). One of the largest outbreaks of VEEV occurred in 1960s where more than 200,000 known human infections and 100,000 equine deaths occurred in Central Colombia. Vigorous immunization of equine population and anti-mosquito spray contained further spread of this outbreak (Zehmer et al 1974, Calisher and Maness 1974). From 1973 to 1992, no VEEV was documented, raising speculations of extinction of VEEV strains from natural circulation. It was also hypothesized that death of large number of equines, reduced mosquito population,

development of a long lasting immunity after the 1969 VEEV outbreak and vigorous vaccination of equines may have helped in eradication of VEE disease.

After a gap of almost two decades, several outbreaks occurred. In 1992, a small outbreak of VEEV was documented with twenty four equine and four human reported cases in Trujillo and Zulia state of western Venezuela (Rico-Hesse et al 1995). During the following year small equine outbreak was described in Pacific coastal communities of the Chiapas and Oaxaca states of Mexico (Oberste et al 1998). An outbreak occurred during June 1994 among Peruvian army troops in Northern Peru causing febrile illness characterized by headache, ocular pain, myalgia, and arthralgia in 8 soldiers and another 24 having afebrile illness (Watts et al 1997). The viral isolates were found to be closely related to VEE ID viruses previously isolated in Peru, Colombia, and Venezuela regions. Two years later, another equine outbreak occurred in the adjacent Oaxaca State but no human cases were recorded. Further spread of these outbreaks was controlled by vaccination of equine population, limiting the equine transportation and use of insecticides to control the mosquito population. Another major VEEV outbreak occurred in 1995 in Venezuela and Colombia during which an estimated 75000 to 100,000 human cases and nearly 20 deaths were reported (Weaver et al 1996). Cases were detected in eastern Falcon State, Venezuela and in Carabobo, Yaracuy, and Lara States, Colombia (Navarro et al 2005). Immediate measures such as restricting the transport and vaccination of equine population, and spray of insecticides to control the mosquito population prevented further spread of this outbreak into more populated areas of Colombia. This outbreak was found to be remarkably similar to the 1962–1964 outbreaks from the same region (Briceno Rossi AL 1965). Since 1996, sporadic cases of unconfirmed equine encephalitis have occurred in Mexico and Central America. A serological survey conducted in 2000 to 2001 in Chiapas State, Mexico within human as well as

animal populations identified seroprevalence levels of 18% to 75% and that medical personnel had a high risk for VEEV exposure (Estrada-Franco et al 2004). The study also indicated cotton rats to be the reservoir hosts in the region. Focal outbreaks also occurred in equines during December'1999 and February'2000 in Carabobo and Barinas States and in Barinas State during October 2003 in Venezuela of VEE subtype IC suggesting persistent infection in the region (Navarro et al 2005). During 2006, nearly 63 confirmed cases of human VEEV type-ID infection were reported in Iquitos, Peru which was a 5-fold higher number than the regularly reported cases (Morrison et al 2008). Serological studies revealed that more than 23% population in the Iquitos region carried neutralizing antibodies against VEEV indicating persistence of VEEV infection in that region. Occassional cases of VEEV infection are recorded in the VEEV endemic areas at regular intervals (Vilcarromero et al 2010). As a result of these recent outbreaks, VEEV infection is now recognized as an emerging infectious disease.

VEEV complex viruses transmit through perennial active cycles in the tropical and subtropical regions of Americas. Wild birds can get infected but mammals e.g. cotton rats, spiny rats, bats and opossum are likely to be the hosts. Some of the enzootic strains infect equines but cause very low titered viremia and thus little or no illness. Epizootic VEEV complex viruses, however, cause severe infection in horses. Humans can get infected with both epizootic as well as some enzootic strains of VEEV (reviewed by Weaver et al 2004 a, b).

C. Virus-host interactions

Alphaviruses display a wide range of host tropism both *in-vivo* and *in-vitro*. Within a host, they can infect a wide variety of cells including neurons and glial cells, striate and smooth muscle cells, lymphoid cells, synovial cells, and even brown fat cells. Due to such a broad host

range, two different types of hypothesis have been proposed regarding the nature of receptor utilized by the virus to bind to the host cell (Strauss et al 1994). First hypothesis is that a ubiquitously present receptor is utilized by alphaviruses so that the same receptor throughout a wide host range will be sufficient. The second hypothesis is that the virus E2 glycoprotein has several distinct sites for binding to different types of receptors on different cell types. Supporting evidences are available for both the hypothesis and it may be a combination of both which facilitates alphaviruses with such a diverse host tropism. VEEV has been suggested to utilize laminin binding protein as a receptor for entry into the cell via receptor mediated endocytosis (Ludwig et al 1996, Weaver et al 2004 a, b). Mutations in E2 protein especially at the glycosylation site have been considered to be important in VEEV virulence determination and enzootic phenotype evolution (Kinney et al 1998, Anishchenko et al 2006). Similarly, amino acid changes in the E2 protein region of Sindbis virus have been shown to alter viral entry and affect early steps in viral replication (Griffin DE 1999).

D. Structure, entry and replication of alphaviruses

Structure

Alphaviruses are enveloped viruses containing an icosahedral nucleocapsid and a single stranded plus-sense RNA genome. VEEV virions are 65-70nm in diameter. The nucleocapsid is enclosed in a host plasma membrane derived lipid bilayer envelope (Figure-1). Two virus encoded glycoprotein, E1 and E2 are incorporated in the envelope. E1 and E2 glycoprotein heterodimers make spike like trimer on the surface of the virus. 240 copies of each glycoprotein interact with 240 copies of capsid protein. Envelope is composed of 80 protein trimers [three heterodimers of E1 and E2 glycoprotein] with each trimer forming a part of overlapping series of

pentons and hexons capsomer arranged on a T=4 icosahedra lattice (Paredes et al 2001, Strauss and Strauss 1994).

Genome

VEEV genome is a positive sense, single-stranded RNA with a 5'cap, 3'polyadenylated tail and non-translated regions at both ends. The RNA is 11.44 Kb in length and infectious. The message sense RNA genome of VEEV is infectious under suitable conditions (Griffin DE 1999, Guzman et al 2005) and cDNA copies of the genomic RNA can be used to transcribe infectious RNA (Kolykhalov et al. 1992, Pratt et al 2003). The 5' region of the genome encodes for 4 nonstructural proteins (nsP1-nsP4) comprising of two third of the total RNA (Figure-2). The 3'region encodes for the capsid protein, E1, E2, 6K and E3 (Hardy et al 1990). The non-structural proteins are expressed as one or two polypeptides (P123 + nsP4 or P1234) due to the presence of a leaky opal stop codon (UGA) following nsP3. The polypeptides are cleaved into nsP1, nsP2, nsP3 and nsP4 by the proteolytic activity of nsP2 (Table-2). The structural proteins are expressed as a single polypeptide (p130) which finally cleaves into C, 6K, E1, E2 and E3 proteins. E1 and E2 proteins have multiple N-linked glycosylation sites and both the proteins undergo post-translational glycosylation in the endoplasmic reticulum. Location of these glycosylation sites vary between the different alphaviruses (White and Fenner 1994, Griffin DE 1999).

Virus entry into the host cell

Generally alphaviruses are believed to enter the host cell via receptor-mediated endocytosis and low-pH-mediated virus membrane-cell membrane fusion; however an alternative mechanism of virus entry has also been suggested which does not require endocytosis or low-pH-mediated membrane fusion and instead releases the viral genome directly into the cell

cytoplasm through generation of ion-permeable pores (reviewed by Brown and Hernandez 2012).

Receptor mediated entry of virus into the host cell occurs by attachment of the virus to host cell receptors, internalization via endocytic pathways which induces conformational changes in the viral glycoproteins and thus triggering virus fusion and release in the cell cytoplasm (Figure-3) (Helenius et al 1980, DeTulleo and Kirchhausen 1998). VEEV has been suggested to utilize laminin binding protein as a receptor for entry into the cell (Ludwig et al 1996, Weaver et al 2004). Binding of virus to heparan sulfate has been shown not to induce such a conformational change in the viral glycoproteins (reviewed in Kielian et al 2006). Entry of virus particles is facilitated by interaction of E2 with the surface receptors and internalization in coated vesicles via clathrin-dependent pathway (Table-2) (Paredes et al 2003). The VEEV particles are delivered from early endosomal compartments to the late endosomes and finally to lysosomes. During this delivery process virus particles are continuously exposed to reduced pH due to increase in amount of acidification of each compartment by vacuolar ATPase which triggers fusion of viral and cellular membranes facilitating the release of nucleocapsid core into the cytoplasm (Schmid et al 1989). In presence of low pH (less than 6.0), the E1-E2 heterodimers are destabilized in the early endosome compartments, dissociating the dimer and exposing the E1 glycoprotein region (fusion peptide) required to form the fusion pore in a cholesterol-dependent manner (Table-2) (Waarts et al 2002, Brown and Hernandez 2012). Upon release into the cytoplasm, uncoating of the nucleocapsid occurs either by interaction with ribosomes or due to priming by low pH in the endosomes (Wengler and Wengler 2002). In the alternative entry mechanism, the virus entry occurs at the plasma membrane and involves a pore complex made from virus and host proteins that connects the interior of the virus to the host cell

cytoplasm via a protein channel. E1 glycoprotein has been suggested to play an important role in the formation of these ion-permeable pores (Wengler et al 2003, 2004). Upon formation of the pores in the membrane, the viral genome is released into the cell cytoplasm.

Replication of the genome

The genomic RNA of alphaviruses serves as the mRNA being a positive sense ssRNA. Therefore, once the viral genome is released into the cytoplasm, structural and non-structural proteins are translated using the host translation machinery (reviewed by Strauss and Strauss 1986, 1994, Shope 1976). Non-structural proteins (nsPs) are expressed as polypeptides (P1234 or P123) and later cleaved into individual nsP1 to nsP4). Due to presence of an opal codon at the end of nsP3 region, most of the time translation is terminated giving rise to P123 polypeptide whereas a read through of this leaky stop codon also results in synthesis of P1234 polypeptide. The polypeptide is processed through the protease activity of the nsP2 protein. P1234 is cleaved in *cis* between nsP3 and nsP4 to yield P123 and nsP4 (the viral RNA-dependent RNA polymerase) early in the infection cycle (reviewed by Strauss and Strauss 1986, 1994, Shope 1976). Three types of viral RNA species are synthesized in alphavirus infected cells which includes 26S subgenomic mRNA for expression of structural proteins, negative strand RNA and the full length positive strand genomic RNA (Figure-2 and 3). The processing of polypeptide and the levels of subsequent precursor and end-product nsPs regulate the synthesis of different viral RNA species on the cytoplasmic surface of the endosomes and lysosomes on structures called as cytopathic vacuoles. P123 or P23 and nsP4 interact to form the replication complex required to synthesize the negative strand of the genome. After complete cleavage to nsP1, nsP2, nsP3, and nsP4, negative-strand synthesis is inactivated and the now stable replication complex switches to the synthesis of positive-strand genomic and subgenomic RNA. The minus strand synthesis level

is about 2-5% of the amount of positive strand synthesized. The subgenomic RNA is synthesized up to 3-fold higher concentration than the genomic RNA.

Structural proteins are expressed later during the infection cycle from the 26S subgenomic RNA as a single polypeptide. It is then cleaved into individual proteins capsid, 6K, E1 and precursor E2 (pE2) co-translationally by the autoproteolytic activity of the capsid protein, cellular signalase protein and a furin-like protease (Shope 1976, White and Fenner 1994). The capsid protein associates with newly synthesized RNA, recognizing specific packaging signals in the 5′ half of the genome, such that only full-length genomic RNA is packaged into nucleocapsid-like particles. Capsid protein has also been shown to inhibit nuclear import in the host cells (Atasheva et al 2008). In parallel to nucleocapsid formation, E1 and pE2 undergo post-translation modifications, interact to form the heterodimers and are translocated from endoplasmic reticulum to cell surface via golgi complex (Figure-3). During later stages of translocation, pE2 is cleaved into E2 and E3 by host furin-like protease. As a result of this cleavage, only E2 reaches the plasma membrane and thus E3 glycoprotein is not packaged in the mature VEEV virion particles (reviewed in Strauss and Strauss 1994, Weaver et al 2004). Proper interaction of the capsid protein (present in the nucleocapsid) with the cytoplasmic domain of E2 glycoprotein (present as a hetrodimer with E1 glycoprotein on the plasma membrane) initiates the budding process of the mature virion. Studies have shown an important role of E3 peptide in E1-pE2 hetrodimer formation and translocation to the ER due to presence of a translocation signal in E3 (Lobigs et al 1990). Presence of nucleocapsid is not necessary for budding process as lateral interactions between E1 and E2 glycoproteins and their interaction with the capsid protein alone are sufficient to initiate budding process. 6K has been suggested to assist in virus assembly, though is not essential and also gets incorporated into the virion in small amounts

(Strauss and Strauss 1994, Griffin DE 1999). While releasing from the host cell, virions acquire a membrane bilayer derived from the host cell plasma membrane (Figure-3).

E. Site-directed mutant strains of VEEV

These strains were generated by site-directed mutagenesis in the two glycoprotein genes of the viral envelop and the details of which are described by Grieder et al 1995 (Table-3). V3000 is a full length cDNA clone of the Trinidad Donkey (TrD) strain of VEEV. It is a neuroinvasive strain that is as neurovirulent and infectious as the wild type virus (TrD strain) both *in-vitro* as well as *in-vivo* (Charles et al 2001). V3034 is also a neuroinvasive but a partially-neurovirulent mutant of VEEV. V3034 has a single site mutation in E1 glycoprotein at position-272 resulting in a change from alanine to threonine (272, Ala→Thr) (Table-3). V3014 is a non-neuroinvasive strain of VEEV which is however, fully neurovirulent when injected intracerebally (Table-3) (Grieder et al 1995). V3014 also possesses the mutation from alanine to threonine (272, Ala→Thr) at position-272 in E1 glycoprotein along with two more mutations in E2 glycoprotein with lysine in place of glutamic acid at codon-209 (209, Glu→Lys) and asparagine instead of isoleucine at codon-239 (239, Ile→Asn). It has been suggested that V3014 is rapidly cleared off from the blood stream due to its higher binding capacity to heparan sulfate, a glucosaminoglycan, ubiquitously present on most cell surfaces (Figure-4) (Bernard et al 2000). Though the two mutants, V3034 and V3014 have different degree of neuroinvasiveness compared to the wild type clone, V3000, these induce robust cytokine and antibody responses respectively in the host (Schoneboom et al 2000, Steele et al 2006, Davis et al 2001, Charles et al 1997).

F. Animal Model to Study VEEV Pathogenesis

Several species of animals have been used as models for studying VEEV pathogenesis (Jackson et al 1991, Pratt et al 2003). Horses and other equines are highly susceptible to epizootic strains of VEEV. Morbidity in epizootics is estimated at 40%–60% of susceptible horses and mortality rates of around 50% (Monlux and Luedke 1973). Horses are important to study the natural transmission caused by VEEV, but, since these are large animals, their use as an experimental model of VEEV is expensive and thus very limited.

VEEV infection in humans displays a biphasic febrile illness with damage to lymphoid tissues followed by CNS manifestations. Most *in-vivo* studies with VEEV have primarily focused on characterization of the early pathogenesis of VEEV, the route of neuroinvasion and the host and viral factors that contribute to the development of encephalitis. Several laboratory animals e.g. hamsters, rabbits, and guinea pigs have been shown to develop an acute, fulminant VEE disease marked by massive necrosis of lymphoid tissues (Gleiser et al 1962, Jackson et al 1991, Pratt et al 2003). Since these animals usually die before the onset of CNS disease, they cannot be ideal models for studying the neurological phase of VEEV infection in humans. Mice and nonhuman primates (NHPs) mimic the biphasic infection of VEEV in humans and thus are considered most relevant animal models to study VEEV infection *in-vivo* (Jackson et al 1991, Grieder et al 1995, Reed et al 2005). Several murine strains such as BALB/c, C3H/Hen and Swiss CD-1 have been studied and described as models for studying VEEV infection (Paessler et al 2003, Bigler et al 1974). Both BALB/c and CD-1 mice initiate humoral antibody response following subcutaneous vaccination with TC-83 vaccine and are a commonly used model to study VEEV pathogenesis (Hart et al 1997, Steele et al 2006).

G. Potential Bio-Weapon and Military Relevance

VEEV is an NIAID Category B priority pathogen, since it can grow to high titers in cell cultures and thus it is easy and inexpensive to be produced in large quantity. It is highly stable in nature both in liquid and dried forms and is highly infectious when aerosolized (Richmond and McKinney 1993, Pratt et al 2003, Guzman et al 2005). The United States and the former Soviet Union weaponized VEEV as an offensive incapacitating agent before terminating their biological weapons programs in 1969. Soviet scientists cloned VEEV genome into a smallpox virus backbone which resulted in a recombinant smallpox-VEEV chimera virus that resembled smallpox under a microscope but produced different symptoms in its hosts (Shubladze et al 1959).

VEEV is highly infectious and infection with 10-100 particles has the potential to cause disease in a person (www.globalsecurity.org/wmd/intro/bio_vee.htm). It has caused more laboratory-acquired disease than any other arbovirus. Since its discovery, at least 150 symptomatic laboratory infections have been reported, most of which have been attributed to aerosol exposure (Hanson et al 1967, Richmond and McKinney 1993). In 1959, at least 20 individuals developed disease within 28 to 33 hours after a small number of vials containing lyophilized virus were dropped and broken in a stairwell at the Ivanovskii Institute in Moscow, in the former Soviet Union (Shubladze et al 1959). Thus, considering its infectious nature, VEEV has been characterized as a BSL-3 agent.

VEEV is endemic to the tropical and subtropical regions of America with frequent outbreaks occurring at regular intervals. The military personnel's deployed to these regions are at a greater risk of acquiring natural VEEV infection as observed during 1997 where nearly 32 soldiers developed febrile or afebrile VEE disease (Watts et al 1998, Estrada-Franco et al 2004).

Although the rate of mortality upon VEEV infection is low but it may lead to wide spread morbidity among the deployed soldiers.

Additionally, there is no licensed vaccine or therapeutic drug against VEEV in humans at present. TC-83 and C-84 are only given to at-risk laboratory personnel and have high number of non-responders or low level of neutralizing response in the host (Pittman et al 1996). In such a scenario with no effective vaccination or treatment program against VEEV, use of VEEV as a bio-weapon would cause a wide spread morbidity and mortality in the masses as well as the military forces. This may lead into a major setback for the country's economy due to reduced productivity of the nation's workforce, expenditure on hospitalization, prophylaxis and out-patient visits by the patients etc as observed in past due to Anthrax attack in USA. Studies have shown that the economic impact of a bioterrorist attack by aerosol exposure can range from an estimated $477.7 million per 100,000 persons exposed (brucellosis scenario) to $26.2 billion per 100,000 persons exposed (anthrax scenario) (Kaufmann et al 1997). Due to these reasons, there is an urgent need to develop effective vaccines and therapeutics against VEEV infection. In order to identify potential drug development targets, we need to have a complete understanding of the type of host responses exploited by VEEV for its replication and virulence.

H. MicroRNA

MicroRNAs (miRNAs) are small (~22 nucleotide long) endogenous non coding RNA molecules and were first discovered in 1993 in C. elegans (Lee et al 1993). MiRNAs play an important role in numerous cellular processes like development, differentiation, cell proliferation, tumorigenesis, neuronal development and hematopoiesis. MiRNAs work mostly by binding to the complementary sequences on the mRNA molecules to introduce a translational

block or accelerated decay of the target sequence. At other times they can even enhance the translation of the target gene (reviewed by O'Carroll and Schaefer 2012, Junn and Mouradian 2011).

MiRNAs are encoded within the host genome with almost 40% of miRNA genes localized within the introns. They are autonomously expressed and possess their own enhancer and promoter elements. MiRNAs are first transcribed as a long primary miRNA (pri-miRNA) by RNA polymerase II (Pol II) (Figure-5). Thereafter, they are processed sequentially by the RNase III enzymes, Drosha in the nucleus and Dicer in the cytoplasm to generate ~ 22 nucleotide duplex RNA. This duplex miRNA is then loaded into the RNA-induced silencing complex, RISC, where the guide strand gets incorporated to regulate the functions whereas the other strand is discarded. The RISC complex is composed of a multitude of proteins including TAR RNA-binding protein (TRBP) and TRBP-associated factors (reviewed by O'Carroll and Schaefer 2012). MiRNAs are highly stable and non-immunogenic due to which these are considered excellent therapeutic and intervention tool. Two miRNA-based therapeutic strategies have been explored: miRNA mimics (small RNA molecules that resemble miRNA precursors) and antagomirs (synthetic inhibitors of miRNAs). Studies have also demonstrated that introduction of mimics as well as synthetic inhibitors of miRNAs into diseased tissues/cells can be an effective way to restore normal cellular and molecular processes (Scherr et al 2007, Junn and Mouradian 2011).

Table-1 Different members of VEEV antigenic complex

Species	Serotype	Transmission pattern	Equine virulence	Location	Vector
Subtype I					
VEE Virus	AB	Epizootic	Yes	Central, South and North America	*Ochlerotatus, Psorophora spp.*
VEE Virus	C	Epizootic	Yes	South America	*Ochlerotatus, Psorophora spp.*
VEE Virus	D	Enzootic	No	Central and South America	*Culex (Mel.) akenii s.l. (ocossa, panocossa), vomerifer, pedroi, adamesi*
VEE Virus	E	Enzootic	Variable	Central America and Mexico	*Culex (Mel.) taeniopus*
Mosso das Pedras Virus	F	Enzootic	Unknown	Brazil	Unknown
Subtype II					
Everglades Virus		Enzootic	No	Southern Florida	*Culex (Mel.) cedecei*
Subtype III					
Mucambo Virus	A	Enzootic	No	Southern America	*Culex (Mel.) portesi*
Tonate Virus	B (also Bijou Bridge Virus)	Enzootic	Unknown	South and North America	*Unknown*, Ceciacus vicarious (cliff swallow bug)
Mucambo Virus	C (strain 71D1252)	Enzootic	Unknown	Western Peru	Unknown
Mucambo Virus	D (strain V407660)	Enzootic	Unknown	Western Peru	Unknown
Subtype IV					
Pixuna Virus		Enzootic	Unknown	Brazil	Unknown
Subtype V					
Cabassou Virus		Enzootic	Unknown	French Guyana	Unknown
Subtype VI					
Rio Negro Virus		Enzootic	Unknown	Northern Argentina	*Culex (Mel.)delpontei*

(Adapted from Weaver and Barret, nature reviews, 2004)

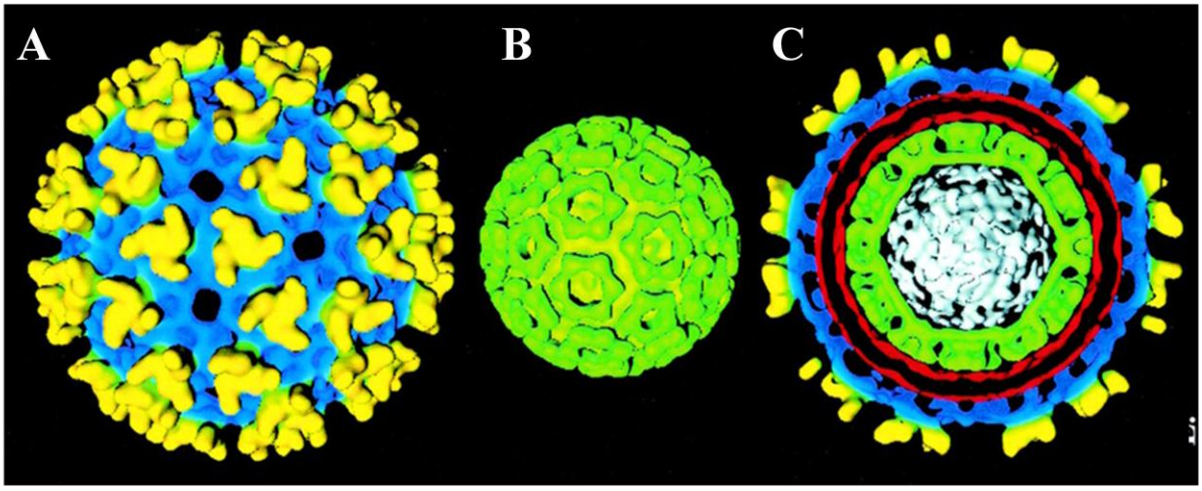


Figure-1 Structure of VEEV: Isosurface view of (A) VEEV mature virion, (B) VEEV nucleocapsid and (C) Cross-sectional view of VEEV mature virion. The structural components of the virus are color-coded:: Yellow indicates the outer spike trimers (E1-E2), blue indicates the skirt region of the outer envelope, red indicates the virus membrane derived from the host cell, green indicates the nucleocapsid, and white indicates the RNA genome. Scale bar corresponds to 100 Å. (Paredes et al 2001)

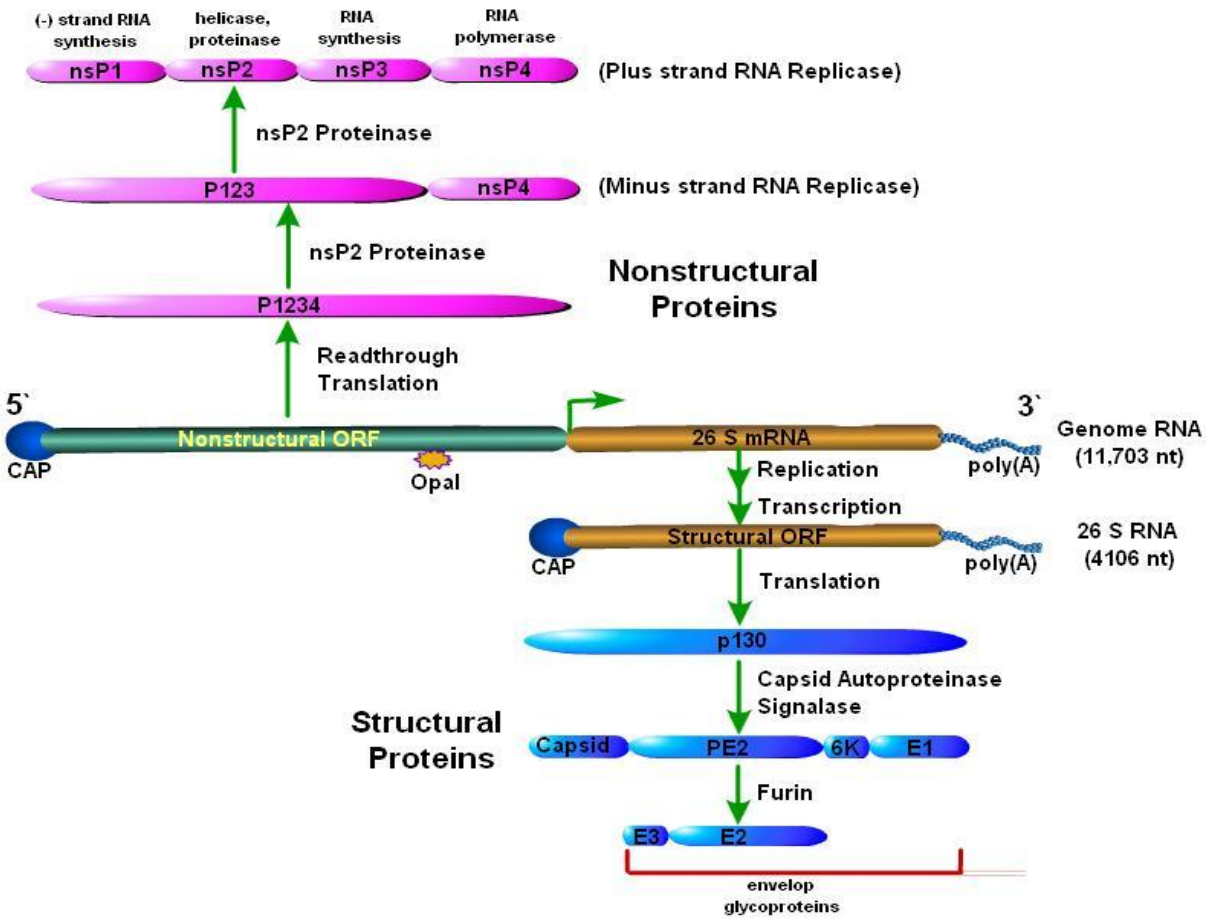


Figure-2 Genome structure of VEEV: The 49S genomic RNA of VEEV is illustrated schematically in the center. The nonstructural polyproteins and their procesed products are shown above. Termination at the opal codon (denoted in orange color) produces P123. Readthrough of the opal stop codon produces P1234, which can form an active replicase. The 26S subgenomic mRNA is expanded below to show the structural ORF and its translation products.

Table-2: Translation products of alphavirus genome and their functions

Name of Protein	Size (No. of Amino acid)	Suggested Function(s)	Category
nsP1	540	Methyltransferase and guanylyltransferase	Non structural proteins
nsP2	807	NTPase, helicase, RNA triphosphatase, protease responsible for processing of the nonstructural polyprotein	Non structural proteins
nsP3	556	Phosphoprotein with unknown function(s) but important for minus strand synthesis	Non structural proteins
nsP4	610	RNA dependent RNA polymerase (RdRp)	Non structural proteins
Capsid (C)	264	Encapsidates genomic RNA to form nucleocapsid core carboxyl domain is a serine protease	Structural proteins
E1	439	Membrane fusion activity	Structural proteins
E2	423	Carries the major neutralizing epitopes and is responsible for receptor binding	Structural proteins
E3	64	Part of the uncleaved PE2	Structural proteins
6K	55	Leader peptide for E1, enhance particle infectivity	Structural proteins

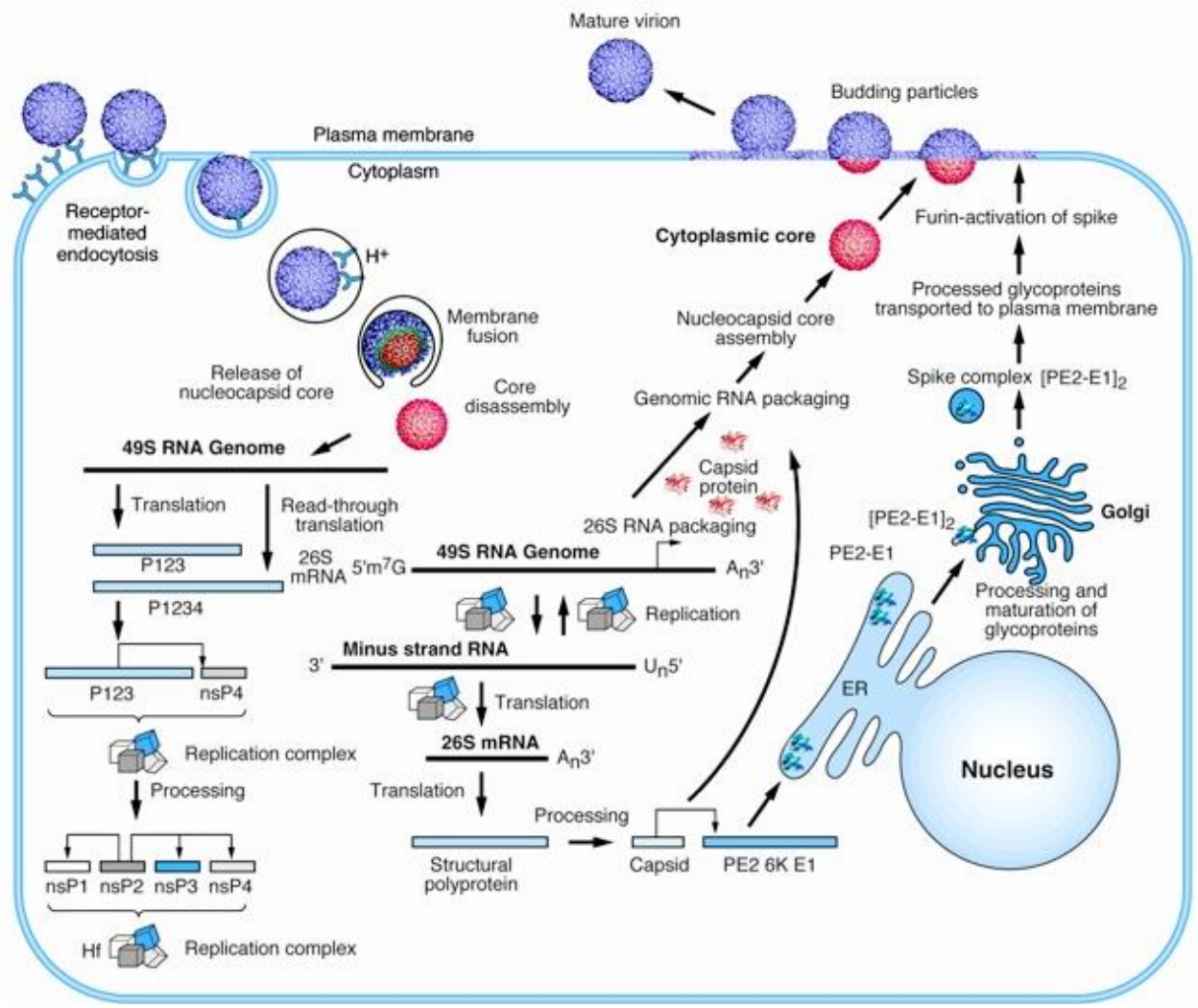


Figure-3 Life cycle of an alphavirus: Alphaviruses enter target cells by attachment of the virus to host cell receptors, internalization via endocytic pathways which induces conformational changes in the viral glycoproteins and thus triggering virus fusion and release of core and release of the viral genome into the cell cytoplasm. Non-structural proteins (nsP1-4) are first syntheses which assemble to form the viral replication complex required for synthesis of structural proteins (E1-3, 6k and Capsid). E1 and pE2 undergo maturation and glycosylation the Golgi and are transported to the host cell membrane for the viral assembly and budding process along with capsid and the genomic RNA copies. The assembled alphavirus particle, with an icosahedral core, buds at the cell membrane. (adapted from Fields Virology, Togaviridae)

Table-3 VEEV mutant strains and their extent of pathogenecity

Strain	E1region (272)	E2 region		%Neuro-invasion	% Mortality	
		(209)	(239)		i.p.*	i.c.*
V3000	Alanine	Glutamic acid	Isoleucine	100	100	100
V3034	Threonine	Glutamic acid	Isoleucine	40	11	11
V3014	Threonine	Lysine	Asparagine	0	0	100

Different site-directed mutations and their locations for different VEEV mutant strains are listed above. The resulting difference in pathology is also listed here. Neuroinvasion is defined as the percentage of mice in which virus can be detected in the brain after peripheral (footpad or subcutaneous) infection from 48 hours pi onward. (Compiled from Gieder et al 1995, Hart et al 1997, Steele et al 1998, Ludwig et al 2001)

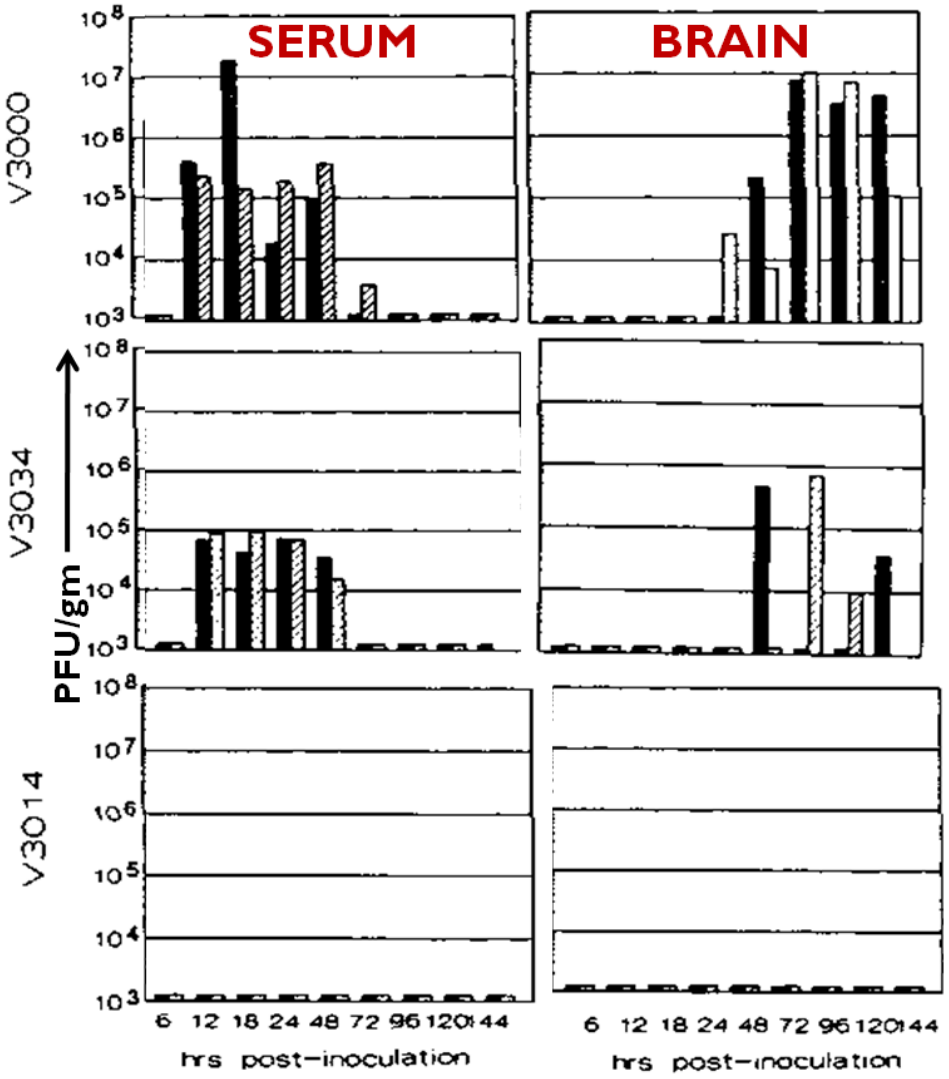


Figure-4 Kinetics of replication of VEEV mutant strains: The histogram summarizes the VEEV titers (PFU/gm tissue or PFU/ml serum) in CD1 mice inoculated with 1000PFU of virus in the left rear footpad. Each bar is representative of 1 animal. Detection level was 1.25×10^3 PFU/gm tissue or 33 PFU/ml serum. (Adapted from Grieder et al 1995)

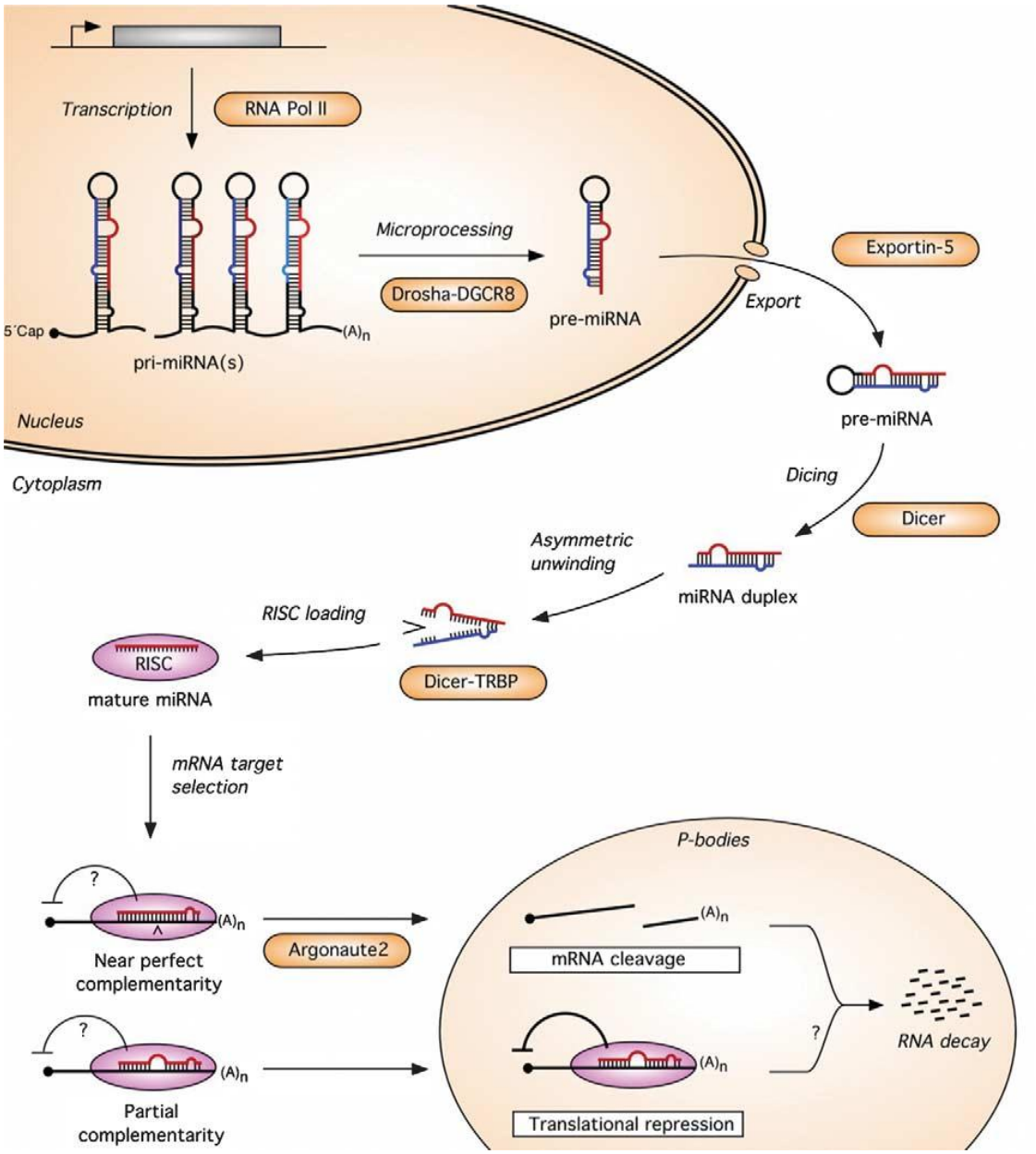


Figure-5 MicroRNA biogenesis: The canonical pathway of miRNA synthesis and maturation is illustrated here. MiRNAs are first transcribed as a long primary miRNA (pri-miRNA) by RNA polymerase II and are processed by the RNase III and Drosha enzymes in the nucleus and Dicer in the cytoplasm to generate ~ 22 nucleotide duplex RNA. This duplex miRNA is then loaded into the RNA-induced silencing complex, RISC, together with Argonaute (Ago2) where it guides RISC to silence target mRNAs through mRNA cleavage, translational repression or deadenylation. (Adapted from O'Carroll and Schaefer 2012)

Chapter-1.2: Review of Literature

A. VEEV Pathogenesis and Host Immune Responses

VEEV Pathogenesis:

Humans are highly susceptible to aerosolized form of VEEV as indicated by a number of cases of infection in laboratory personnel who are accidentally exposed while working with aerosolized VEEV. Since its discovery, at least 150 symptomatic laboratory infections have been reported, most of which have been attributed to aerosol exposure (Shubladze et al 1959, Hanson et al 1967, Richmond and McKinney 1993). Natural VEEV infection in humans is generally a nonlethal illness with fever, headache, malaise, myalgia, sore throat and vomiting as the most common symptoms. Clinical symptoms start appearing after 2-5 days of VEEV infection in humans and subside within 4–6 days after the onset in case of an acute infection (Johnson and Martin 1974, Bowen et al 1976). Lymphopenia and elevated hepatic enzymes are common during acute illness whereas CNS infection is apparent in a very small percentage of VEEV cases (0.5% of adults and up to 4% of children) which is usually apparent a few days following the acute febrile phase. The neurological disease may lead to somnolence, mild confusion, photophobia, seizures, ataxia, paralysis and even coma causing long-term neurological deficits, abortions, and teratogenic effects depending upon the severity of disease. Mortality rates in neurological cases are as high as 35% in children and 10% in adults (Bowen et al 1976). The pathogenesis of VEEV in humans is not well characterized mainly due to insufficient data obtained during various outbreaks. As mentioned above, VEEV infection in humans may cause a biphasic febrile illness with damage to lymphoid tissues followed by CNS manifestations. The

major tissues affected in fatal cases in humans with documented infection and exhibiting clinical encephalitis were found to be the brain, lymph nodes, spleen, gastrointestinal (GI) tract, liver, and lung along with infiltrating lymphocytes, mononuclear cells, and neutrophils (de la Monte et al 1985, Steele and Twenhafel 2010).

Mice are the most commonly used model animal for understanding the VEEV pathogenesis. During the lymphatic phase, virus is initially taken up by the Langerhans's cells (Skin dendritic cells) present at the site of inoculation and is transported to the lymph nodes draining the site. During this phase, VEEV infects monocyte macrophages and fibroblasts, causes viremia and disseminates to other lymphatic organs like spleen, GALT, thymus, bone marrow and nondraining lymph nodes. VEEV is effectively cleared from blood and lymphoid tissues by 3–4 days p.i., but by then the virus starts appearing in the brain. This marks the beginning of the neurotropic phase of infection where the major targets are neurons and microglia cells (Charles et al 1995). VEEV infection causes cell death, reactive gliosis and severe inflammatory response in the brain which is characterized by perivascular cuffing and intestinal mononuclear infiltration (Grieder et al 1995, Schoneboom et al 2000). The extensive neurodegeneration occurring during VEEV infection is attributed to both the virus induced neuronal death as well as inflammation induced death of neurons which are not even infected with the virus (Grieder et al 1995, Schoneboom et al 2000, Ludwig et al 2001).

Entry of virus to the brain is not well understood but is suggested to occur through olfactory neuroepithelium or trigeminal nerve routes (Charles et al 1995, Steele et al 2006). Blood brain barrier plays an important role in VEEV pathogenesis but the detailed mechanism is not well understood till now (Sharma et al 2011, Schafer et al 2011). It has been suggested that active replication of VEEV in the nasal mucosa facilitates opening up of the BBB and

subsequent multifocal spread of the virus into the host brain (Schafer et al 2011). The neurotropic phase is marked by apoptosis of neuronal cells in the brain and spinal cord. Microglia cells also show evidence of infection (Vogel et al 1996). Astrocytes are infected with VEEV *in-vitro* but are not a major target *in-vivo* (Schoneboom et al 2000). Encephalitis is accompanied by a wide range of histopathology, from mild neutrophilic infiltration to neuronal degeneration, necrotizing vasculitis, and Purkinje cell destruction (Charles etal 1995, Davis et al 1994). Both necrotic as well as apoptotic neurons are suggested to be involved during VEEV induced neuronal damage depending upon the type of neurons involved in infection (Schoneboom et al 2000, Steele et al 2006, Steele and Twenhafel 2010). For example, neurons in the pyriform cortex, Purkinje neurons in the cerebellum, pyramidal neurons of the hippocampus and large neurons in the frontal cortex displayed necrosis features while damaged granule-type neurons in the dentate gyrus of the hippocampus and cerebellum displayed apoptosis like features (Steele et al 2006). In addition to neuronal and glial cell damage, lymphocytolysis and astrocytosis is also observed in VEEV infected mice brain (Schoneboom et al 2000, Steele and Twenhafel 2010).

Host immune responses against VEEV infection:

Interferon (IFN) response is a very important part of host antiviral responses. Alphaviruses induce differing levels of type I (α/β) interferon response in the host and have varying levels of IFN sensitivity which may or may not depend upon the level of virulence of the virus. VEEV induces a very strong IFN response in the host (Ryman and Kimstra 2008). Antiviral activity of IFN and IFN regulatory factors (IRF) -1 & 2 early during VEEV infection has been well established (Grieder et al 1999, White et al 2001). Treatment of cell with IFN does not interfere with attachment and entry of alphaviruses but the infection is inhibited.

Alphaviruses including VEEV limit the ISG induction in infected neurons through host transcription and translation shutoff and such an effect of VEEV infection is also resistant to IFN priming (Yin et al 2009). Mutations in the genomic regions encoding for nsP1, nsP2 and 5' UTR have been shown to be associated with altered sensitivity to IFN (White et al 2001). IFN α/β receptor knock out (IFNAR1$^{-/-}$) mice show reduction in mean survival time (MST) after VEEV infection and the spread of virus to the brain is also accelerated (Schoneboom et al 2000, White et al 2001). These mice also exhibit much higher level of inflammation in spleen and brains after VEEV infection. Several pro-inflammatory cytokines like IFN-γ, IL-6, IL-12 and TNF-α and anti-inflammatory cytokine, IL-10 are induced against VEEV infection in the lymph nodes as early as 6 hr p.i. (Grieder et al 1997) as well as in blood in macaques by 72 hr p.i. (Hammamieh et al 2007).

Inflammation plays a very important role during VEEV pathogenesis especially in the brain tissue (Schoneboom et al 2000). Konopka et al have nicely shown that the VEEV infected cells release factors which in turn activate the surrounding yet uninfected cells. Apart from the marked encephalitis, astrogliosis and cerebral edema resulting due to the VEEV infection in host brain, a lot of neuronal death also occurs due to the high level of inflammatory cytokines such as interleukins (IL), IFN, iNOS and TNF-α (Schoneboom et al 1999, 2000, Charles et al 2001). Anti-inflammatory drugs have been shown to enhance the host survival time after SINV infection (Irani and Prow 2007). Studies from our laboratory have also shown that although treatment with anti-inflammatory drug, naproxen initially showed reduction in the clinical signs of VEEV disease but failed to protect the animals against VEEV infection (Sharma et al 2011). While the degeneration of neurons and the role of astrocytes are well accepted during VEEV infection, the role of microglia cells in VEEV neuropathology is unclear (Jackson and Rossiter

1997, Sconeboom et al 1999). Nevertheless, multiple pathogen recognition receptor pathways are triggered in microglia upon sindbis virus infection (Esen et al 2012).

VEEV is a neurotropic virus and thus a lot of emphasis has been laid on understanding the molecular changes that occur in the host brain upon VEEV infection. The mechanism(s) underlying the inflammatory immune responses to VEEV infection in brain and subsequent neurodegeneration are poorly understood. Studies from our laboratory have demonstrated that genes involved in important host immune pathways such as antigen presentation, inflammation, apoptosis and response to virus (*Cxcl10, CxCl11*, *Ccl5*, *Ifr7*, *Ifi27 Oas1b*, *Fcerg1*, *Mif*, *Clusterin and MHC class II)* are upregulated against a virulent VEEV infection in brain (Sharma et al 2008). VEEV infection was also shown to upregulate *Toll Like Receptors (1*, *2*, *3, 7* and *9)* as well as several IFN regulatory genes such as *Mcp1, Cxcl10, IL12, IFN-β, IRF-1, IRF-7, Fos, Jun, MyD88, Nfkb, Cd14* and *Cd86 (*Sharma et al 2008, 2009*)*. On the other hand, it was recently shown that host survival post Sindbis virus infection is independent of TLR-mediated responses (Esen et al 2012). VEEV has also been shown to suppress S100b, a factor associated with brain injury, and Myelin oligodendrocyte glycoprotein (MOG, expressed on oligodendrocytes and serves as a necessary "adhesion molecule" to provide structural integrity to the myelin sheath) gene expression in the host brain (Koterski et al 2007). Recently, it was also shown that the host complement system plays a critical role in protecting the host from VEEV-induced encephalitis by clearance of the peripheral virus infection (Brooke et al 2012). Although a number of studies have focused at understanding the different aspects of the host-virus interactions and the responses triggered following that but still a lot remains to be understood and explored.

B. Chemotherapeutic Agents and Virus Infections

***Tunicamycin*:**

Tunicamycin (TM) is a mixture of homologous nucleoside antibiotics and is produced by several species of bacterium *Streptomyces*. The structure of TM consists of uracil, N-acetylglucosamine, an 11-carbon 2-aminodialdose sugar and an amide-linked fatty acid (Tsvetanomva et al 2002). TM blocks the N-linked glycosylation of proteins in the cell and causes cell cycle arrest in G1 phase. Due to this property, TM is widely used in cell biology to study post translational modification and transportation of glycoproteins. A group of toxins called corynetoxins are products of *Rathayibacter Toxicus* and are very closely related to TM in structure and biological activity (Takatsuki et al 1971). These toxins may contaminate *Lolium rigidum* (annual ryegrass) and are ingested by the cattle and sheep leading to fatal hepatocerebral disorder or a disease known as annual ryegrass toxicity in the affected animals (Vogel et al 1981).

Antimalarials:

Quinine is a naturally occurring white crystalline alkaloid with antimalarial, anti-analgesic, anti-inflammatory and anti-pyretic properties. It occurs naturally in the bark of the cinchona tree however, it can be easily synthesized in laboratory as well. Quinine has been used against *P. falciparum* since 17^{th} century (reviewed in Achan et al 2011). Chloroquine (7-chloro-4-(4-diethylamino-1-methylbutylamino) quinoline, CHL) was originally synthesized by Bayer Corporation in 1934 as a cheaper alternate to quinine. It has a quinoline ring like that of the quinine and a side chain identical to that of quinacrine; and the chloride atom in the seventh position appears to be crucial to its antimalarial activity. CHL is generally taken orally and is well absorbed from the gastrointestinal tract. It acts by interfering with the malarial parasite's

hemoglobin digestive pathway and prevents development of malaria parasite in the host blood. Being more effective than other antimalarial like quinidine and quinine against different malarial parasites, CHL was developed as the drug of choice for prophylaxis and treatment of all types of malaria. Due to its ability to block pro-inflammatory cytokine release, CHL has also been used as an anti-inflammatory drug in the treatment of rheumatoid arthritis, discoid lupus erythematosus and amoebic hepatitis. Several studies advocate the use of CHL as an anti-viral drug as well as for the treatment of cancer. CHL is one of the most successful and widely used antimalarial drugs (Cooper and Magwere 2008).

The major drawbacks associated with use of CHL are adverse effect on liver functioning, vomiting, headache and ineffectiveness against the dormant liver stage of parasitic infection resulting in relapse of malaria. Therefore, malaria treatment often involves a combination therapy using CHL and primaquine. Primaquine is mainly used to treat the *P. vivax* or *P. ovale* malaria and is effective against the dormant liver stage of parasitic infection (hypnozoites) in host. Once the parasite has been eliminated from the bloodstream using sulfadoxine, CHL or other quinine derivatives, the remaining hypnozoites are eradicated from the liver using primaquine. However, primaquine cannot be given to pregnant women, children and Glucose-6-Phosphate Dehydrogenase deficient people. Primaquine also has some side effects which may include vomiting, nausea, headache, stomach cramps and itching (Galappathy et al 2007).

Effect of chemotherapeutics on viral infections in-vitro and in-vivo:

CHL inhibits the replication of several viruses *in vitro* (Ooi et al 2006, Inglot AD 1969). This inhibitory effect may be related to the inhibition of acidification of the endosome thereby preventing the conformational changes in the envelop glycoproteins of the viral envelop for facilitating fusion and thereafter release of nucleocapsid into the cytosol or due to inhibition of

low pH dependent proteases important for the glycosylation of nascent viral proteins in the Golgi. CHL and TM also potentiate the antiviral activity of IFN and inhibit enveloped virus replication *in-vitro* (Maheshwari et al 1983 and 1991, Singh et al 1996). However, when used *in-vivo*, in animals and humans against several important viruses including HIV, SARS, Influenza, Hendra and Nipah, and Chikungunya, similar results were not observed (Ooi et al 2006, Inglot et al 1969, Seth et al 1999, Maheshwari et al 1991, Vigerust et al 2007, Pallister et al 2009, Freiberg et al 2010, Tricou et al 2010, Sperber et al 1997). TM and CHL had an opposite effect and interfered with the antiviral activity of IFN and augmented viral replication and pathogenesis of SFV, EMCV and VEEV. Treatment with CHL was also shown to inhibit the antimalarial activity of IFN in rhesus monkeys (Maheshwari et al 1991). Inhibitory activity of TM and CHL was concomitant with increased mortality and shortening of MST (Maheshwari et al 1983). A similar augmentation of viral pathogenesis was observed with other antimalarial drugs like quinine sulphate, primaquine and pyrimethamine (Seth et al 1999). Along with increased viral pathogenesis, treatment with these antimalarial drugs enhanced inflammatory cytokine production (such as IL1β and TNFα) and reduced natural killer cell activity (Singh et al 1987). Therefore, the ineffectiveness or enhancement of virus replication by CHL may be related to the immune system activation or by the induction of pro-inflammatory cytokines by viral antigen. Similar worsening of disease and elevation in levels of inflammatory cytokine expression was also observed after exposure of mice infected with SFV or EMCV to heavy metals like cadmium, manganese and lead (Cd, Mn and Pb respectively). Early appearance of virus induced symptoms, increased mortality, greater tissue pathology and significantly elevated virus titers were observed in the brain and other organs of these animals (Gupta et al 2002; Seth et al 2003). Similar

observations were made during fungal infection (*Aspergillus fumigatus*) in mice. We have also reported that the antiviral activity of IFN is also attenuated in the presence of CHL and TM.

CHL treatment has been shown to inhibit the human corona virus OC43 infection in new born mice (Keyaerts et al 2009). Clinical trial in patients with HIV-1 has shown the inhibitory effect of CHL. However, no effect of CHL was seen against Influenza, Nipah, and Hendra viruses in various animal models including mice, ferrets and hamsters. CHL in combination with antiviral drug ribavirin also could not protect the hamsters against virus infection suggesting an unfavorable drug-drug interaction. CHL treatment failed to prevent influenza virus infection in human subjects in a recent randomized double blinded clinical trial (Paton et al 2011). During another randomized double blinded clinical trial, CHL treatment was shown to increase the incidence of chikungunya virus induced arthralgia symptoms in human subjects (De Lamballerie et al 2008).

These studies, along with the results from our laboratory clearly suggest that the antiviral effect of CHL *in vitro* failed to translate *in vivo* in animals and humans against several viruses. Thus, the paradoxical antiviral potential of CHL *in vitro* and *in vivo* has been intensively debated over the years (Ooi et al 2006, Inglot et al 1969, Seth et al 1999, Maheshwari et al 1991, Viguret et al 2007, Pallister et al 2009, Cooper and Magwere 2008, Delogu and de Lamballerie 2011). CHL has been suggested to play an important role in the development of Burkitt's lymphoma by enhancing the Epstein-Barr virus expression (Olweny et al 1977) and dramatically increase the trans-activation of Tat protein purified from HIV (Frankel and Pabo 1988). Incidence of Herpes zoster virus infection, which is normally uncommon in younger children, was markedly enhanced in children treated with CHL following malarial infection (Cook IF 1985). These observations suggest that CHL treatment instead of being antiviral can fuel the spread of some

viruses. These results are particularly important from a public health perspective, since the use of CHL has been advocated against viral infections in humans (Cooper and Magwere 2008, Savarino et al 2003). Therefore, in depth studies are warranted before recommending the use of CHL against important virus infections in humans especially in malaria endemic area.

Chapter-1.3:
Research Gaps, Hypothesis and Study Design

The interactions occurring between any pathogen and the host are reflected as specific changes in the gene-expression pattern of the host. These changes are often evident in the form of altered transcription patterns and changes in signal transduction pathways. Pathogenesis of different strains of VEEV is well studied and understood but the types of host responses triggered are poorly explored. Why a certain strain is completely lethal for the host whereas the other strain with a point mutation in the genome fails to cause any disease altogether. How the host immune responses are bypassed during infection by V3000 whereas V3034 is successfully cleared from the brain most of the times by the host. More specifically, since neuroinvasion plays a vital role during VEEV pathogenesis, what are the host factors associated with VEEV neuroinvasion and neurovirulence.

Based on this we hypothesized that if we compare the host responses triggered against neuro-invasive and non neuro-invasive strains of VEEV in the host blood before the virus has entered the brain, we may be able to identify the host factors playing critical role during the neuroinvasion of VEEV which can be exploited for drug development against VEEV infection in hosts. Similarly, by comparing the host responses triggered against the neurovirulent and non neurovirulent VEEV infections and gain an understanding of the differential host responses triggered against different strains of VEEV, we decided to compare the host responses triggered against each of these strains at different time points. We have performed whole genome microarray using total RNA from blood, spleen and brain tissues of CD-1 mice infected with V3000, V3014, or V3034 at 24hr, 48hr, 72hr and 96hr post infection. After comparing the host

responses among different strains used, genes specifically involved in combating or facilitating virus neuroinvasion and neurovirulence were identified in the host. The list of these genes was then used to characterize the different signaling pathways involved in host-virus interactions after infection. Results obtained were validated by using qRT-PCR.

Detailed information about the host gene expression patterns after virus exposure will help to provide the means for early detection of surrogate biomarkers of the impending illness and thus help in developing strategies for the treatment. Understanding and identification of the critical host factors exploited by the virus after infection in the host for its own survival will also help in identifying potential novel host derived drug targets for developing therapeutics against VEEV infection. This will be very helpful for the prevention and emergency preparedness against a natural outbreak of VEEV infection in human population. Since, VEEV (and other members of alphavirus family) presents a good model for studying the enveloped virus structure, replication and pathogenesis, the knowledge about host gene response against VEEV will lead to improved understanding of the host responses to other enveloped RNA viruses as well.

Virus-host interactions are complex and dynamic and a number of studies have underscored the importance of early host innate immune response in dictating the course, severity, and outcome of the infection. Co–administration of many prophylactic drugs like CHL may adversely affect the equilibrium between the innate immune responses of the host and virus survival strategies. We have previously demonstrated that treatment with prophylactic antimalarial drugs such as CHL, quinine, primaquine and pyrimethamine, antibiotics like TM and environmental toxins such as Cd, Mn and Pb increase the pathogenesis, morbidity and mortality caused by viruses such as SFV, EMCV, HSV and VEEV in mice as well as enhance the virus load and inflammatory cytokines production (e.g., IL1β and TNFα) in the host brain.

The exact mechanism by which the antimalarial drugs and TM augment viral pathogenesis is not clearly understood. Based on these observations, we propose that there may be common mechanism(s) mediating the enhanced viral pathogenesis in presence of chemical agents like TM, antimalarials and environmental toxins. To test this hypothesis, we performed global gene expression profiling to identify potential host gene determinants that may be involved in potentiating the viral infection in mice. MiRNA profiling was performed to evaluate the levels of modulation of miRNA regulating expression of genes identified during microarray studies. The results from this analysis were further confirmed by qRT-PCR in samples with CHL treatment and SFV infection or $CdCl_2$ treatment and VEEV infection.

Chapter-1.4: Materials and Methods

Virus strains:

Three different strains of VEEV generated by site-directed mutations in the wild type VEEV, the Trinidad Donkey (TrD) strain were used in the studies: V3000, V3034 and V3014. V3000 is a full length cDNA clone of the TrD strain. V3034 was generated by a single site mutation in E1 glycoprotein at position-272 resulting in a change from alanine to threonine (272, Ala→Thr). V3014 also possesses the mutation of alanine to threonine (272, Ala→Thr) at position-272 in E1 glycoprotein along with two more mutations in E2 glycoprotein with lysine in place of glutamic acid at codon-209 (209, Glu→Lys) and asparagine instead of isoleucine at codon-239 (239, Ile→Asn) (Table-3) (Grieder et al 1995). Virus stocks used in this study were obtained from Dr. Franziska B. Grieder, USUHS, Bethesda, MD.

Animals studies:

Neuroinvasion and neurovirulence studies: Groups of 6-10 weeks old male CD-1 mice (10-12gms) were infected with any one of the above mentioned strains of VEEV. Mice were given mild anesthesia using isoflurane and 1000 pfu of virus in 25µl volume was inoculated in the left rear footpad using sterile 26G(3/8) intradermal bevel needle on 1cc sterile syringe (Becton Dickinson and Company, Franklin Lakes, NJ). Working dilutions of virus were made by diluting stock virus solution in 1X Dulbecco's phosphate buffered saline (DPBS) (Gibco, Invitrogen Corporation, Carlsbad, CA). Control mice were inoculated with 25µl of sterile 1X DPBS in left rear footpad. Blood samples were collected at 24hr, 48hr and 72hr post infection (p.i.) by cardiac puncture and were immediately stored in RNA later at -80°C for RNA isolation. Spleen samples

were collected at 24hr, 48hr and 72hr p.i. and brain samples were collected at 48hr, 72hr and 96hr p.i. and were immediately stored in trizol at -80°C for RNA extraction or in formalin at room temperature for histological evaluations (Figure-6). All the experiments were carried out in bio-safety level-3 facility at USUHS.

Studies with chemotherapeutic agents and virus infection: Total RNA from tissue samples collected during previous studies was used for microarray and qPCR. Brain RNA samples for TM alone and VEEV infected-TM treated- mice at 48hr p.i. were used from Steele et al 2006, brain RNA samples from Cd treated and SFV infected mice brain at 48hr p.i. were used from Seth et al 2003 and brain RNA samples from mice treated with CHL, quinine, primaquine or sulfadoxine and infected with SFV at 48hr p.i. were used from *Seth et al 1999*. $CdCl_2$, CHL, primaquine and quinine sulfate used in the above mentioned studies were of highest purity grade procured from Sigma Chemicals Co (St. Louis, MO). Sulfadoxine was a gift from the Central Drug Research Institute (Lucknow, India). TM was obtained from Calbiochem Inc (San Diego, CA).

Ethics Statement: All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, NIH Publication No. 86-23, revised 1996). The experiment protocols were approved by the USUHS IACUC committee keeping in view to minimize suffering by the animals.

Total RNA isolation and RNA amplification:

RNA isolation and purification from brain and spleen tissues: Total RNA was isolated using TriZol method (Invitrogen Inc., Carlsbad, CA) according to the manufacturer's protocol. Total

RNA was then purified using the RNeasy mini kit and DNaseI (Qiagen Inc., Valencia, CA) treatment to remove any DNA contamination. Purified RNA was quantified spectrophotometrically using Beckman DU640 spectrophotometer (Beckman Instruments Inc., Columbia, MD) and stored at -80°C. RNA quality was determined by running it on 1% denaturing formaldehyde agarose gel.

RNA isolation and purification from blood samples: Total RNA was isolated using Blood RNA pure kit (Ambion Inc., Austin, TX) according to the manufacturer's protocol. Briefly, supernatant was removed from blood samples collected in RNA later by high speed centrifugation and cells were lysed using the lysis buffer and sodium acetate. Acid-Phenol:Chloroform was added to the lysate and aqueous phase was collected after high speed centrifugation. Absolute ethanol was added to the aqueous phase and loaded onto the filter cartridges provided in the kit for RNA isolation. RNA was recovered in RNase DNase free water after filtration. RNA purification and quality control was done as mentioned above.

Blood RNA amplification: Due to low yield of total RNA obtained from the blood after purification and DNaseI treatment, the RNA samples were amplified *in-vitro* using the RampUP kit (Genisphere Inc., Hatfield, PA) The amplification protocol was followed as described by the manufacturer (Figure-7). Briefly, cDNA was synthesized from 100ng purified total RNA using SS-II reverse transcription enzyme. First round of *in-vitro* transcription using this cDNA was carried with T7 RNA polymerase at 37°C overnight. Round-1 amplified RNA was used to synthesize cDNA which was then used for round-2 *in-vitro* transcription using T3 RNA polymerase. The RNA obtained was polyadenylated, purified through Qiagen columns and quantified using Beckman DU640 spectrophotometer (Beckman instruments Inc., Columbia, MD).

Microarray Experiments:

Two step hybridization and labeling using 3DNA Array 900 expression array detection kit from Genisphere Inc, Hatfield, PA was performed as described in the manufacturer's protocol. Briefly, cDNA was synthesized from 200ng of amplified blood RNA or 2µg of purified total RNA from brain and spleen; at 42°C using SS-II reverse transcription enzyme (Invitrogen Inc.) and RT primers with specific leader sequences complimentary to the "capture sequence" on cy5 and cy3 dye dendrimers (Genisphere Inc.). Reaction was stopped at 80°C followed by RNAse H treatment to degrade the RNA-DNA duplexes from the reaction.

The high quality mouse microarrays containing approximately 37,000 oligonucleotides were used in this study, and were produced in the laboratory at Center for Biologics Evaluation & Research, Food and Drug Administration (**CBER, FDA**). The detailed information regarding array printing, post-printing processing, and testing array quality is described by Yang et al 2006.

The cDNAs were first hybridized with microarray slides overnight at 42°C in MAUI microarray hybridization chambers (Biomicro Systems Inc. Salt Lake City, UT) followed by stringent washes to remove any non-specifically bound probes. Slides hybridized with cDNA were then end labeled with Cy5/Cy3 dyes containing dendrimers at 65°C for 5 hrs in MAUI microarray hybridization chambers followed by stringent washes (Figure-8). The end-labeling method followed here provides a more reliable and consistent signal than the conventional direct or indirect labeling techniques. Firstly, since the fluorescent dye is not incorporated during the cDNA preparation, it prevents any dye bias during the reverse transcription process. Secondly, since each 3DNA molecule contains an average of about 850 fluorescent dyes, the signal generated from each cDNA molecule is independent of base the composition or length of the

transcript. The slides were spin dried at 1000rpm at room temperature and scanned using the Axon GenePix 4000B scanner (Axon Instruments Inc.,Foster City, CA).

Microarray data analysis:

Microarray slides were scanned on an Axon GenePix 4000B scanner (Axon Instruments, Inc., Foster City, CA) with a 10-micron resolution. Scanned microarray images were analyzed and data files were generated with GenePix Pro 5.1 software. For advanced data analysis, data files (in gpr format) and image (in jpeg format) were imported into mAdb (microarray database), and analyzed by the software tools in the mAdb database provided by Center for Information Technology (CIT), National Institutes Health (NIH). The advanced filters were applied before data analysis to select only the good quality spots e.g. spot size at least 10μm and ≤300μm, ≤80% signal saturation, minimum fluorescent intensity of 150 and signal ≥2SD (standard deviation) above background in both Cy3 and Cy5 channels. A global normalization approach utilizing the Loess normalization method was used for each experiment.

All the biological replicates (duplicates for each time point) shared significant homology in gene expression pattern with correlation coefficients of ≥ 0.80. The genes with mean fold difference of ≥ 1.5 fold in comparison to the controls were considered significant. Gene ontology analysis was performed with significantly modulated genes using software tools in mAdb database CIT/NIH. Furthermore, the significantly modulated genes were exported into the Ingenuity Pathway Analysis (IPA) system (Ingenuity Systems Inc., Redwood City, CA). Biological functions and relevant pathways were constructed by the web software provided by IPA.

MicroRNA expression profiling and analysis:

Reverse transcription (RT), real-time PCR and their data analysis were carried out as mentioned earlier in detail (Bhomia et al 2010). Briefly, RT was performed using total RNA from VEEV infected and VEEV infected-TM treated-mice brain samples with megaplex pools of stem-loop RT primers and TaqMan microRNA reverse transcription kit (Applied biosystems Inc., Carlsbad, CA). qPCR reaction was carried out using 6µl RT product, and TaqMan Universal PCR master mix containing no AmpErase UNG (Applied biosystems Inc., Carlsbad, CA) as per manufacturer's protocol. High throughput profiling of 692 miRNAs was carried out using TaqMan® rodent microRNAs array set v2.0 (Applied Biosystems Inc., Carlsbad, CA). The array was run in AB 7900 HT (Applied Biosystem Inc., Carlsbad, CA) with default thermal-cycling conditions. Real-time PCR data were analyzed using STATMINER (a statistical analyses package from Integromics, Philadelphia, PA) and the data were normalized to all the six endogenous control genes present in the array cards (namely Mamm-U6, snoRNA135, snoRNA202, U87, Y1 and ath-miR159a).

Quantitative RT-PCR:

Expression levels of selected genes from the microarray analysis were measured by quantitative RT-PCR using SYBR Green technique (Applied Biosystems Inc., Carlsbad, CA). SuperScript-III First-Strand Synthesis System (Invitrogen Inc., Carlsbad, CA) for RT-PCR was used to prepare cDNA from 400ng of amplified RNA samples. cDNA was then diluted to 1:20 in RNase DNase free water. Reactions consisted of 200nM forward and reverse primers, 2.5µl of diluted cDNA, 10µl SYBR Green PCR master mix (Applied biosystems Inc., Carlsbad, CA) and water to obtain a 20µl volume. Reactions were run on 7900HT fast real time PCR machine (Applied biosystems Inc., Carlsbad, CA) using the following cycle: 95°C for 10 min, followed

by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The data obtained was analyzed with ABI RQ manager software (Carlsbad, CA) by the $2^{-\Delta\Delta Ct}$ method and normalized against housekeeping gene, GAPDH. Data is representative of at least 2 technical replicate for each biological replicate. The details of primer sets used are mentioned in Table-2.

Chapter-1.5: Results and Discussion

A. Identification of host responses associated with neuroinvasive and non-neuroinvasive VEEV infection in mouse blood.

Abstract:

Since these mutations have such drastic variations in the pathology of these strains, it is hypothesized that there would be some host factors or mechanisms that are playing important roles in controlling virus infection in one case whereas fail to do so in the other case. In this study, gene signature common to all the VEEV strains irrespective of their virulence level and those specific to neuroinvasive or non-neuroinvasive strains were identified. These include *Ifitm3, Mst1, Sox10, Mier2* etc. Genes uniquely expressed against neuroinvasive and non neuroinvasive VEEV infections were also identified. Since V3000 and V3034 also differ in their neuroinvasiveness and pathogenecity, differential expression of some commonly modulated genes was also observed against these two strains. The signaling responses modulated against the different strains of VEEV correlated well with the observed pathology. Pathways like Granzyme A signaling and tight junction signaling were only modulated against neuroinvasive VEEV infection. Difference in the kinetics of some pathways modulated against all the strains was also observed. To our knowledge, this is the first study evaluating the differential host responses against different types of VEEV infections so far. The results from this study will help in identification of novel drug targets against VEEV.

Results:

VEEV strains induce differential host gene expression kinetics post infection.

To investigate the difference in the kinetics of host gene responses against neuroinvasive and non-neuroinvasive strains of VEEV, we performed whole genome microarrays using total RNA isolated from the blood samples collected at 24hr, 48hr and 72hr p.i. These time points were selected based on the replication kinetics of the VEEV strains in the host blood. The presence of VEEV in the samples at each time point was measured by qRT-PCR against NsP-4 gene of the VEEV genome which encodes for the viral RNA polymerase. V3000, the pathogenic strain of VEEV was detected in the blood till 72hr p.i. V3034 was detected till 48hr p.i. **(Figure-9, Table-12)**. V3014 showed weak amplification at 24hr p.i.

RNA isolated from the blood samples from uninfected saline-treated mice were used as controls. All the biological replicates showed good correlation as confirmed by hierarchical clustering of the arrays at different time point p.i. (Figure-10). The total number of genes differentially modulated were maximum in case of V3034 infection at all time points (5137, 634 and 7767 genes were differentially up-regulated at 24hr, 48hr and 72hr p.i. respectively whereas 4393, 196 and 10317 genes were differentially down-regulated at 24hr, 48hr and 72hr p.i. respectively; **Figure-11**). During V3000 infection 2594, 2657 and 3719 genes were differentially up-regulated whereas 1762, 1387 and 4793 genes were down-regulated at 24hr, 48hr and 72hr p.i. respectively. V3014 infection resulted in the least number of significantly modulated genes amongst the three VEEV strains (1814, 571 and 3363 genes were differentially up-regulated at 24hr, 48hr and 72hr p.i. respectively whereas 910, 449 and 2272 genes were differentially down-regulated at 24hr, 48hr and 72hr p.i. respectively). Overall, the number of

differentially expressed genes decreased at 48hr p.i. followed by significant increase with the maximum number of genes being modulated at 72 p.i. by all strains of VEEV. However, similar proportion of genes were found in different functional categories as identified by gene ontogoly classification analysis (Figure-12).

A subset of genes was modulated against all the three strains of VEEV irrespective of the level of their neuroinvasiveness and neurovirulence.

The gene expression profiles against each of the three strains were compared amongst each other at each time point to identify the common genes differentially expressed during infection (**Figure-13**). Six hundred thirty nine, 148 and 1537 genes were found to be commonly modulated between all the three strains at 24hr, 48hr and 72hr p.i. respectively. Select genes that were modulated at all the time points against all the strains are listed in **table-4**. Of these, Nt5c2 and Samd9l were also validated with quantitative RT-PCR for 24hr p.i. and were found to have similar expression patterns as seen in the microarrays (Figure-15, Table-12).

Although many genes were modulated against all the strains, not all genes had similar expression pattern. For example, i*fitm3* (IFN induced trans-membrane protein 3) was significantly down-regulated against infection with the non-neuroinvasive VEEV (V3014) strain at 24hr and 72hr p.i. while in sharp contrast, the neuroinvasive strains (V3000 and V3034) caused up-regulation of *ifitm3* at all the time points. The level of gene expression also varied between V3000 and V3034. For example, in case of V3000, the fold up-regulation of *ifitm3* increased to maximum at 48hr p.i. and remained high at 72hr p.i. whereas in case of V3034, the maximum fold up-regulation was observed at 24hr p.i. followed by decrease in the expression

level to 2 fold. Therefore, the expression kinetics of *ifitm3* correlated with the presence of VEEV (Grieder et al 1995).

Mier2 was the only gene that was down-regulated at all the time points against V3000 and V3034. In case of V3014 infection, however, *Mier2* was down-regulated initially at 24hr and 48hr p.i. and up-regulated by 1.5 folds at 72hr p.i. The exact function of *Mier2* is not well understood but it is categorized under nucleic acid metabolism by gene ontological classification. VEEV is known to interfere with the host transcription machinery (Garmashova et a 2007). Thus, down-regulation of this gene might be a virus strategy to curb the host transcription, which seems to be more efficient in case of neuroinvasive VEEV infection.

Gene ontological analysis was also performed on this subset of differentially expressed genes at different time points to better understand the type of biological functions represented by these genes. A large proportion of the common genes belonged to biological functions like cell death and immune system processes (97 and 94 respectively). Interestingly, the number of genes involved in antigen presentation pathway decreased with time in this subset.

A subset of genes was differentially modulated only against the neuroinvasive strains of VEEV (V3000 and V3034).

Comparison of host genes modulated against V3000 and V3034 infections identified 1115, 159 and 2860 genes at 24hr, 48hr and 72hr p.i. respectively that were commonly modulated against both the neuroinvasive strains of VEEV. Out of these common genes, 19 genes were differentially modulated at all the time points. Significantly modulated genes against both the neuroinvasive VEEV strains, V3000 and V3034, were further compared to identify the genes with similar expression pattern and those with opposite expression pattern (**Table-5 and 6**

respectively list some of these genes with more than 1.5-folds modulation during at least one of the time points). Genes like *Casp3, Ncf1, Cdkn2a* and *Psma1* were down-regulated against V3034 infection but up-regulated against V3000 infection at different time points. Genes like *Brd7*, *Vegfa* and *Cfp* were found to be down-regulated during V3000 infection unlike V3034 infection at different time points. These genes were further categorized based on their molecular functions (GO classification). Genes related to cell death like *Ncf1* and *Bmp2* were down-regulated against V3000 infection unlike V3034 infection at 72hr p.i. However, cell proliferation related genes like *Cdkn2a*, *Ccdc88a* and *Brd7* were found up-regulated against V3000 infection but down-regulated in case of V3034 infection at different time points.

A subset of genes was differentially modulated only against the non-neuroinvasive strain of VEEV (V3014).

Comparison of microarray data identified 864, 442 and 1599 host genes that were differentially expressed only against V3014 infection in host blood at 24hr, 48hr and 72hr p.i. respectively. These significantly modulated genes were further analyzed for their role in host protective responses during non-neuroinvasive VEEV infection. This subset of genes is referred to as non-neuroinvasion specific genes in **figure-13**. The significantly up-regulated and down-regulated genes were further categorized based on the gene ontology and are listed in **Table-7 and 8**. Most of the GO categories e.g., biological adhesion, death, immune system process, viral reproduction, cell proliferation and regulation of biological processes, which included the majority of genes, were similar to those observed against the infection with neuroinvasive strains of VEEV (V3000 and V3034). However, the genes under these categories were different from the genes modulated against the neuroinvasive VEEV infection and may reflect a protective host response against non-neuroinvasive VEEV infection.

Pathway analysis of gene expression showed unique host responses against neuroinvasive and non-neuroinvasive strains of VEEV.

To further compare the differences between the hosts signaling responses against the different VEEV strains, the above selected subsets of genes were subjected to pathway analysis using Ingenuity pathway analysis software as explained in the methods section. Some of the significantly modulated canonical pathways representative of the neuroinvasion specific genes included IL-2 signaling, Jak/Stat, PI3K/Akt, apoptosis, notch, Granzyme A signaling, axonal guidance signaling, calcium signaling, tight junction signaling and integrin signaling pathways (**Figure-14a**). In general, immune response related pathways were significantly modulated during initial time points (24hr and 48hr p.i.). At 48hr p.i. cell death related pathways were significantly represented. At 72hr p.i., there was a significant involvement of protein ubiquitination pathway which may be suggestive of an active degradation of viral proteins by the host. Calcium signaling and tight junction signaling pathways were also uniquely modulated in this subset of genes at 72hr p.i.

In contrast, the analysis of non-neuroinvasion specific genes revealed that protein ubiquitination pathway was significantly involved at 24hr p.i. Some other pathways such as CCR5 signaling in macrophages, activation of IRFs by cytosolic pattern recognition, GABA receptor signaling, MIF regulation of innate immune response, Wnt/β-catenin signaling pathway, PI3K/Akt signaling, integrin signaling and mTOR pathways were also involved at 24hr p.i. (**Figure-14b)**. In addition, most of the pathways involved in immune responses were significantly modulated at 24hr p.i. only. At later time points, protein translation and cell development related function pathways were involved. Similarly, signaling pathway analysis was done using the VEEV specific gene subset as shown in **figure-14c** where we observed a

significant modulation of several immune response related pathways like CCR3 signaling in eosinophiles, IL-22 signaling, Fc-γ receptor and phagocytosis in macrophages and monocytes and protein ubiquitination pathway at 24hr and 48hr p.i.

Vomeronasal and olfactory receptors are differentially modulated against all the three strains of VEEV.

Apart from the other genes and signaling pathways, several vomeronasal and olfactory receptors were also found to be differentially modulated against VEEV infection with all the three strains. Detailed lists of the different vomeronasal receptors modulated at different time point against V3000, V3034 and V3014 have been listed in **Tables 9, 10 and 11** respectively.

Discussion:

VEEV has been weaponized due to its highly infectious nature and ease of spread through aerosol. Due to these reasons, VEEV is considered a potential bio-threat agent. Severe infection of humans by VEEV can cause neurological complications and death (Rivas et al 1997, Watts 1998, Aguilar et al 2004, Quiroz et al 2009). Absence of any licensed drug or vaccine against VEEV infection presents an area that needs immediate attention. During recent years, growing focus has been on the understanding of the host response and disease pathology caused by a pathogenic versus a non-pathogenic infection (Hayashi et al 2011, Lederer et al 2009). Pathogenesis caused by different strains of VEEV is well studied; however, the basis of restriction in infectivity and pathogenicity by different strains of VEEV is still uncharacterized. Understanding of the molecular changes induced in the host upon VEEV infection resulting in either restriction or progression of viral replication can identify potential host derived targets for drug development. In addition, since neuroinvasion by VEEV is a critical step causing

significant morbidity and mortality in the host, identification of neuroinvasion factors will be a step forward in the field of drug development against VEEV infection.

In this study, we have compared the host gene responses against three different strains of VEEV which are neuroinvasive (V3000 and V3034) or non-neuroinvasive (V3014) in mice (Grieder et al 1995). V3000 which is a fully virulent strain of VEEV, gets cleared from the blood by 72hr p.i.; V3034 is cleared by 48hr p.i. whereas V3014, the non-pathogenic strain, does not replicate to detectable titers and is cleared off from blood even before 24hr p.i. (Grieder et al 1995). Different time points post infection: 24hr, 48hr and 72hr p.i.; were studied to understand the kinetics of host gene expression. Comparison of the host gene responses against these strains at each time point generated different subsets of genes. We focused on the following three subsets of genes: 1) genes commonly modulated by all the three strains, 2) genes commonly modulated by neuroinvasive strains (V3000 and V3034) and 3) genes exclusively modulated by non-neuroinvasive strain infection (V3014). Interestingly, V3014 infection triggered differential expression of least number of genes in comparison to the other two strains which may explain the restricted replication of V3014. However, more number of host genes were modulated against V3034, a less pathogenic strain, in comparison to V3000, a highly virulent strain. Though the underlying reason for such a discrepancy is not clear, it may be due to more active host cellular response to control replication of the less pathogenic V3034 strain of VEEV which achieves detectable levels of replication *in-vivo*. Whereas, in case of V3000 infection, virus achieves higher titer levels and the lesser number of significantly modulated genes may reflect inhibited host cellular response *in-vivo*. A unique expression kinetics was observed with all the VEEV strains where the number of genes modulated was lowest at 48hr p.i. in comparison to 24hr and 72hr p.i.

A group of genes were significantly modulated against infection with all the three strains of VEEV irrespective of the level of their neurovirulence. Of these, *Ifitm3* has been shown to interfere with entry of a number of different viruses like filoviruses, coronaviruses, HCV, HIV-1, VSV and influenza virus (Huang et al 2011, Lu et al 2011, Weidner et al 2010). However, role of other genes such as *Mst1, Sox10, Mier2, Zfp456, Hist2h2ac, Nt5c2* and *Samd9l* has not been explored during virus infections. In addition to these genes some yet uncharacterized transcripts like *A530023O14Rik, 1700009J07Rik* and a transcribed locus (Mm.426889) were also found to be up-regulated to high levels. Since most of these genes have not been implicated during other viral infections at present, these transcripts present with a potential cohort that can be further explored as diagnostic biomarkers against VEEV.

Neuroinvasion by VEEV is major milestone during the virus infection in the host which may ultimately lead to encephalitis (Rivas et al 1997, Aguilar et al 2004, Quiroz et al 2009). The mechanism of VEEV entry to the brain is not well understood. Previous studies suggest that VEEV enters the brain primarily through olfactory neuroepithelium infection but may also enter the brain through an altered BBB (Schafer et al 2011, Sharma et al 2011). Identification of host factors critical for neuroinvasion can be helpful in developing strategies to limit the virus dissemination to the brain. V3034 has been shown to enter the CNS but fails to actively replicate in the brain (Grieder et al 1995). In this study, several host genes that were commonly modulated against the neuroinvasive strains of VEEV (V3000 and V3034) were identified. Some of these genes showed similar expression patterns against the two strains while others had opposite expression pattern. For example, *Casp3* was up-regulated against V3000 infection but down-regulated against V3034 infection. Influenza-A virus infection has been shown to induce Casp3-mediated proteolytic processing of pro-IL-18 and thus leading to secretion of active IL-18, a pro-

inflammatory cytokine (Rintahaka et al 2008). Several studies in past have emphasized on the contribution of inflammation mediated secondary neuronal death and alterations in the BBB during VEEV disease pathology (Schoneboom et al 2000, Charles et al 2001, Sharma et al 2008 and 2011). Immuno-deficient mice were also shown to survive longer than immuno-competent animals infected with VEEV (Charles et al 2001). Thus, up-regulation of Casp3 during V3000 infection may be related to the enhanced inflammation and neuropathology during V3000 infection.

Pathway analysis of the neuroinvasion specific subset showed an interesting pattern of gene expression at different time points. Significant modulation of host immune response pathways e.g., IL-2 signaling and Jak-stat signaling was observed at 24hr p.i. and cell death related pathways e.g., apoptosis signaling and Granzyme-A signaling were significantly modulated at 24hr and 48hr p.i. It is known that V3000 and V3034 replicate to peak titers in the blood during initial 48hr p.i. (Grieder et al 1995). These observations correlate well with the pathology of the strains observed in the host and suggest that an active host immune response is triggered during active virus replication which is also accompanied by activation of cell death pathway. Additionally, a significant modulation of protein ubiquitination pathway was observed at 72hr p.i. Earlier studies have reported active clearance of both the neuroinvasive strains from the host blood by 72hr p.i. (Grieder et al 1995). Activation of protein ubiquitination pathway thus may be involved in active clearance of the viral antigens from the blood. Previously, it has been shown that by 72hr p.i. V3000 and V3034 start appearing in the brain. It has also been suggested that other than the olfactory tract, compromised BBB structure might also be exploited by VEEV to enter the host brain (Schafer et al 2011, Sharma et al 2011). Calcium and tight junction signaling pathways, important in the maintenance of BBB, were found to be significantly

modulated at 72hr p.i. *Mlck*, which increases tight junction permeability (Vandenbroucke et al 2008, Yu et al 2010), was found to be up-regulated at all time points during V3000 infection unlike V3014 infection. Since VEEV has been suggested to induce BBB compromise to enter the host brain, difference in expression pattern of *Mlck* might be connected to maintenance of tight junction and thus preventing the virus entry in case of V3014. Taken together, these observations suggest an active engagement of the host CNS and BBB in VEEV neuroinvasion.

Another set of genes that can help in improving the understanding of the host factors involved in preventing neuroinvasion during VEEV infection is the genes exclusively expressed against V3014 infection. This subset is important since it represents the host response that controlled and cleared the virus infection from disseminating to other organs post viremia. Pathway analysis of this subset identified distinct differences in the host response over the neuroinvasive strains. Protein ubiquitination pathway was found to be significantly modulated at 24hr p.i. during V3014 infection unlike the response in the neuroinvasion specific subset where it was modulated at 72hr p.i. This suggests an active clearance of viral antigen very early by the host which also corresponds with the previously reported viremia levels of V3014 (Grieder et al 1995). Moreover, in this subset most of the host innate responses pathways such as CCR2 signaling in macrophages, activation of IRF by cytosolic PRRs and MIF regulation of innate immunity were significantly modulated during 24hr p.i. which then quickly went down to non-significant levels by 48hr and 72hr p.i. suggesting that some host immune responses were triggered by the presence of virus in the blood, but quickly subsides due to successful clearance of virus from the blood.

Two conclusions can be drawn from the present study. First, there is a qualitative difference in the type of host response involved in the two subsets, for example, the delayed and

early appearance of protein ubiquitination response against neuroinvasive and non-neuroinvasive VEEV infections respectively. Second, unique signaling response patterns were observed in each subset represented by the pathways such as tight junction, calcium and granzyme A signaling pathways in the neuroinvasion specific subset whereas CCR5 signaling in macrophages and MIF regulation of innate immunity in the non-neuroinvasion specific subset. These differences between the neuroinvasive and non-neuroinvasive VEEV infection may help in predicting the prognosis of the disease once a patient is found infected with VEEV. This would in turn help in identifying the correct therapeutic interventions for more effective treatment of VEEV infections.

Additionally, modulation of a large number of olfactory and vomeronasal receptors was also observed against infection of all the three strains (Tables- 9, 10 and 11). Utilization of olfactory neuroepithelium for CNS invasion by VEEV is well known but the role of vomeronasal organ during VEEV pathogenesis has not been explored in detail. Presence of VEEV antigen in the vomeronasal neuroepithelium has been reported during aerosol challenge with the virulent TrD strain but not in case of infections with attenuated strains (TC83 or V3526) (Steele et al 1998). However, presence of VEEV antigen in vomeronasal neuroepithelium during subcutaneous or intraparitoneal infections has not been reported. Other than VEEV, herpes simplex virus has been shown to utilize vomeronasal organ in absence of olfactory neuroepithelium to enter the host brain (Mori et al 2005). Although VEEV is known to appear in the brain by 48hr p.i., modulation of these receptors was observed as early as 24hr p.i. and during V3014 infection as well. But lack of sufficient knowledge about the function of different vomeronasal receptor homologs limits the conclusion about their role in VEEV pathogenesis and thus needs to be further explored.

Robust IFN response against VEEV infection is well known and has been studied extensively. Even after a strong IFN response, the host antiviral mechanisms fail to control the disseminating virus. The microarray data showed differences in the interferon-γ receptor (IFNγR1) expression against the three strains. There was a down-regulation of IFNγR1 against V3000 and V3034 infection at 24hr and 72hr p.i. respectively whereas during V3014 infection IFNγR1 was up-regulated by 72hr p.i. Similarly, Kaposi's sarcoma-associated herpesviruses have also been shown to down-regulate host IFNγR1 expression (Li et al 2007). A similar expression pattern was also observed during quantitative RT-PCR validation. A difference in expression levels of Stat molecules was also observed where Stat1 expression was down-regulated in V3000 infection whereas during V3034 it was found up-regulated. Previously, VEEV has been suggested to interfere with Stat1 activation and thus disrupts the IFN signaling (Simmons et al 2009). Taken together these observations suggest that inefficient disruption of type- II IFN receptor expression by V3014 may result in inhibition of viral spread by IFN signaling. However, the exact mechanism will need to be further explored.

To our knowledge this is the first comprehensive study to evaluate the host gene expression responses in the blood during infection with various strains of VEEV that exhibit differential pathology. The results identify several host factors that might be involved in the neuroinvasion of VEEV in the host and thus can be further explored for targeted drug development against VEEV infection. The VEEV specific genes also provide as a potential VEEV blood biomarker gene signature.

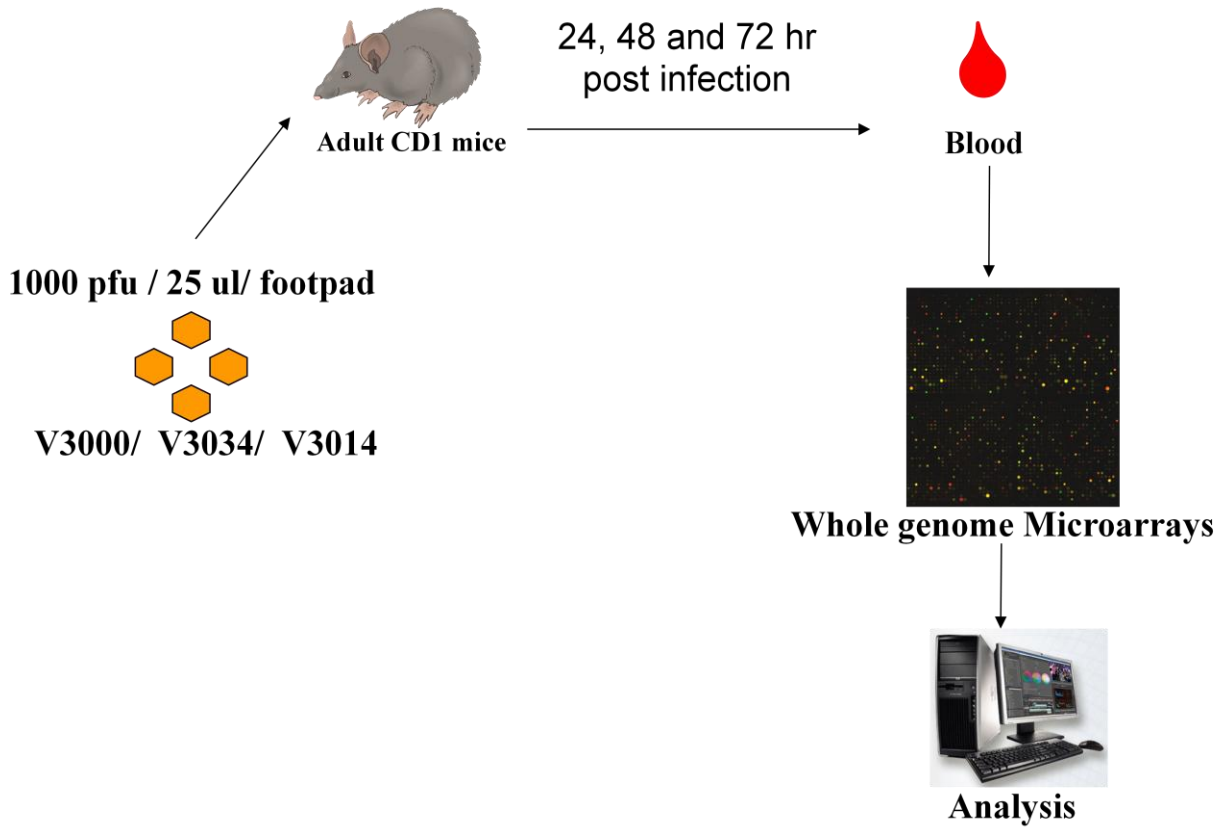


Figure-6 Study design to identify the neuroinvasion and neurovirulence associated host responses against VEEV infection:

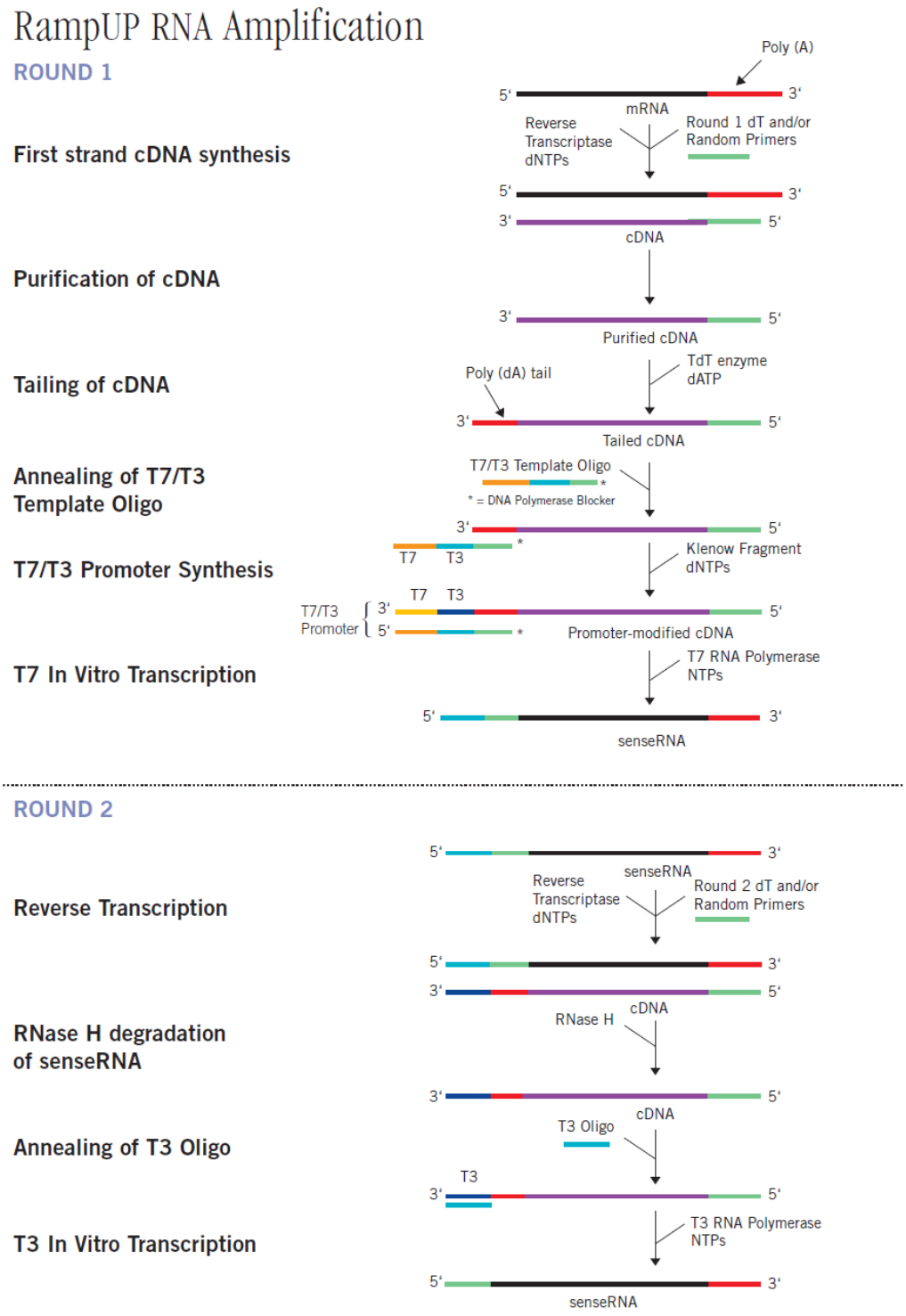


Figure-7 Work-flow followed for blood RNA amplification: (adapted from Genisphere Inc.)

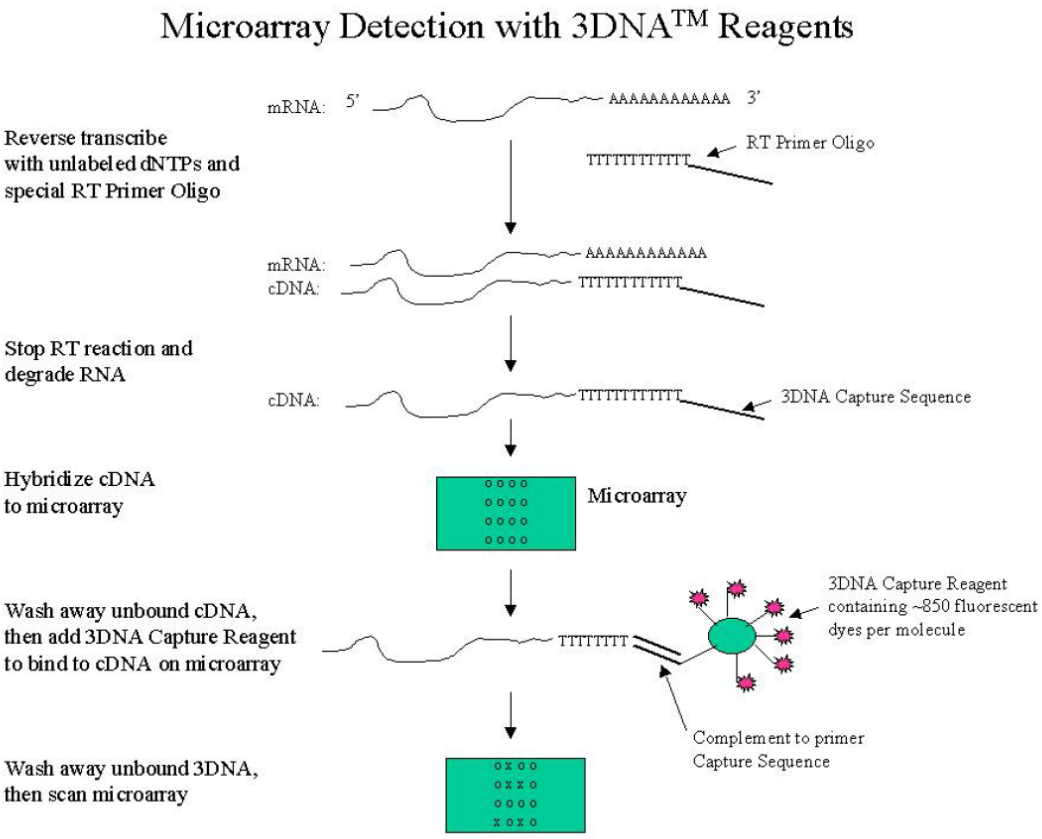


Figure-8 Microarray hybridization protocol followed: (adapted from Genisphere Inc.)

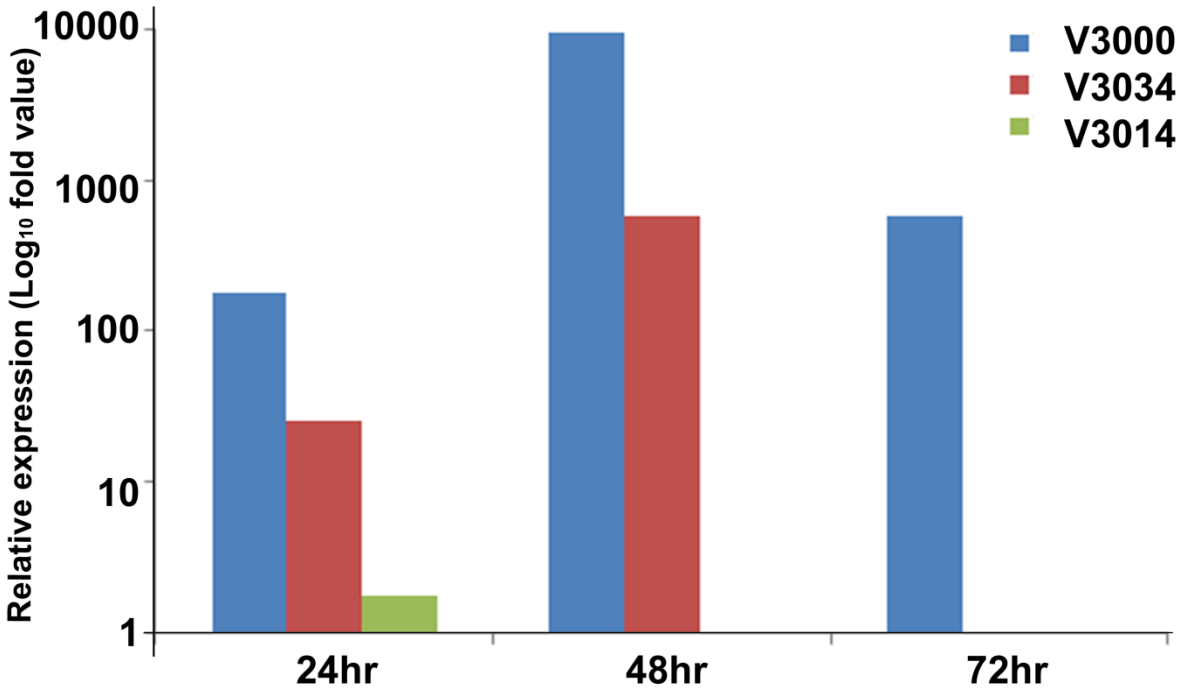


Figure-9 Presence of viral genome in the host blood at different time points: Total RNA isolated from blood of mice infected with V3000, V3034 or V3014 was used for performing quantitative RT-PCR against the viral nsP4 gene encoding for the viral RNA polymerase. Expression values of all the genes were normalized with the house keeping gene, GAPDH. The results here are representative of 2 biological replicates and 2 technical replicates for each biological replicate. Sequences of primer sets used are given in supplementary table-9.

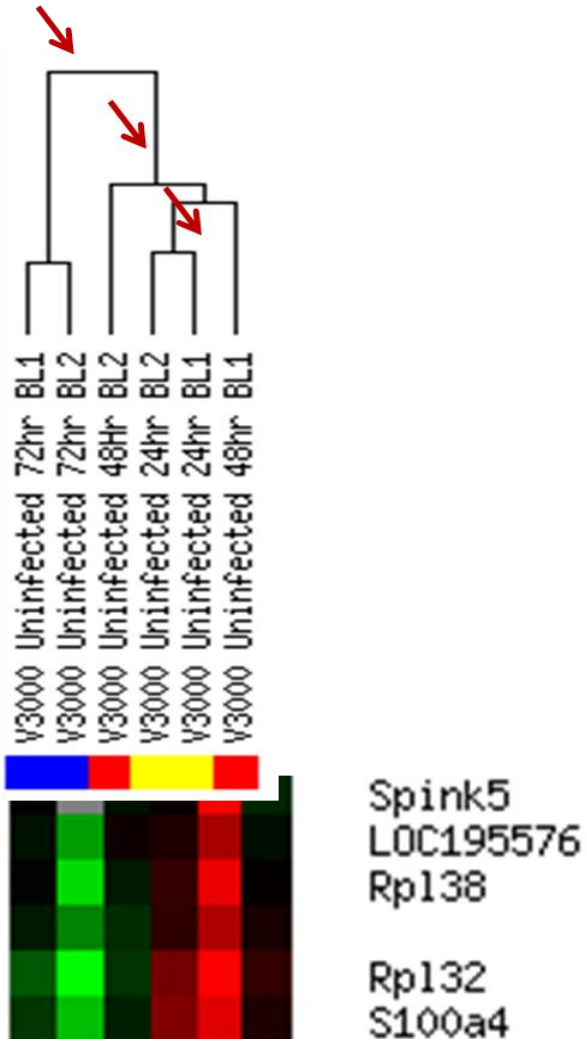


Figure-10 Heirarchical clustering analysis between biological replicates at different time points: The different biological replicates for each sample clustered together during hierarchical clustering analysis.

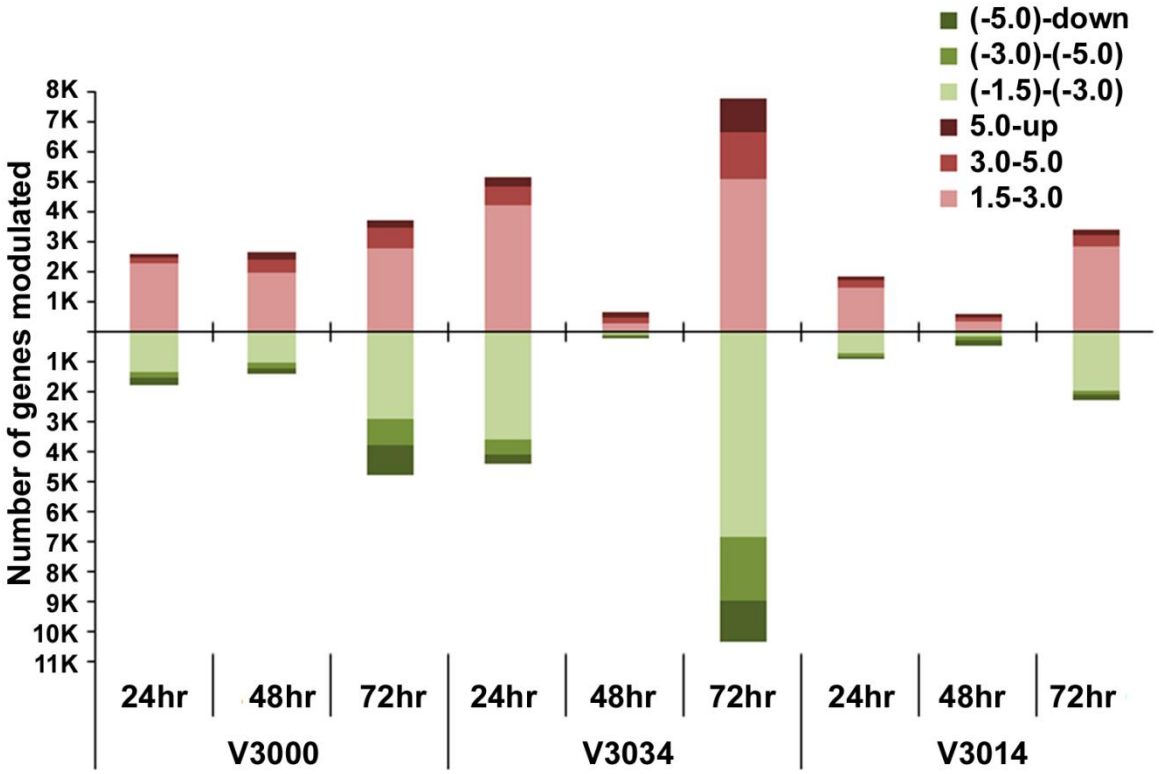


Figure-11 Total number of genes modulated against infection by different VEEV strains at different time points in blood: Total RNA isolated from blood of mice infected with different strains was used for performing whole genome microarray as explained in the material and methods. The genes having ≥ 1.5 fold change in expression level (up-regulation as well as down-regulation) in comparison to uninfected controls were selected. Maximum host genes were significantly modulated at 72hr p.i. against infection by all the VEEV strains. Majority of genes at each time point were modulated by 1.5-3.0 folds. Numbers on the y-axis are in thousands.

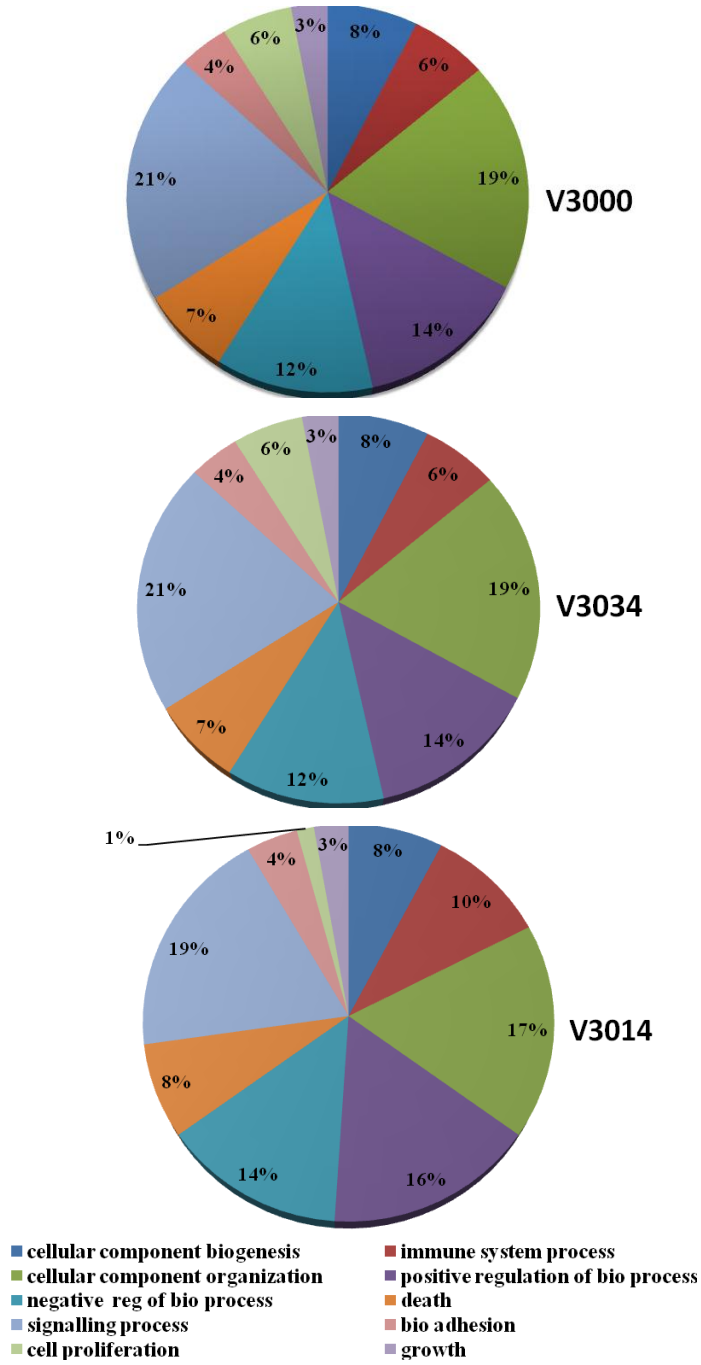


Figure-12 Gene ontological classification of the differentially modulated genes against VEEV infections in blood: The differentially modulated genes against infection with each strain of VEEV were subjected to gene ontological classification using mAdb. The overall proportion of genes each category were similar against all the strains except cell proliferation related genes.

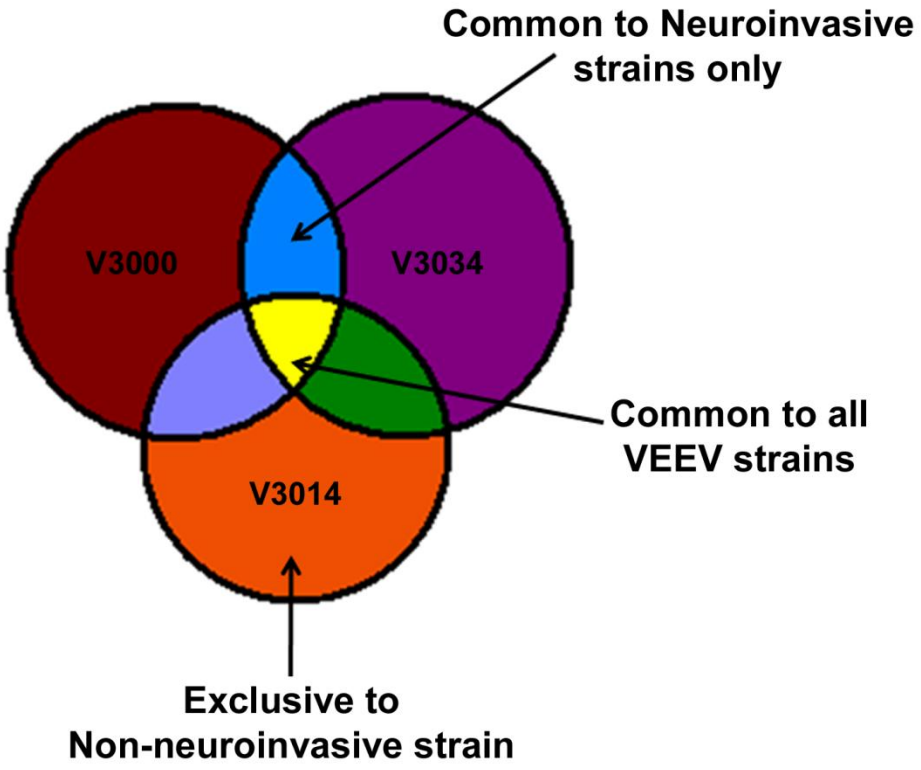


Figure-13 Analysis strategy followed for identification of host-associated neuroinvasion factors: Genes significantly modulated (fold change ≥ ±1.5) against the different strain of VEEV at each time point (24hr, 48hr and 72hr p.i.) were filtered and compared to generate subsets of genes comprising of genes that: (i) modulated against all the 3 VEEV strains (V3000, V3034 and V3014), (ii) modulated against both the neuroinvasive strains of VEEV (V3000 and V3034) but not against the non-neuroinvasive strain of VEEV, V3014 and (iii) modulated against the non-neuroinvasive strain of VEEV (V3014) only.

Table-4 Genes differentially modulated against infection by all the three strains of VEEV

UniGene Id	Gene (Description)	Fold Expression Values								
		V3000			V3034			V3014		
		24hr	48hr	72hr	24hr	48hr	72hr	24hr	48hr	72hr
Mm.461583	**Zfp456** (Zinc finger protein 456)	47.5 ± 2.1	61.1 ± 4.3	4.6 ± 2.8	19.5 ± 2.4	17/-	11.6 ± 1.6	39.1 ± 1.8	8.1/-	32.4 ± 1.3
Mm.40965	**Nt5c2** (5'-nucleotidase, cytosolic II, transcript variant 3)	24.2/-	54.4 ± 5.1	4.3 ± 3.3	25.6 ± 2.6	9.1/-	7.8 ± 1.7	40.9/-	6.2/-	40.5 ± 1.2
Mm.276739	**Sox10** (SRY-box containing gene 10)	14.7 ± 1.5	12.3 ± 1.9	5.7 ± 1.9	7.3 ± 1.1	8.1/-	2.5/-	12.6/-	10.3/-	8.5/-
Mm.347647	**A530023O14Rik** (RIKEN cDNA A530023O14 gene)	12.9	9.7	4.6	9.5	7.5	5.9	19.2	2.8	18.3
Mm.426889	**Transcribed locus**	12.8 ± 1.5	5.2/-	1.9 ± 1.6	10.4 ± 1.6	22.4/-	3.7 ± 1.3	5.0/-	7.5/-	13.3
Mm.8369	**Mst1** (macrophage stimulating 1 (hepatocyte growth factor-like))	9.9 ± 1.6	57.7 ± 5.1	15 ± 3.4	14.3 ± 3.7	6.8/-	18 ± 1.9	15 ± 1.1	4.9/-	27.5 ± 1.7
	1700009J07Rik (RIKEN cDNA 1700009J07 gene)	8.1 ± 1.1	43.9 ± 4.1	5 ± 2.8	13 ± 3.5	13.1/-	11.2 ± 1.7	18.6 ± 2.4	21.6 ± 2.2	32 ± 2.1
Mm.196013	**Samd9l** (sterile alpha motif domain containing 9-like)	3.1 ± 2.0	2.9/-	4.6/-	5.9/-	2.9/-	3.1 ± 1.5	2.3/-	3.7/-	1.9/-
Mm.358954	**Hist2h2ac** (histone cluster 2, H2ac)	2.3/-	2.9 ± 1.3	1.7 ± 1.1	1.6 ± 1.1	1.8/-	1.6 ± 1.1	2.6/-	1.6/-	1.8 ± 1.4

Mm.141021	**Ifitm3** (interferon induced transmembrane protein 3	**4 ± 0.9**	**17.3 ± 0.9**	**8.3 ± 0.6**	**10.8 ± 1.2**	**2.2 ± 0.03**	**2 ± 0.8**	**-3.3/-**	**2.1/-**	**NS**
Mm.334193	**Mier2** (mesoderm induction early response 1, family member 2)	**NS**	**-4.5 ± 2.4**	**-36.9 ± 2.2**	**-12.3/-**	**-14.5/-**	**-6.5 ± 4.2**	**-3 ± 1.6**	**-8.3/-**	**NS**

Gene subsets common to all the three strains of VEEV from figure 4 were further compared amongst each other and the genes expressed at all the time points (24hr, 48hr and 72hr p.i.) were selected. These genes are differentially modulated against VEEV infection irrespective of the type of strain and time p.i. and thus may be explored for identifying VEEV- specific blood biomarkers. NS represents non-significant expression values in microarrays.

Table-5 Genes similarly modulated against neuroinvasive strains of VEEV (V3000 and V3034)

UniGene Id	Gene Description	V3000			V3034		
		24hr	48hr	72hr	24hr	48hr	72hr
Viral reproduction							
Mm.326477	Zinc finger protein 445 (Zfp445)	6.37 / -	7.01 / -	5.08 / -	6 ± 1.147	A	2.55 ± 1.17
Mm.284248	Chemokine (C-C motif) ligand 5 (Ccl5)	NS	NS	2.99 ± 0.67	NS	2.04 / -	2.82 ± 0.674
Mm.290906	Nuclear antigen Sp100 (Sp100)	NS	NS	2.03 ± 0.283	1.21 ± 0.346	A	0.48 ± 0.219
Cell killing							
Mm.14874	Granzyme B (Gzmb)	3.53 / -	4.59 / -	3.66 ± 0.228	3.5 ± 0.149	3.31 ± 0.793	3.02 ± 0.714
Mm.281805	Mannose-binding lectin (protein A) 1 (Mbl1)	-1.13 ± 0.714	-1.63 ± 0.831	-4.24 ± 0.617	-2.24 ± 0.169	A	-4.38 / -
Mm.163	Beta-2 microglobulin (B2m)	NS	2.38 ± 0.566	3.01 ± 0.483	1.53 ± 0.958	2.09 ± 1.401	1.9 ± 0.318
Mm.442442	UL16 binding protein 1 (Ulbp1)	NS	-0.89 / -	-1.42 / -	-2.11 / -	A	-2.99 / -
Mm.8217	Killer cell lectin-like receptor subfamily K, member 1 (Klrk1)	A	A	0.86 / -	2.11 / -	A	3.13 ± 0.348
Biological adhesion							
Mm.386931	Extracellular matrix protein 2 (Ecm2)	3.53 ± 0.104	6.17 / -	2.04 ± 0.92	3.65 / -	3.88 / -	2.24 ± 0.828
Mm.971	Plasminogen (Plg)	1.43 / -	A	3.76 ± 0.2	2.23 ± 0.531	A	0.93 ± 0.188
Mm.371552	CD63 antigen (Cd63)	-1.55 / -	-1.72 ± 1.092	-4.54 ± 0.573	A	A	-2.63 ± 1.371
Mm.32741	Integrin, alpha 10 (Itga10)	NS	1.12 ± 0.717	1.12 ± 0.34	2.3 ± 0.218	A	NS
Mm.66222	Coxsackie virus and adenovirus receptor (Cxadr)	NS	-0.74 ± 0.2	2.38 ± 1.744	2.62 ± 0.115	A	2.95 ± 1.171
Mm.390589	Osteomodulin (Omd)	A	A	-6.03 / -	-3.82 ± 0.488	-3.26 / -	-5 ± 0.047
Cell proliferation							
Mm.158143	Nuclear receptor subfamily 2, group F,	2.16 ± 1.259	A	NS	2.04 ± 0.708	A	1.15 ± 0.305

	member 2 (Nr2f2)						
Mm.333406	Cyclin D2 (Ccnd2)	1.4 ± 0.599	1.75 ± 0.426	2.01 ± 0.735	2.42 ± 0.396	0.92 / -	NS
Mm.339812	Fibroblast growth factor 18 (Fgf18)	-0.75 ± 0.558	-1.77 ± 1.381	-5.09 ± 1.176	-2.35 / -	A	-3.51 ± 0.723
Mm.168789	Cyclin-dependent kinase inhibitor 1C (P57) (Cdkn1c)	-2.51 / -	-1.85 / -	A	-2.08 / -	A	-2.03 / -
Mm.292729	TNF receptor-associated factor 6 (Traf6)	-2.88 / -	A	-1.99 ± 0.588	-1.09 ± 0.046	A	-1.88 ± 0.5
Mm.299647	Lipase, endothelial (Lipg)	NS	0.77 / -	3.05 ± 0.189	NS	A	0.86 / -
Mm.461296	Mucin 2 (Muc2)	NS	-0.51 / -	-5.00 / -	-3.15 ± 0.112	A	-2.7 ± 0.493
Mm.77697	Interleukin 34 (Il34), transcript variant 2	NS	A	1.2 / -	0.9 ± 0.285	A	1.58 ± 0.5
Mm.998	Presenilin 1 (Psen1)	NS	NS	-1.96 / -	-0.72 ± 0.393	1.65 / -	-1.52 ± 0.396
Mm.273695	Interferon regulatory factor 6 (Irf6)	NS	A	A	-2.59 ± 0.427	A	-3.51 ± 1.782
Immune response							
Mm.196581	Mitogen-activated protein kinase 1 (Mapk1)	5.80 / -	7.06 / -	1.73 / -	5.08 ± 0.579	3.75 / -	3.59 ± 2.023
Mm.248478	SAM domain and HD domain, 1 (Samhd1)	1.47 ± 0.339	1.77 / -	2.16 ± 0.088	2.42 ± 1.061	A	0.22 / -
Mm.676	Activating transcription factor 1 (Atf1)	1.36 / -	NS	NS	1.63 ± 0.739	1.09 ± 0.234	2.8 ± 1.151
Mm.17484	Synuclein, alpha (Snca)	NS	-0.7 ± 0.379	-0.92 ± 0.01	NS	A	-2.05 ± 0.617
Mm.182359	Interleukin 33 (Il33)	NS	-1.39 ± 0.91	-2.24 ± 0.12	NS	A	-1.12 ± 0.273
Mm.296457	Pellino 2 (Peli2)	NS	-1.78 / -	-1.88 ± 1.155	NS	A	-2.53 ± 0.432
Mm.3951	Thymus cell antigen 1, theta (Thy1)	NS	NS	1.46 ± 0.122	-0.36 ± 0.033	3.42 ± 1.246	-0.72 ± 0.524

Mm.27431	TNF receptor-associated factor 3 (Traf3)	NS	A	-1.39 / -	-1.4 / -	A	-1.69 ± 1.045
Mm.247623	Complement component 5a receptor 1 (C5ar1)	NS	A	-1.47 / -	-1.9 / -	A	-2.03 / -
Mm.287226	Mitochondrial antiviral signaling protein (Mavs)	NS	NS	-2.86 ± 0.057	-1.36 ± 0.173	A	-2.19 ± 1.238
Death							
Mm.7454	Immunoglobulin (CD79A) binding protein 1 (Igbp1)	4.63 / -	4.83 / -	1.53 ± 0.632	3.31 ± 0.955	3.73 / -	1.9 ± 0.94
Mm.290476	NLR family, apoptosis inhibitory protein 5 (Naip5)	1.48 / -	1.96 ± 0.034	NS	3.13 ± 0.884	A	NS
Mm.29820	BCL2/adenovirus E1B interacting protein 3-like (Bnip3l)	-1.02 ± 0.269	1.02 / -	-2.64 ± 0.434	-0.81 ± 0.03	A	-2.55 ± 0.63
Mm.234204	p21 protein (Cdc42/Rac)-activated kinase 2 (Pak2)	-5.35 / -	-3.02 / -	-5.35 ± 2.112	-3.37 ± 0.353	-2.0 / -	-7.03 ± 3.789
Mm.257266	Eph receptor A7 (Epha7)	NS	A	-5.25 ± 0.778	-0.83 / -	A	-2.33 ± 0.376
Immune system process							
Mm.323595	Transducer of ERBB2, 2 (Tob2)	3.47 / -	3.91 / -	A	2.05 ± 0.62	A	-1.36 ± 0.774
Mm.260325	Bone marrow stromal cell antigen 2 (Bst2)	2.17 ± 0.217	A	0.62 ± 0.194	2.74 ± 1.017	A	1.77 ± 1.455
Mm.302724	Aminolevulinic acid synthase 2, erythroid (Alas2)	0.84 ± 0.516	-0.69 ± 0.418	-3.58 ± 0.443	NS	A	-3.25 ± 0.271
Mm.294826	Homeobox A7 (Hoxa7)	-0.97 / -	NS	-4.14 ± 1.063	-2.19 / -	A	-2.09 ± 1.768
Mm.138792	Chromodomain helicase DNA binding protein 7 (Chd7)	-4.42 / -	A	-2.04 ± 1.231	-1.79 ± 0.566	A	NS
Mm.30533	Grb2-binding adaptor, transmembrane (Gapt)	-5.51 / -	A	-6.88 / -	-3.11 ± 0.546	-3.19 / -	-6.09 ± 0.406
Mm.154457	TAP binding protein (Tapbp)	NS	-1.04 ± 0.817	-3.12 ± 0.404	NS	A	-2.28 ± 0.459

Mm.277072	Leptin (Lep)	NS	A	-2.94 ± 0.884	1.49 / -	A	-2.56 / -

Genes with similar expression trend by V3000 and V3034 infections were selected from the gene subsets common to both V3000 and V3034 explained in figure-3. The table represents $\log_2$ transformed values of fold change in the expression of genes ($\log_2(1.5) = 0.59$). "A" indicates that the gene expression was not detected in microarrays and "NS" indicates that the expression level was not significant. Values represent fold change $\pm$ SD

Table-6 Genes differentially modulated between neuroinvasive strains of VEEV (V3000 and V3034) at different time points.

UniGene ID	Gene Description	V3000			V3034		
		24hr	48hr	72hr	24hr	48hr	72hr
Viral reproduction							
Mm.2647	Profilin 1 (Pfn1)	A	A	0.98 ± 0.371	NS	A	-0.67 ± 0.374
Biological adhesion							
Mm.4733	Cyclin-dependent kinase inhibitor 2A (Cdkn2a), transcript variant 1	1.33 ± 0.296	A	4.17 / -	2.35 ± 0.322	A	1.99 ± 0.172
Mm.254515	Digeorge syndrome critical region gene 2 (Dgcr2)	-1.29 / -	A	1.15 / -	NS	A	-2.01 ± 0.762
Mm.171736	Roundabout homolog 2 (Drosophila) (Robo2)	A	0.64 / -	-2.61 / -	A	A	0.77 ± 0.019
Mm.38993	Calsyntenin 1 (Clstn1)	NS	-1.24 ± 0.628	0.69 / -	-1.6 ± 0.433	A	-2.14 ± 0.715
Cell proliferation							
Mm.338284	Coiled coil domain containing 88A (Ccdc88a)	1.16 / -	A	-1.35 ± 0.681	-1.9 / -	A	-1.64 ± 0.903
Mm.34405	Caspase 3 (Casp3)	0.37 / -	NS	2.05 / -	0.77 ± 0.426	A	-0.59 / -
Mm.5400	Bromodomain containing 7 (Brd7)	-4.48 / -	-2.26 / -	-1.89 ± 0.381	-1.04 / -	1.94 / -	-3.48 / -
Mm.240396	Protein phosphatase 2, regulatory subunit B (B56), gamma isoform (Ppp2r5c), transcript variant 2	NS	-0.61 ± 0.354	1.44 ± 0.568	-1 ± 0.028	A	-2.04 ± 0.517
Mm.264889	Myeloid/lymphoid or mixed-lineage leukemia 2 (Mll2)	A	A	1.19 ± 0.105	1.47 ± 0.091	A	-0.92 ± 0.826
Mm.291442	Secreted acidic cysteine rich glycoprotein (Sparc)	A	A	1.15 / -	0.98 / -	A	-2.01 / -
Mm.21974	C-src tyrosine kinase (Csk)	A	A	0.9 / -	-0.62 ±	A	-2.57 / -

					0.473		
Mm.287100	Nuclear receptor subfamily 2, group E, member 1 (Nr2e1)	A	A	-0.71 / -	A	A	1.85 / -
Immune response							
Mm.282184	Vascular endothelial growth factor A (Vegfa), transcript variant 1	1.06 ± 0.78	A	-3.06 / -	-1.54 ± 0.958	2.79 / -	1.45 ± 0.842
Mm.121265	Proteasome (prosome, macropain) subunit, alpha type 1 (Psma1)	0.9 / -	A	-1.78 / -	-1.29 ± 1.099	A	-1.29 ± 0.547
Mm.271814	Nuclear receptor co-repressor 1 (Ncor1)	-1.19 ± 0.537	2.6 ± 0.735	NS	2.26 ± 0.388	0.46 / -	2.98 ± 1.499
Mm.3064	Complement factor properdin (Cfp)	A	A	-2.39 / -	1.36 / -	A	0.83 ± 1.824
Death							
Mm.425296	Neutrophil cytosolic factor 1 (Ncf1)	A	A	0.69 / -	-0.44 ± 0.135	A	-0.79 / -
Mm.103205	Bone morphogenetic protein 2 (Bmp2)	NS	A	-2.63 / -	-0.75 ± 0.138	A	2.82 / -

Genes with opposite expression trend against V3000 and V3034 infections were selected from the gene subsets common to both V3000 and V3034 explained in figure-3. The table represents $\log_2$ values of fold change in the expression of genes ($\log_2(1.5) = 0.59$). "A" indicates that the gene expression was not detected in microarrays and "NS" indicates that the expression level was not significant. Values represent fold change $\pm$ SD.

Table-7 Genes specifically up-regulated against non-neuroinvasive strain of VEEV (V3014).

UniGene ID	Gene Description	V3014		
		24hr	48hr	72hr
Cell killing				
Mm.203747	Arrestin, beta 2 (Arrb2)	NS	A	1.53 ± 0.088
Viral reproduction				
Mm.260456	Vesicle-associated membrane protein, associated protein B and C (Vapb)	1.07 ± 0.191	A	NS
Mm.4341	Poliovirus receptor-related 2 (Pvrl2), transcript variant 1	A	A	1.12 / -
Mm.267998	TAF11 RNA polymerase II, TATA box binding protein (TBP)-associated factor (Taf11)	A	A	0.76 ± 0.531
Biological adhesion				
Mm.247203	Protocadherin gamma subfamily A, 6 (Pcdhga6)	1.89 ± 0.937	A	0.79 ± 0.201
Mm.560	Lymphocyte antigen 9 (Ly9)	1.05 ± 0.239	A	NS
Mm.12862	Protocadherin 12 (Pcdh12)	1.04 / -	A	0.69 / -
Mm.31903	Integrin alpha 4 (Itga4)	0.62 / -	A	0.64 / -
Mm.4911	Contactin 1 (Cntn1), transcript variant 2	A	2.34 / -	1.49 / -
Mm.3519	Glycoprotein 5 (platelet) (Gp5)	A	2.09 / -	A
Mm.25568	Scribbled homolog (Drosophila) (Scrib)	A	1.64 / -	0.59 / -
Mm.157591	Fermitin family homolog 3 (Drosophila) (Fermt3)	-0.8 ± 0.134	1.57 / -	1.59 ± 0.254
Mm.249146	Fibulin 2 (Fbln2), transcript variant 2	A	1.54 / -	A
Mm.3819	Collagen, type XII, alpha 1 (Col12a1)	A	A	2.84 / -
Mm.271745	Neuropilin 1 (Nrp1)	A	A	1.89 ± 1.331
Mm.1296	Cysteine-rich secretory protein 2 (Crisp2)	A	A	1.63 / -
Mm.45127	Cell growth regulator with EF hand domain 1 (Cgref1), transcript variant 2	A	A	1.5 / -
Mm.279437	Craniofacial development protein 1 (Cfdp1)	A	A	1.41 / -
Mm.288694	G protein-coupled receptor 98 (Gpr98)	A	A	1.36 / -
Mm.458684	Alkaline ceramidase 2 (Acer2)	A	A	1.33 / -
Mm.332387	Protocadherin 7 (Pcdh7), transcript variant 1	A	A	1.27 / -
Death				

Mm.264255	TNFRSF1A-associated via death domain (Tradd)	2.99 / -	A	A
Mm.436667	Autophagy/beclin 1 regulator 1 (Ambra1), transcript variant 2	1.83 / -	A	NS
Mm.440704	Ceramide kinase-like (Cerk1)	1.8 / -	A	NS
Mm.250866	Aldehyde dehydrogenase family 1, subfamily A1 (Aldh1a1)	NS	2.06 / -	NS
Mm.386878	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, delta (Nfkbid)	NS	NS	3.89 / -
Mm.779	CD5 antigen (Cd5)	A	A	2.05 / -
Mm.249873	CD38 antigen (Cd38)	A	A	1.78 / -
Mm.287901	DEAD (Asp-Glu-Ala-Asp) box polypeptide 19a (Ddx19a)	A	A	1.76 / -
Immune system process				
Mm.323057	Leucine rich repeat containing 32 (Lrrc32)	3.08 / -	A	NS
Mm.389903	Non-catalytic region of tyrosine kinase adaptor protein 2 (Nck2)	1.94 / -	A	NS
Mm.155583	Interleukin 1 receptor-like 2 (Il1rl2)	A	A	2.57 / -
Mm.386776	Estrogen related receptor, alpha (Esrra)	A	A	1.94 / -
Immune response				
Mm.676	Activating transcription factor 1 (Atf1)	0.81 ± 0.023	0.96 ± 0.296	0.88 ± 0.355
Mm.325757	MAD homolog 6 (Drosophila) (Smad6)	1.5 / -	A	A
Mm.436922	Immunoglobulin heavy chain 6 (heavy chain of igm) (Igh-6)	1.32 / -	A	NS
Mm.116739	Chemokine (C-C motif) ligand 20 (Ccl20), transcript variant 2	1.12 / -	A	0.85 / -
Mm.303231	Chemokine (C-X-C motif) ligand 12 (Cxcl12), transcript variant 3	1.11 / -	A	NS
Mm.1001	Endonuclease, polyu-specific (Endou), transcript variant 1	1.03 / -	A	A
Mm.343610	Polymerase (RNA) III (DNA directed) polypeptide A (Polr3a)	0.96 / -	A	A
Mm.39253	Mitogen-activated protein kinase 10 (Mapk10), transcript variant 2	0.93 / -	A	1.15 / -
Mm.21495	Mitogen-activated protein kinase 8 (Mapk8)	0.82 / -	NS	1.01 / -
Mm.296049	Calcium binding and coiled-coil domain 2 (Calcoco2), transcript variant 1	0.67 / -	A	NS
Mm.2326	Macrophage migration inhibitory factor (Mif)	A	1.4 / -	1.3 ± 0.686
Mm.41171	Tumor necrosis factor (ligand) superfamily, member 9 (Tnfsf9)	A	A	1.41 / -

Mm.190	Chemokine (C motif) ligand 1 (Xcl1)	A	A	1.26 / -
Mm.24006	S100 calcium binding protein A14 (S100a14), transcript variant 3	A	A	1.16 / -
Mm.290320	Chemokine (C-C motif) ligand 2 (Ccl2)	A	A	0.74 / -
Mm.14190	Interleukin 2 (Il2)	A	A	0.71 / -

Genes significantly up-regulated more than 1.5 fold and that were only expressed against V3014 infection were categorized by GO based analysis. The table represents $\log_2$ values of fold change in the expression of genes ($\log_2 (1.5) = 0.59$). "A" indicates that the gene expression was not detected in microarrays and "NS" indicates that the expression level was not significant. Values represent fold change $\pm$ SD.

Table-8 Genes specifically down-regulated against non-neuroinvasive strain of VEEV (V3014)

UniGene ID	Gene Description	V3014		
		24hr	48hr	72hr
Viral reproduction				
Mm.328831	THO complex 6 homolog (Drosophila) (Thoc6)	A	-2.39 / -	-0.71 / -
Biological adhesion				
Mm.299254	Par-3 (partitioning defective 3) homolog (C. Elegans) (Pard3), transcript variant 3	-1.09 / -	-2.25 / -	0.8 / -
Mm.42249	Neogenin (Neo1), transcript variant 2	-0.59 / -	-1 / -	-0.72 ± 0.205
Mm.27681	A disintegrin and metallopeptidase domain 17 (Adam17)	-0.61 / -	A	-0.87 / -
Mm.289682	Disabled homolog 1 (Drosophila) (Dab1)	-0.63 / -	A	NS
Mm.29729	Tweety homolog 1 (Drosophila) (Ttyh1)	-0.72 / -	A	NS
Mm.195010	FAT tumor suppressor homolog 3 (Drosophila) (Fat3)	-0.79 ± 0.276	A	-0.85 ± 0.493
Mm.373589	Discoidin, CUB and LCCL domain containing 2 (Dcbld2)	-1.05 / -	A	NS
Mm.57734	LIM and senescent cell antigen-like domains 1 (Lims1), transcript variant 3	-1.84 / -	A	-2.38 / -
Mm.3951	Thymus cell antigen 1, theta (Thy1)	-2 / -	A	NS
Mm.22842	CD2 antigen (Cd2)	-4.57 ± 1.386	A	A
Mm.1123	Wingless-related MMTV integration site 1 (Wnt1)	A	-0.89 / -	A
Mm.294826	Homeobox A7 (Hoxa7)	NS	-2.25 ± 0.093	NS
Mm.331784	Utrophin (Utrn)	NS	-2.27 / -	2.31 ± 1.607
Mm.119714	Immunoglobulin superfamily, member 5 (Igsf5), transcript variant 3	A	-4.41 / -	A
Mm.326247	Atpase, Ca++-sequestering (Atp2c1)	A	A	0.59 / -
Mm.308500	Protocadherin alpha 3 (Pcdha3)	A	A	-1.58 ± 0.642
Death				
Mm.23670	FAST kinase domains 3 (Fastkd3)	-0.57 / -	-2.9 / -	A
Mm.246550	POU domain, class 4, transcription factor 1 (Pou4f1)	-1.18 / -	-2.87 / -	0.46 ± 0.031
Mm.196006	Mutl homolog 1 (E. Coli) (Mlh1)	-1.48 / -	-4.1 / -	-0.56 / -
Mm.461296	Mucin 2 (Muc2)	-2.03 / -	-2.79 / -	-0.11 ±

				0.035
Mm.26768	SAM pointed domain containing ets transcription factor (Spdef)	-3.21 / -	A	A
Mm.215173	Pre T-cell antigen receptor alpha (Ptcra)	NS	-1.62 ± 0.846	0.7 ± 0.446
Mm.17	B-cell receptor-associated protein 31 (Bcap31)	NS	-1.71 ± 0.127	0.78 / -
Mm.1605	Programmed cell death 4 (Pdcd4), transcript variant 2	NS	-1.78 / -	A
Mm.211838	Solute carrier family 5 (sodium/glucose cotransporter), member 11 (Slc5a11)	A	-2.01 / -	0.54 / -
Immune system process				
Mm.30837	N-myc downstream regulated gene 1 (Ndrg1)	0.63 / -	-2.11 / -	NS
Mm.296457	Pellino 2 (Peli2)	-2.02 / -	A	NS
Mm.20466	Interleukin 18 receptor accessory protein (Il18rap)	A	-1.88 / -	A
Mm.12932	Bloom syndrome, RecQ helicase-like (Blm), transcript variant 2	A	A	-2.41 / -
Immune response				
Mm.90154	Interleukin 25 (Il25)	-0.65 / -	A	NS
Mm.331	Ubiquitin C (Ubc)	-0.99 / -	1.2 / -	NS
Mm.249142	Transcription factor E3 (Tcfe3), transcript variant 2	NS	A	-0.96 / -
Mm.1741	Avian reticuloendotheliosis viral (v-rel) oncogene related B (Relb)	A	-0.73 / -	NS
Mm.221227	Interleukin 23 receptor (Il23r)	A	A	-0.9 / -
Mm.116844	Lymphocyte antigen 96 (Ly96), transcript variant 1	A	A	-0.92 ± 0.294

Genes significantly down-regulated more than 1.5 fold and that were only expressed against V3014 infection and not against V3000 or V3034 infections were filtered and categorized by GO based analysis. The table represents $\log_2$ transformed values of fold change in the expression of genes ($\log_2(1.5) = 0.59$). "A" indicates that the gene expression was not detected in microarrays and "NS" indicates that the expression level was not significant. Values represent fold change $\pm$ SD.

Table-9 Different vomeronasal receptor genes modulated against neuroinvasive strain of VEEV (V3000) in the host blood

Gene	UniGene Id	24hr pi		48 hr pi		72 hr pi	
		Log2 (Fold expression)	Std Dev	Log2 (Fold expression)	Std Dev	Log2 (Fold expression)	Std Dev
Vmn1r1	Mm.479992					-0.33	0.00
Vmn1r100	Mm.484979					-1.02	0.00
Vmn1r118	Mm.480258	0.59	0.64	2.25	0.00	1.23	0.00
Vmn1r124	Mm.480250	0.65	0.19			0.68	0.12
Vmn1r139	Mm.484972			0.63	0.00		
Vmn1r15	Mm.377160					-0.26	0.00
Vmn1r151	Mm.480281	0.21	0.00				
Vmn1r16	Mm.431971	-0.57	0.00				
Vmn1r168	Mm.480247	1.23	0.00			1.55	0.00
Vmn1r171	Mm.160377					1.18	0.00
Vmn1r174	Mm.160375	-2.83	0.00			-2.04	0.79
Vmn1r18	Mm.377177	-0.06	0.00				
Vmn1r180	Mm.377249	0.87	0.00			0.49	0.03
Vmn1r181	Mm.279185	1.61	0.00				
Vmn1r183	Mm.377233	0.19	0.00				
Vmn1r19	Mm.377178			0.96	0.00	0.75	0.00
Vmn1r191	Mm.479517	-0.86	0.25	-2.40	0.31	-4.15	0.00
Vmn1r193	Mm.432535	1.69	0.00				
Vmn1r195	Mm.377211			0.90	0.00		
Vmn1r198	Mm.377208	1.02	0.00	2.34	0.00		
Vmn1r200	Mm.377202	-0.12	0.00				
Vmn1r201	Mm.377209	0.21	0.00	2.25	0.00	-0.21	0.00
Vmn1r202	Mm.377212			0.18	0.00		
Vmn1r206	Mm.377205	0.51	0.00			0.79	0.15
Vmn1r207-ps	Mm.480282	-0.54	0.00			0.88	0.26
Vmn1r209	Mm.377950	-0.61	0.00				
Vmn1r211	Mm.377228	0.20	0.00			0.28	0.00
Vmn1r216	Mm.377229			-0.29	0.00		
Vmn1r217	Mm.377225	-0.02	0.00	-0.41	0.00	-1.54	0.00
Vmn1r23	Mm.377176					-1.17	0.00
Vmn1r230	Mm.377188	1.41	0.00				
Vmn1r234	Mm.377189	0.11	0.00				
Vmn1r235	Mm.222636	-0.59	0.62	-1.15	0.90	-4.69	1.19
Vmn1r236	Mm.432089			-0.05	0.00		
Vmn1r27	Mm.389781					-1.25	0.00

Vmn1r32	**Mm.222748**					0.21	0.00
Vmn1r33	**Mm.451648**					-0.80	0.00
Vmn1r36	**Mm.377165**					0.10	0.00
Vmn1r4	**Mm.222754**			1.23	0.00	1.42	0.00
Vmn1r4	**Mm.222754**					-0.41	0.00
Vmn1r40	**Mm.377152**			1.16	0.00		
Vmn1r41	**Mm.377154**					-1.13	0.00
Vmn1r42	**Mm.349358**	-0.04	0.27			0.80	0.26
Vmn1r45	**Mm.431981**	-0.38	0.00				
Vmn1r48	**Mm.425336**	0.90	0.00				
Vmn1r5	**Mm.377172**	0.04	0.00				
Vmn1r56	**Mm.160380**	0.40	0.53	0.00	0.58	-1.97	1.04
Vmn1r59	**Mm.377928**			1.19	0.00		
Vmn1r62	**Mm.160381**	0.71	0.00			0.14	0.00
Vmn1r63	**Mm.160382**					-0.93	0.00
Vmn1r64	**Mm.377929**	-0.01	0.00				
Vmn1r65	**Mm.160378**	0.22	0.98	1.63	0.00	-3.65	1.77
Vmn1r70	**Mm.222634**			0.96	0.00	-3.67	0.00
Vmn1r71	**Mm.451639**	-2.12	0.00				
Vmn1r72	**Mm.377264**	0.13	0.25	0.02	0.00	-0.77	0.50
Vmn1r73	**Mm.377193**	0.82	0.00				
Vmn1r75	**Mm.377197**	1.32	0.00				
Vmn1r76	**Mm.377195**	0.21	0.33	0.01	0.00	0.28	0.16
Vmn1r78	**Mm.377198**	-0.42	0.04	0.51	0.00	1.08	0.04
Vmn1r79	**Mm.484984**	0.28	0.55	0.31	0.00	0.33	0.24
Vmn1r8	**Mm.222638**	0.10	0.15	0.10	0.00	0.69	0.13
Vmn1r82	**Mm.377220**	-0.10	0.00			0.88	0.00
Vmn1r83	**Mm.377199**	-0.96	0.00			-1.08	0.00
Vmn1r85	**Mm.377268**					-1.37	0.00
Vmn1r87	**Mm.377215**	0.99	0.00	-0.26	0.00	1.54	0.00
Vmn1r89	**Mm.377214**					-0.50	0.00
Vmn1r90	**Mm.372763**			-0.14	0.00	0.02	0.00
V1ra8	**Mm.377146**					-1.88	0.00
Vmn2r101	**Mm.469857**	-0.12	0.00	-0.12	0.00		
Vmn2r105	**Mm.379363**	-0.12	0.00				
Vmn2r106	**Mm.469854**	0.20	0.00			-0.52	0.00
Vmn2r109	**Mm.469852**	0.16	0.00	0.65	0.00	0.90	0.29
Vmn2r112	**Mm.390947**					-0.99	0.00
Vmn2r114	**Mm.469838**	0.75	0.00				
Vmn2r115	**Mm.469849**	0.05	0.00			0.18	1.00
Vmn2r118	**Mm.461682**	-0.22	0.00	0.60	0.00		

Vmn2r16	**Mm.469880**	-0.44	0.32	0.19	0.00	1.30	0.00
Vmn2r16	**Mm.469880**					-1.15	0.00
Vmn2r19	**Mm.469878**	-1.53	0.00			-1.74	0.00
Vmn2r23	**Mm.469874**	0.47	0.00				
Vmn2r24	**Mm.469873**	0.56	0.00				
Vmn2r25	**Mm.484595**					-0.06	0.00
Vmn2r29	**Mm.387947**	-0.08	0.00				
Vmn2r37	**Mm.302158**	-0.49	0.00				
Vmn2r4	**Mm.425135**	-0.28	0.16				
Vmn2r4	**Mm.425135**					-0.83	0.00
Vmn2r42	**Mm.359403**	0.83	0.00			1.40	0.00
Vmn2r56	**Mm.469869**	1.35	0.00	0.38	0.00	0.85	0.00
Vmn2r56	**Mm.469869**					-2.04	0.00
Vmn2r57	**Mm.483785**			0.16	0.00		
Vmn2r58	**Mm.469902**					-0.07	0.00
Vmn2r65	**Mm.389926**	0.13	0.00				
Vmn2r68-ps	**Mm.389945**	0.62	0.00				
Vmn2r7	**Mm.477092**	1.15	0.00			0.56	0.00
Vmn2r7	**Mm.477092**			0.00	0.00		
Vmn2r75	**Mm.469835**					0.64	0.00
Vmn2r76	**Mm.469834**			1.01	0.00		
Vmn2r84	**Mm.359168**	0.24	0.00			0.21	0.00
Vmn2r87	**Mm.469844**			0.28	0.00	-1.88	0.00
Vmn2r88	**Mm.377117**	-0.52	0.08			0.17	0.56
Vmn2r89	**Mm.482572**	0.26	0.19	-0.71	0.00	-1.70	0.00
Vmn2r89	**Mm.482572**					-1.15	0.00
Vmn2r9	**Mm.387745**	0.16	0.00				
Vmn2r90	**Mm.404019**	0.57	0.09				
Vmn2r94	**Mm.469861**					1.20	0.00
Vmn2r122	**Mm.377117**	-0.62	0.15	-0.22	0.00	-0.42	0.00
Vmn2r122	**Mm.377117**	-0.48	0.00				

Different vomeronasal receptor homologue genes that were significantly modulated more than 1.5 fold against V3000, the neuroinvasive strain of VEEV, infection were filtered and listed above. The table represents $\log_2$ transformed values of fold change in the expression of genes ($\log_2(1.5) = 0.59$). Values represent average fold change $\pm$ SD.

Table-10 Different vomeronasal receptor genes modulated against partially-neuroinvasive strain of VEEV (V3034) in the host blood

Gene	UniGene Id	24hr pi		48 hr pi		72 hr pi	
		Log2 (Fold expression)	Std Dev	Log2 (Fold expression)	Std Dev	Log2 (Fold expression)	Std Dev
V1ra8	**Mm.377146**	-1.63	0.00			-0.12	0.85
V1rd19	**Mm.377931**					-0.19	0.00
Vmn1r1	**Mm.479992**	0.28	0.00			-0.16	0.22
Vmn1r10	**Mm.436285**					0.42	0.00
Vmn1r10	**Mm.436285**					-0.22	0.00
Vmn1r100	**Mm.484979**					0.76	0.00
Vmn1r118	**Mm.480258**	0.18	0.59			-0.90	0.71
Vmn1r12	**Mm.475092**					-0.04	0.00
Vmn1r121	**Mm.480259**	0.09	0.00			-0.41	0.00
Vmn1r124	**Mm.480250**	0.44	0.05			-0.26	1.01
Vmn1r13	**Mm.431976**					0.58	0.00
Vmn1r139	**Mm.484972**	-0.63	0.01			0.23	0.23
Vmn1r14	**Mm.431974**					0.67	1.25
Vmn1r151	**Mm.480281**	-1.23	0.34			-3.62	0.00
Vmn1r16	**Mm.431971**					0.23	0.00
Vmn1r168	**Mm.480247**	0.56	0.24			1.67	0.00
Vmn1r17	**Mm.377169**	0.47	0.00			0.45	0.00
Vmn1r170	**Mm.480274**					0.09	0.00
Vmn1r171	**Mm.160377**	0.67	0.36	2.64	0.00	-0.04	0.00
Vmn1r174	**Mm.160375**	-0.64	1.14			-2.03	0.90
Vmn1r178	**Mm.377248**					0.78	0.00
Vmn1r179	**Mm.377930**					-0.15	0.00
Vmn1r18	**Mm.377177**	-0.10	0.00			-0.60	0.00
Vmn1r180	**Mm.377249**	-0.60	0.37			-1.29	0.00
Vmn1r181	**Mm.279185**	0.92	0.41			-0.01	0.43
Vmn1r183	**Mm.377233**	-0.46	0.52			-1.15	0.14
Vmn1r184	**Mm.480246**	-0.56	0.00			0.33	0.00
Vmn1r188	**Mm.377269**					1.35	0.00
Vmn1r189	**Mm.377265**	0.96	0.00				
Vmn1r19	**Mm.377178**	-0.16	0.00			2.83	0.00
Vmn1r191	**Mm.479517**	-0.20	0.11			-2.16	0.00
Vmn1r192	**Mm.435548**	-0.18	0.00			1.48	0.00
Vmn1r193	**Mm.432535**					0.19	0.00
Vmn1r195	**Mm.377211**	-0.08	0.00			1.88	0.00
Vmn1r198	**Mm.377208**	0.51	0.00			-0.45	0.48
Vmn1r199	**Mm.377203**					2.13	0.00
Vmn1r200	**Mm.377202**	0.39	0.00			-0.12	1.20
Vmn1r201	**Mm.377209**	0.50	0.25			0.35	1.54

Vmn1r203	**Mm.377222**	0.03	0.00				
Vmn1r205	**Mm.377206**					0.01	0.00
Vmn1r206	**Mm.377205**	-0.31	0.49			-1.04	0.00
Vmn1r207-ps	**Mm.480282**	-0.69	0.30			-0.40	1.70
Vmn1r208	**Mm.390562**	0.65	0.00			-0.29	0.00
Vmn1r209	**Mm.377950**	1.62	0.00			-1.93	0.47
Vmn1r21	**Mm.377179**					0.26	0.00
Vmn1r210	**Mm.377221**					-1.44	0.00
Vmn1r211	**Mm.377228**	0.66	0.00			-0.64	0.00
Vmn1r213	**Mm.378580**	1.30	0.00				
Vmn1r214	**Mm.377204**	1.12	0.00				
Vmn1r217	**Mm.377225**	-0.01	0.20			-3.26	0.00
Vmn1r218	**Mm.377210**	0.75	0.00				
Vmn1r219	**Mm.377224**	-0.10	0.00			0.49	0.00
Vmn1r226	**Mm.377184**					0.31	0.00
Vmn1r227	**Mm.377187**					-0.17	0.12
Vmn1r228	**Mm.222639**	0.39	0.00			0.69	0.00
Vmn1r23	**Mm.377176**					-0.60	0.88
Vmn1r230	**Mm.377188**					-0.01	1.35
Vmn1r231	**Mm.222642**					-0.27	1.16
Vmn1r233	**Mm.377192**					0.24	0.00
Vmn1r234	**Mm.377189**	-0.86	0.00			-1.48	0.00
Vmn1r235	**Mm.222636**	-1.45	0.00	-4.10	0.00	-3.32	0.00
Vmn1r236	**Mm.432089**	0.17	0.44				
Vmn1r237	**Mm.405416**	0.50	0.00				
Vmn1r24	**Mm.377171**	0.53	0.00			-1.58	0.83
Vmn1r25	**Mm.431972**					0.53	0.03
Vmn1r26	**Mm.377170**					1.12	0.00
Vmn1r27	**Mm.389781**					-0.87	0.00
Vmn1r28	**Mm.222640**					0.00	0.00
Vmn1r29	**Mm.377156**					0.56	0.39
Vmn1r30	**Mm.389764**					-0.83	0.00
Vmn1r31	**Mm.480268**					1.83	0.00
Vmn1r32	**Mm.222748**	0.51	0.00			-0.03	0.03
Vmn1r33	**Mm.451648**	0.01	0.00				
Vmn1r36	**Mm.377165**					0.52	0.78
Vmn1r37	**Mm.377164**					-0.13	0.00
Vmn1r38	**Mm.377167**					1.23	0.00
Vmn1r4	**Mm.222754**	0.25	0.53			1.37	0.09
Vmn1r4	**Mm.222754**					-0.42	0.00
Vmn1r40	**Mm.377152**			1.83	0.00	0.16	0.00
Vmn1r42	**Mm.349358**	-0.63	0.42			-0.68	1.22
Vmn1r43	**Mm.431989**					-1.11	0.00
Vmn1r45	**Mm.431981**	0.31	0.44			0.50	1.40

Vmn1r46	**Mm.377153**					0.94	0.00
Vmn1r48	**Mm.425336**	0.03	0.66			-1.11	0.00
Vmn1r49	**Mm.89985**	-0.24	0.00			-0.70	0.00
Vmn1r50	**Mm.377149**					0.45	0.00
Vmn1r51	**Mm.20471**	0.22	0.00				
Vmn1r53	**Mm.434332**					2.71	0.00
Vmn1r55	**Mm.480286**					-0.11	0.00
Vmn1r56	**Mm.160380**	-1.42	0.37	0.94	0.00	-1.54	1.76
Vmn1r58	**Mm.261621**					0.22	0.32
Vmn1r6	**Mm.377173**					-1.28	0.00
Vmn1r62	**Mm.160381**					-0.43	0.58
Vmn1r62	**Mm.160381**	-0.07	0.57			-1.41	1.72
Vmn1r63	**Mm.160382**					-0.58	0.00
Vmn1r64	**Mm.377929**	-0.68	0.10			-1.34	0.45
Vmn1r65	**Mm.160378**	1.75	0.00	0.54	0.00	-1.44	1.07
Vmn1r66	**Mm.222637**					-0.03	0.00
Vmn1r68	**Mm.480301**					1.21	1.50
Vmn1r70	**Mm.222634**	1.02	0.87			-1.94	1.03
Vmn1r72	**Mm.377264**	-0.60	0.43			-3.11	0.00
Vmn1r73	**Mm.377193**	0.71	0.00			0.62	0.30
Vmn1r75	**Mm.377197**	1.31	0.11			1.31	0.90
Vmn1r76	**Mm.377195**	-0.42	0.26			0.21	0.43
Vmn1r78	**Mm.377198**	-0.70	0.07			-0.16	0.86
Vmn1r79	**Mm.484984**	0.06	0.45			-0.81	0.86
Vmn1r8	**Mm.222638**	-0.12	0.40			0.59	0.84
Vmn1r81	**Mm.377200**					-0.35	0.00
Vmn1r82	**Mm.377220**			-0.32	0.00	0.87	0.00
Vmn1r83	**Mm.377199**					-0.70	0.00
Vmn1r84	**Mm.434341**					0.34	0.00
Vmn1r85	**Mm.377268**	0.14	0.00			1.59	0.00
Vmn1r87	**Mm.377215**	0.06	0.48			-1.54	0.00
Vmn1r90	**Mm.372763**	-0.07	0.00				
Vmn1r-ps103	**Mm.377201**			3.52	0.00	-0.18	0.00
Vmn2r101	**Mm.469857**	0.47	0.00				
Vmn2r101	**Mm.469857**	0.29	0.00				
Vmn2r105	**Mm.379363**	-0.15	0.00			0.66	0.00
Vmn2r106	**Mm.469854**	0.79	0.00			1.19	0.00
Vmn2r109	**Mm.469852**	-0.57	0.05			0.06	0.35
Vmn2r110	**Mm.469851**					-1.93	0.94
Vmn2r112	**Mm.390947**	-0.89	0.00				
Vmn2r114	**Mm.469838**	-0.10	0.00			-1.46	0.00
Vmn2r115	**Mm.469849**	-0.65	0.00			0.16	0.00
Vmn2r118	**Mm.461682**					-0.13	0.00
Vmn2r118	**Mm.461682**					-1.19	0.00

Vmn2r12	**Mm.469884**	-0.89	0.00			2.21	0.00
Vmn2r120	**Mm.469889**	-0.15	0.00			0.84	0.00
Vmn2r120	**Mm.469889**	-0.23	0.00			-0.67	0.00
Vmn2r122	**Mm.377117**	-0.68	0.15			0.28	0.44
Vmn2r122	**Mm.377117**	0.20	0.00			-2.07	0.00
Vmn2r123	**Mm.377117**	1.06	0.35				
Vmn2r13	**Mm.469883**					0.66	0.00
Vmn2r15	**Mm.469881**					0.30	0.00
Vmn2r16	**Mm.469880**					3.25	0.00
Vmn2r16	**Mm.469880**	-0.28	0.38			-0.57	1.80
Vmn2r18	**Mm.469837**	-0.56	0.35			-0.14	0.00
Vmn2r19	**Mm.469878**					1.79	0.00
Vmn2r20	**Mm.469877**					1.34	0.00
Vmn2r25	**Mm.484595**	-0.07	0.00			1.40	0.89
Vmn2r26	**Mm.23795**	0.21	0.00			1.16	0.00
Vmn2r27	**Mm.469872**					2.12	0.00
Vmn2r29	**Mm.387947**	0.42	0.00				
Vmn2r29	**Mm.465200**					-1.14	0.81
Vmn2r30	**Mm.458977**	-4.16	0.00			0.47	0.00
Vmn2r30	**Mm.461092**					1.70	0.00
Vmn2r30	**Mm.461092**	0.53	0.00	2.42	0.00		
Vmn2r37	**Mm.302158**	0.81	0.00				
Vmn2r37	**Mm.302158**	0.43	0.07			0.09	0.00
Vmn2r4	**Mm.425135**	-2.62	0.00			0.77	0.00
Vmn2r4	**Mm.425135**					0.91	1.23
Vmn2r42	**Mm.359403**	0.71	0.00			1.39	0.00
Vmn2r43	**Mm.247383**	1.08	0.00				
Vmn2r5	**Mm.484957**					1.16	0.00
Vmn2r53	**Mm.469871**					0.14	0.00
Vmn2r56	**Mm.469869**	-0.41	0.93			0.99	0.55
Vmn2r57	**Mm.483785**	-0.39	0.00			-0.58	0.00
Vmn2r58	**Mm.469902**					0.30	0.00
Vmn2r59	**Mm.469901**					-0.44	1.60
Vmn2r60	**Mm.469900**	0.40	0.00				
Vmn2r63	**Mm.469897**					-0.60	0.00
Vmn2r65	**Mm.389926**	0.23	0.00			1.56	0.00
Vmn2r66	**Mm.424396**	0.73	0.00				
Vmn2r68-ps	**Mm.389945**	0.16	0.00			-1.76	0.00
Vmn2r69	**Mm.469909**					-0.56	0.30
Vmn2r7	**Mm.477092**	0.62	0.00			0.26	0.00
Vmn2r71	**Mm.389930**					-0.29	0.00
Vmn2r72-ps	**Mm.469907**					-0.09	0.00
Vmn2r73	**Mm.469906**	0.51	0.00	2.15	0.00	0.96	0.00

Vmn2r74	**Mm.469905**	0.18	0.00			1.26	1.05
Vmn2r75	**Mm.469835**	0.29	0.39			-1.81	0.00
Vmn2r76	**Mm.469834**					-1.28	0.00
Vmn2r78	**Mm.389957**					0.14	0.00
Vmn2r8	**Mm.469886**					0.73	1.81
Vmn2r80	**Mm.469843**	1.17	0.00				
Vmn2r81	**Mm.246574**	-0.23	0.00			-1.35	0.59
Vmn2r83	**Mm.469865**	-3.18	0.00			1.13	0.00
Vmn2r84	**Mm.359168**	0.32	0.00			-2.23	0.00
Vmn2r88	**Mm.377117**	-0.16	0.32			0.17	0.85
Vmn2r89	**Mm.482572**	0.16	0.00			-1.16	0.00
Vmn2r89	**Mm.482572**	-1.63	0.24			-3.04	0.00
Vmn2r9	**Mm.387745**					-0.49	1.26
Vmn2r90	**Mm.404019**	-0.55	0.46			-2.05	0.00
Vmn2r94	**Mm.469861**					-0.98	0.00
Vmn2r99	**Mm.469859**	1.14	0.00			2.18	0.00

Different vomeronasal receptor homologue genes that were significantly modulated more than 1.5 fold against V3034, the partially-neuroinvasive strain of VEEV, infection were filtered and listed above. The table represents $\log_2$ transformed values of fold change in the expression of genes ($\log_2(1.5) = 0.59$). Values represent average fold change $\pm$ SD.

Table-11 Different vomeronasal receptor genes modulated against non-neuroinvasive strain of VEEV (V3014) in the host blood

Gene	UniGene Id	24hr pi		48 hr pi		72 hr pi	
		Log2 (Fold expression)	Std Dev	Log2 (Fold expression)	Std Dev	Log2 (Fold expression)	Std Dev
Vmn1r118	**Mm.480258**	1.27	0.00			0.12	0.00
Vmn1r124	**Mm.480250**	0.58	0.00			0.06	0.00
Vmn1r13	**Mm.431976**					2.29	0.00
Vmn1r139	**Mm.484972**					-0.65	0.00
Vmn1r14	**Mm.431974**					0.75	0.00
Vmn1r15	**Mm.377160**					-0.96	0.00
Vmn1r151	**Mm.480281**					0.32	1.00
Vmn1r168	**Mm.480247**	0.86	0.00			0.01	0.00
Vmn1r171	**Mm.160377**					-0.16	0.00
Vmn1r174	**Mm.160375**					-0.77	0.00
Vmn1r178	**Mm.377248**			-0.80	0.00		
Vmn1r180	**Mm.377249**					0.33	0.00
Vmn1r181	**Mm.279185**					-0.14	0.00
Vmn1r183	**Mm.377233**	0.00	0.00				
Vmn1r19	**Mm.377178**					-0.07	0.00
Vmn1r191	**Mm.479517**	-0.14	0.00	2.51	0.00	0.98	0.00
Vmn1r207-ps	**Mm.480282**	-0.05	0.00			-0.06	0.00
Vmn1r230	**Mm.377188**					-0.32	0.00
Vmn1r231	**Mm.222642**					0.23	0.00
Vmn1r232	**Mm.451646**					2.07	0.00
Vmn1r234	**Mm.377189**					0.85	0.00
Vmn1r235	**Mm.222636**	-0.28	0.70	-1.53	0.21	1.12	0.40
Vmn1r27	**Mm.389781**					0.27	0.00
Vmn1r36	**Mm.377165**					-2.93	0.00
Vmn1r38	**Mm.377167**					-0.35	0.00
Vmn1r42	**Mm.349358**	1.33	0.00			0.75	0.00
Vmn1r44	**Mm.377151**					-0.49	0.00
Vmn1r45	**Mm.431981**	-0.65	0.00			0.84	0.00
Vmn1r46	**Mm.377153**					3.24	0.00
Vmn1r48	**Mm.425336**					0.03	0.00
Vmn1r49	**Mm.89985**					0.05	0.00
Vmn1r54	**Mm.377148**					-0.30	0.00

Vmn1r56	**Mm.160380**	0.78	0.07	2.60	0.00	2.48	1.93
Vmn1r59	**Mm.377928**					1.56	0.00
Vmn1r62	**Mm.160381**	-0.95	0.00				
Vmn1r63	**Mm.160382**					-0.22	0.00
Vmn1r64	**Mm.377929**	-0.51	0.00				
Vmn1r65	**Mm.160378**	-4.91	0.00	3.27	0.00	-2.32	0.25
Vmn1r68	**Mm.480301**					0.49	0.00
Vmn1r70	**Mm.222634**					0.43	0.00
Vmn1r72	**Mm.377264**	0.02	0.00				
Vmn1r73	**Mm.377193**					0.38	0.00
Vmn1r76	**Mm.377195**	1.01	0.00			-0.17	0.00
Vmn1r78	**Mm.377198**	1.01	0.00				
Vmn1r79	**Mm.484984**					-0.53	0.00
Vmn1r8	**Mm.222638**	-0.42	0.00			0.12	0.65
Vmn1r80	**Mm.438893**					-0.21	0.00
Vmn1r84	**Mm.434341**					1.36	0.00
Vmn1r87	**Mm.377215**	1.37	0.00			0.44	0.00
Vmn2r101	**Mm.469857**					0.73	0.00
Vmn2r106	**Mm.469854**					0.11	0.00
Vmn2r109	**Mm.469852**					0.11	0.00
Vmn2r115	**Mm.469849**					-1.11	0.00
Vmn2r118	**Mm.461682**					1.47	0.00
Vmn2r12	**Mm.469884**					-0.57	0.00
Vmn2r122	**Mm.377117**	-1.34	0.00			0.10	0.00
Vmn2r16	**Mm.469880**					0.32	0.00
Vmn2r16	**Mm.469880**	0.05	0.00			0.54	0.00
Vmn2r18	**Mm.469837**	-0.12	0.00				
Vmn2r20	**Mm.469877**			-3.29	0.00		
Vmn2r27	**Mm.469872**					0.24	0.00
Vmn2r37	**Mm.302158**					0.11	0.00
Vmn2r4	**Mm.425135**	0.37	0.00			0.49	0.00
Vmn2r42	**Mm.359403**	0.48	0.00			-0.34	0.00
Vmn2r56	**Mm.469869**					2.08	1.86
Vmn2r57	**Mm.483785**					0.60	0.00
Vmn2r59	**Mm.469901**					0.66	0.00
Vmn2r7	**Mm.477092**					0.15	0.00
Vmn2r72-ps	**Mm.469907**					0.68	0.00
Vmn2r74	**Mm.469905**	0.47	0.00				
Vmn2r75	**Mm.469835**	0.54	0.00			0.08	0.00

Vmn2r81	**Mm.246574**					0.31	0.00
Vmn2r83	**Mm.469865**					0.19	0.00
Vmn2r84	**Mm.359168**					-0.03	0.00
Vmn2r88	**Mm.377117**	-1.18	0.00			-0.14	0.00
Vmn2r89	**Mm.482572**	0.94	0.00				
Vmn2r89	**Mm.482572**					0.60	0.00
Vmn2r89	**Mm.482572**					0.42	0.00
Vmn2r90	**Mm.404019**					0.11	0.00
Vmn2r94	**Mm.469861**					0.34	0.00

Different vomeronasal receptor homologue genes that were significantly modulated more than 1.5 fold against V3014, the non-neuroinvasive strain of VEEV, infection were filtered and listed above. The table represents $\log_2$ transformed values of fold change in the expression of genes ($\log_2(1.5) = 0.59$). Values represent average fold change $\pm$ SD.

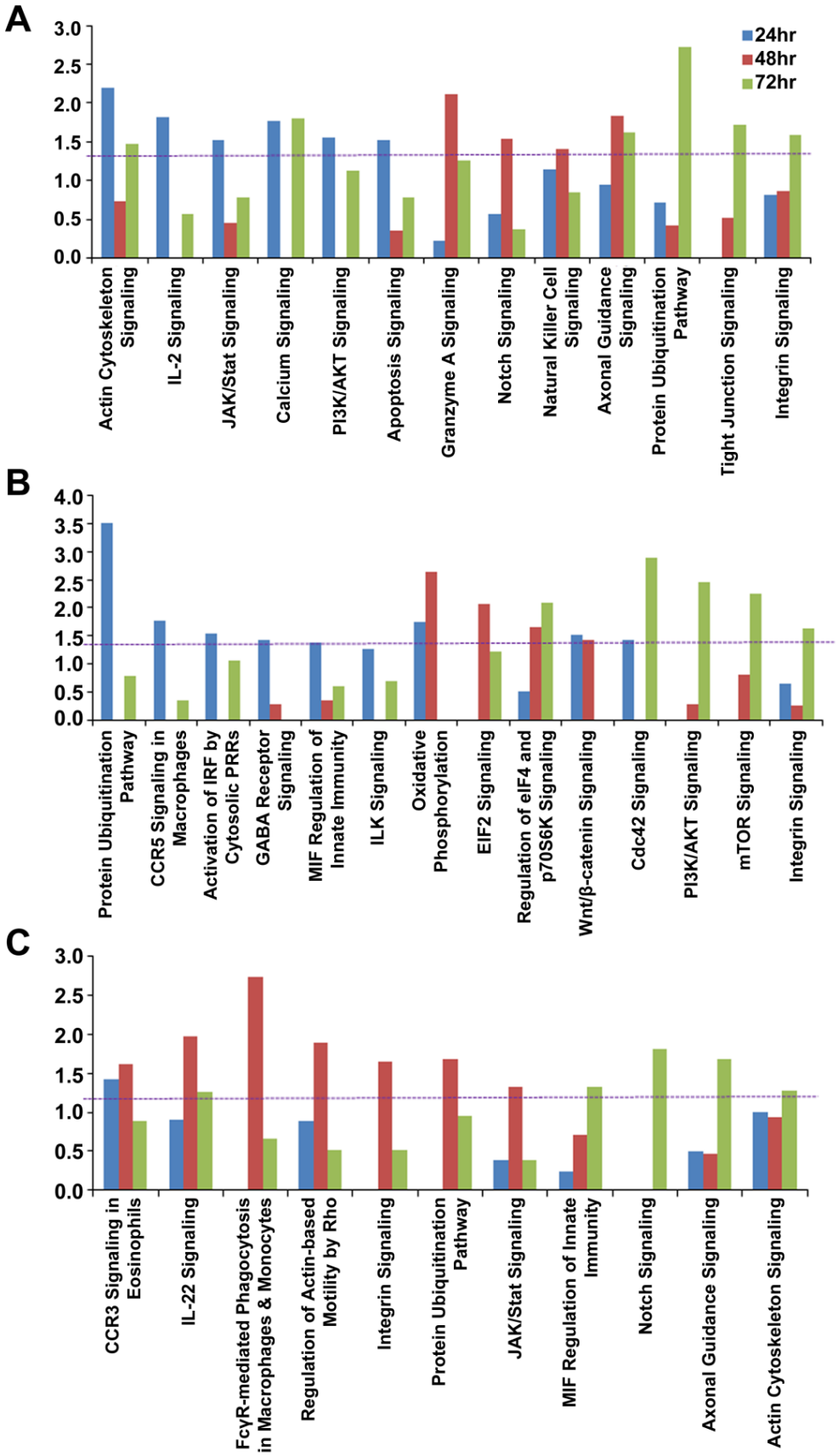

A
3.0
2.5
2.0
1.5
1.0
0.5
0.0
24hr
48hr
72hr
Actin Cytoskeleton Signaling
IL-2 Signaling
JAK/Stat Signaling
Calcium Signaling
PI3K/AKT Signaling
Apoptosis Signaling
Granzyme A Signaling
Notch Signaling
Natural Killer Cell Signaling
Axonal Guidance Signaling
Protein Ubiquitination Pathway
Tight Junction Signaling
Integrin Signaling
B
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Protein Ubiquitination Pathway
CCR5 Signaling in Macrophages
Activation of IRF by Cytosolic PRRs
GABA Receptor Signaling
MIF Regulation of Innate Immunity
ILK Signaling
Oxidative Phosphorylation
EIF2 Signaling
Regulation of eIF4 and p70S6K Signaling
Wnt/β-catenin Signaling
Cdc42 Signaling
PI3K/AKT Signaling
mTOR Signaling
Integrin Signaling
C
3.0
2.5
2.0
1.5
1.0
0.5
0.0
CCR3 Signaling in Eosinophils
IL-22 Signaling
FcγR-mediated Phagocytosis in Macrophages & Monocytes
Regulation of Actin-based Motility by Rho
Integrin Signaling
Protein Ubiquitination Pathway
JAK/Stat Signaling
MIF Regulation of Innate Immunity
Notch Signaling
Axonal Guidance Signaling
Actin Cytoskeleton Signaling

Figure-14 Pathway analysis of genes modulated in response to VEEV infections in blood: Significantly modulated genes in neuroinvasion specific subset (a), non-neuroinvasion specific subset (b) and VEEV infection specific subset (c) described in Figure 6 were analyzed by Ingenuity Pathway analysis software. Some of the pathways with a p-value ≤ 0.05 (marked by the purple line) at least at one of the three time points are shown here.

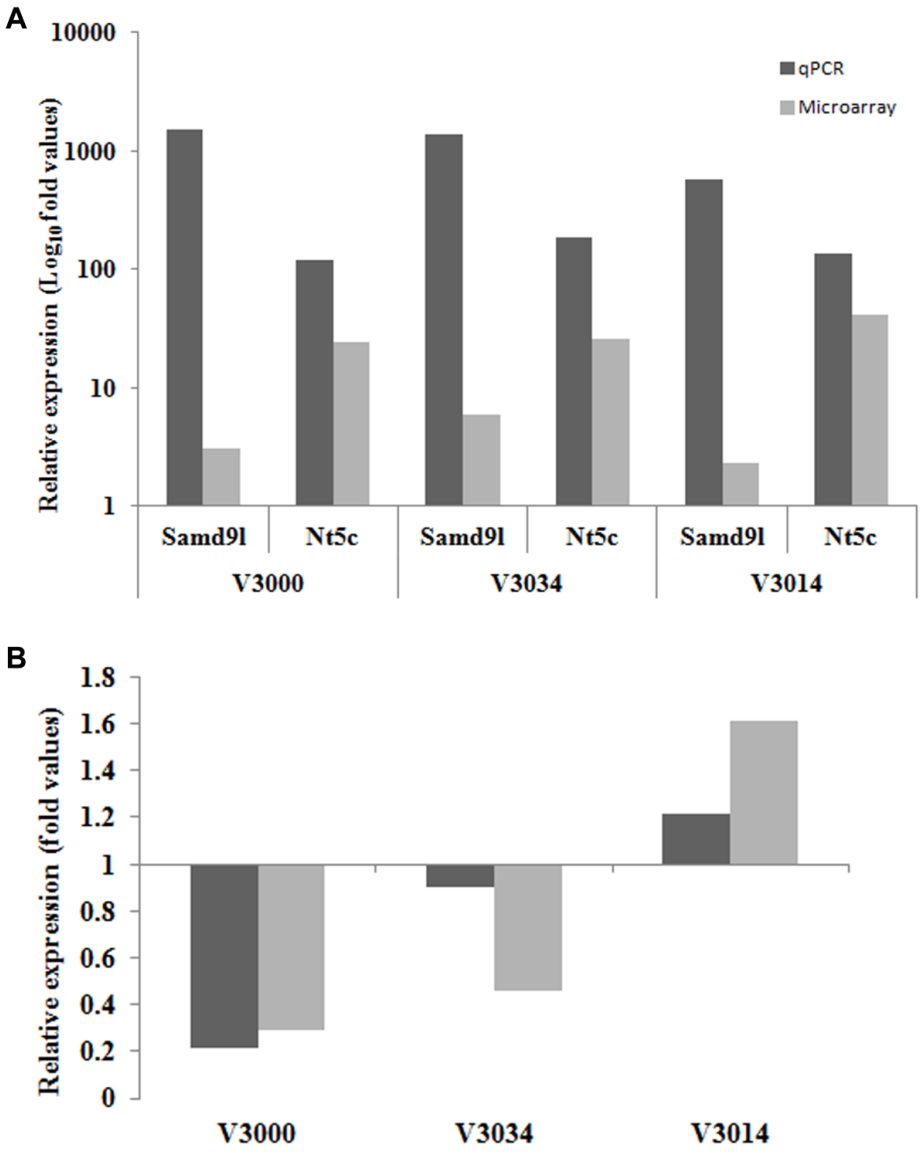


Figure-15 Confirmation of gene expression in blood by qRT-PCR: Quantitative RT-PCR analysis was performed to confirm the microarray results for randomly selected genes e.g., a) Samd9l and Nt5c at 24hr post V3000, V3034 and V3014 infection and b) IFNγR at 24hr and 72hr post V3000 and V3034, V3014 infections, respectively. Expression values of all the genes were normalized with the house keeping gene, GAPDH. The results here are representative of 2 biological replicates and 2 technical replicates for each biological replicate. Details of primer sets used are given in table-12.

Gene	Unigene ID	Forward Primer	Reverse Primer
IFNγR	Mm.549	tcaaaagagttccttatgtgccta	tacgaggacggagagctgtt
Nt5c2	Mm.40965	accgcacgtcagtggatttcaa	tcatggcagtgtgtgatctcct
Nsp4	GI: 25140293	cggtcaagggcatttacaac	tccaattcggtcctctccaaca
Samd9l	Mm.196013	tgttggtgtgcaagtcacca	acaagccctggcttcactgatt

Table-12 Primer sequences used for quantitative RT-PCR

B. Identification of host responses associated with neurovirulent and non-neurovirulent VEEV infection in mouse brain and spleen.

Abstract:

VEEV is a neurotropic virus and a lethal infection causes massive neuronal damage and inflammation in the brain leading to encephalitis in the host. However, in many instances the virus is cleared off by the host immune system before it can lead to the massive neuronal damage and thus preventing mortality. Identification of the host factors which play an important role during a virulent infection as well as those critical during a non-virulent infection can help in identifying potential drug development targets against VEEV infection.

Thus, in this study we have compared the host responses against a neurovirulent (V3000) and a partially neurovirulent (V3034) VEEV infection in mouse model. Whole genome microarrays were performed using the RNA isolated from spleen and brain tissues of these animals and the host factors exclusively modulated against each strain were identified. Pathway based analysis was performed to understand the overall signaling pathways modulated in each case. Comparison of the cytokines involved in each type of infection was also done.

The unique genes identified in the brain microarray can be explored further as host derived drug targets for VEEV therapeutics. The comparative host responses in brain and spleen tissues against neurovirulent and partially neurovirulent VEEV infections will help in improving our current understanding of the virus-host interactions during VEEV pathogenesis.

Results:

VEEV strains induce differential host gene expression kinetics post infection.

To investigate the differences in the kinetics of host gene responses against neurovirulent and non-neurovirulent strains of VEEV, we performed whole genome microarrays using total RNA isolated from the brain and spleen samples collected at 24hr, 48hr, 72hr and 96hr p.i. These time points were selected based on the replication kinetics of the V3000 and V3034 strains in the host spleen and brain. The presence of VEEV in the brain samples at each time point was measured before performing the microarrays by RT-PCR against NsP-4 gene of the VEEV genome which encodes for the viral RNA polymerase. V3000, the pathogenic strain of VEEV was detected in the brain as early as 48hr p.i. in all the animals. V3034 was however, detected in one sample at 48hr p.i. but was present in all brain samples by 96hr p.i. **(Figure-16)**.

RNA isolated from the brain and spleen samples from uninfected saline-treated mice were used as controls. Comparison of host genes modulated against V3000 and V3034 infections identified genes that were significantly modulated only against V3000 and V3034 infections. In order to understand the host responses exclusively involved during virulent and nonvirulent infections, we focused on the genes exclusively modulated against both these strains in the brain tissue (Figure-17). Maximum number of genes was differentially modulated against V3000 infection at 48hr p.i. (6307 genes were specifically modulated against V3000 infection; **Table-13**). In case of V3000 infection specific subset, number of differentially modulated genes decreased at 72 hr p.i. (2848 genes) and then increased marginally by 96hr p.i. (3153 genes). However, in case of V3034 infection, maximum number of genes was significantly modulated at 72 hr p.i. (3538 genes) that decreased marginally to 3007 genes by 96hr p.i. At 48hr p.i., 2837

genes were significantly modulated against V3034 infection in the brain. Unlike V3000 infection, no drastic change in the number of significantly modulated genes was observed against V3034 infection (Table-13). Some of these genes were randomly selected for quantitative RT-PCR based validation of the gene expression data (Figure-20, Table-12)

Pathway analysis of genes differentially modulated exclusively against the neurovirulent (V3000) and partially-neurovirulent (V3034) strains of VEEV.

Pathway analysis was performed on the gene exclusively modulated against V3000 and V3034 infections in brain and spleen. Very few pathways were found to be significantly modulated specifically against V3034 infection in contrast to V3000 infection which resulted in significant modulation of a much larger number of host signaling pathways (Figure-18 and 19). Some of the pathways were found to be commonly modulated among the two subsets e.g. chemokine signaling, IL-2 signaling, NRF-2 mediated oxidative stress and protein ubiquitination pathway. Some endocytic pathways like clathrin-mediated endocytosis signaling and virus entry via endocytic pathways, were also found commonly modulated against both the virus strains however, the level of significance and the kinetics was different. These pathways were modulated much more significantly against V3000 infection as well as were modulated earlier i.e. during 48hr and 72 hr p.i. against V3000. On the other hand, in case of V3034 infection, these pathways were marginally modulated during 72hr and 96hr p.i. (Figure-19). Similar differences were also observed in the kinetics of other significantly modulated pathways against V3000 and V3034 infections. Such a difference in the signaling response can also be a result of amount of virus replication in the host.

Some of the unique pathways involved in each subset were also identified. Some pathways were only modulated against V3000 infection e.g. activation of IRF by cytosolic PRRs, acute phase response signaling, B cell receptor signaling, calcium induced T lymphocyte apoptosis, CCR3 signaling in eosinophils, CXCR4 signaling, IL-3 signaling, PI3K signaling in B lymphocytes, role of NFAT regulation in immune response and T cell receptor signaling (Figure-18). On the other hand, pathways like CTLA4 signaling in cytotoxic T lymphocytes, EIF2 signaling, regulation of eIF4 and p70S6K signaling, granzyme A signaling and PTEN signaling were observed against V3034 infection alone. The pathways specifically modulated against V3000 infection present a very good subset for targeted drug development against virulent VEV infection.

Discussion:

VEEV enters into the CNS primarily through the olfactory neuroepithelium, via brain capillary endothelial cells and trigeminal nerve (Charles et al 1995, Ryzhikov et al 1995). In CNS, VEEV infects neurons and glial cells and causes subsequent cellular degeneration. This results in activation of several host defense pathways which can also have unfavorable effect on the host tissue pathology. Infection by virulent VEEV results in neuronal cell death, active gliosis and intense inflammatory response characterized by perivascular and interstitial mononuclear infiltrate (Jackson et al 1991, Grieder et al 1995, Schoneboom et al 1999, 2000, Steele et al 2006). However, in case of infection with non-virulent VEEV, virus is generally cleared off from the brain tissue and therefore does not result in any disease (Grieder et al 1995). It becomes very important to understand the difference in the type of host responses involved and differing during both virulent and non-virulent infections. Thus, in the present study, we

performed global gene expression analysis against V3000, a neurovirulent infection and V3034, a partially-neurovirulent infection in mouse model.

Although V3000 and V3034 differ only by a single amino acid in their genomic sequence, but their neuropathology is vastly different. V3000 is a fully lethal strain of VEEV causing 100% mortality whereas V3034 results in 30% mortality in mouse model (Grieder et al 1995). V3000 replicates to higher titers in the brain tissue which was also accompanied by a greater number of host genes with increase expression. V3000 infection not only resulted in much more number of differentially modulated genes but a greater fold change in the expression levels of host genes as compared to the partially-neurovirulent VEEV strain (V3034) in our studies. Even among the genes that were modulated against both V3000 and V3034, the extent of modulation was much higher in case of V3000 infection.

Similar observation was made with the results from pathway analysis on the gene exclusively modulated against V3000 and V3034 infections. V3000 modulated much higher number of host pathways as compared to V3034 infection. Some of the pathways commonly modulated among the two subsets were identified; however, the level of significance and the kinetics was different. These pathways were modulated much more significantly against V3000 infection during 48hr and 72 hr p.i. whereas in case of V3034 infection these pathways were modulated during 72hr and 96hr p.i. This difference in the signaling response can be due to delayed appearance of V3034 in the host brain (Grieder et al 1995) which was also shown by only one V3034 infected brain samples being positive for VEEV specific PCR at 48hr p.i. At the same time, the signaling pathways were also not as significantly modulated against V3034 infection during 96hr p.i. where the VEEV specific amplification was observed in all the V3034 and V3000 infected samples. This may be due to difference in the level of virus replication of

these strains in the host brain. Interestingly, endocytic pathways like clathrin-mediated endocytosis signaling and virus entry via endocytic pathways were commonly modulated against both the virus strains but to lesser significance against V3034 infection. This suggests that insufficient modulation of the endocytic pathway may contribute towards lower level of replication and thus efficient clearance of V3034 from host brain. Some of the unique pathways modulated against each strain were also identified. Further analysis of pathways specifically modulated against V3000 infection will help in identifying host-derived drug development targets against virulent VEEV infection.

V3000 and V3034 show very similar pathology in the peripheral system of the host during initial phase of VEEV infection. Therefore, host responses modulated at different time points against V3000 and V3034 infection in spleen during early time points (24hr, 48hr and 72hr p.i.) was also compared in order to identify any difference in the type of cytokine response in the peripheral system of the host against these strains (Tables- 14 and 15). A relatively larger number of cytokines were found to be significantly modulated against V3000 infection in comparison to V3034. Only IL10rb was found to be significantly modulated (downregulated) at 24hr p.i. against V3034 infection whereas several cytokines were found significantly modulated against V3000 infection. This indicates that although pathologically V3000 and V3034 replication is very similar in the peripheral system but V3034 fails to trigger a strong immune response by the host initially. This may be due to relatively lower level of virus titer during V3034 infection as compared to V3000 infection. Several host immune response genes like Ifitm6, IRF7, Igtp, Il2rg and Ccl2 were significantly upregulated against V3000 infection during 24 hr p.i. At the same time genes like Ccr6, Ilf3, Il6ra and Sigirr were significantly downregulated against V3000 infection during initial time points.

Taken together we observed a delayed and milder host response against V3034 infection in comparison to V3000 infection in the mouse model. We also identified some of the pathways exclusively modulated against V3000 and V3034 infections as well as others that were common. However, there was a significant difference between the extent and kinetics of modulation of the pathways common between the two types of VEEV infection. We also identified a much stronger cytokine response against V3000 infection in the spleen. These unique genes and pathways specifically modulated against the V3000 infection can be potential host derived drug targets for developing effective therapeutics against a neurovirulent VEEV infection.

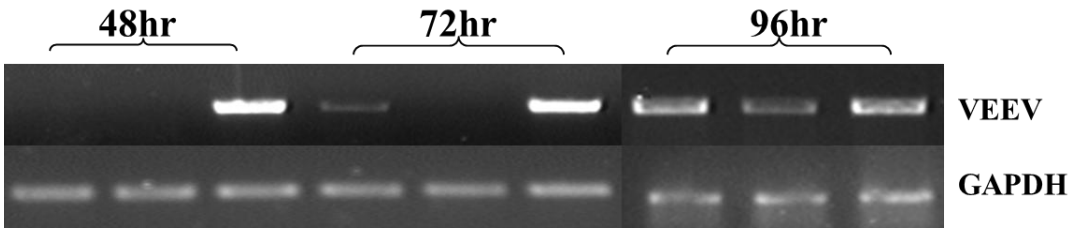


Figure-16 Nsp4 specific PCR on RNA from brain samples from mice infected with V3034:

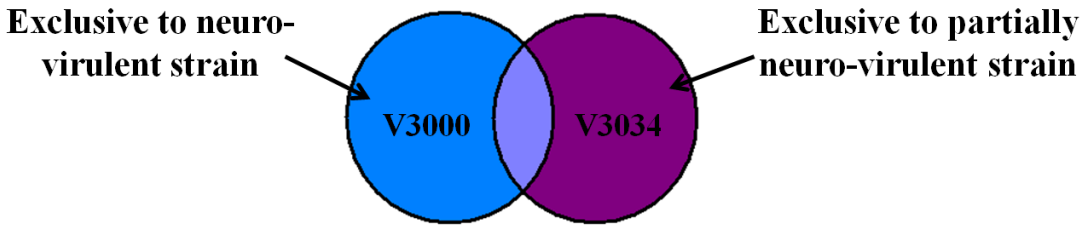


Figure-17 Analysis strategy followed to identify host factors associated with neurovirulence of VEEV:

Table-13 Number of genes significantly modulated at different time points against each strain of VEEV

VEEV strain	48hr p.i.	72hr p.i.	96hr p.i.
V3000	6307	2848	3153
V3034	2837	3538	3007

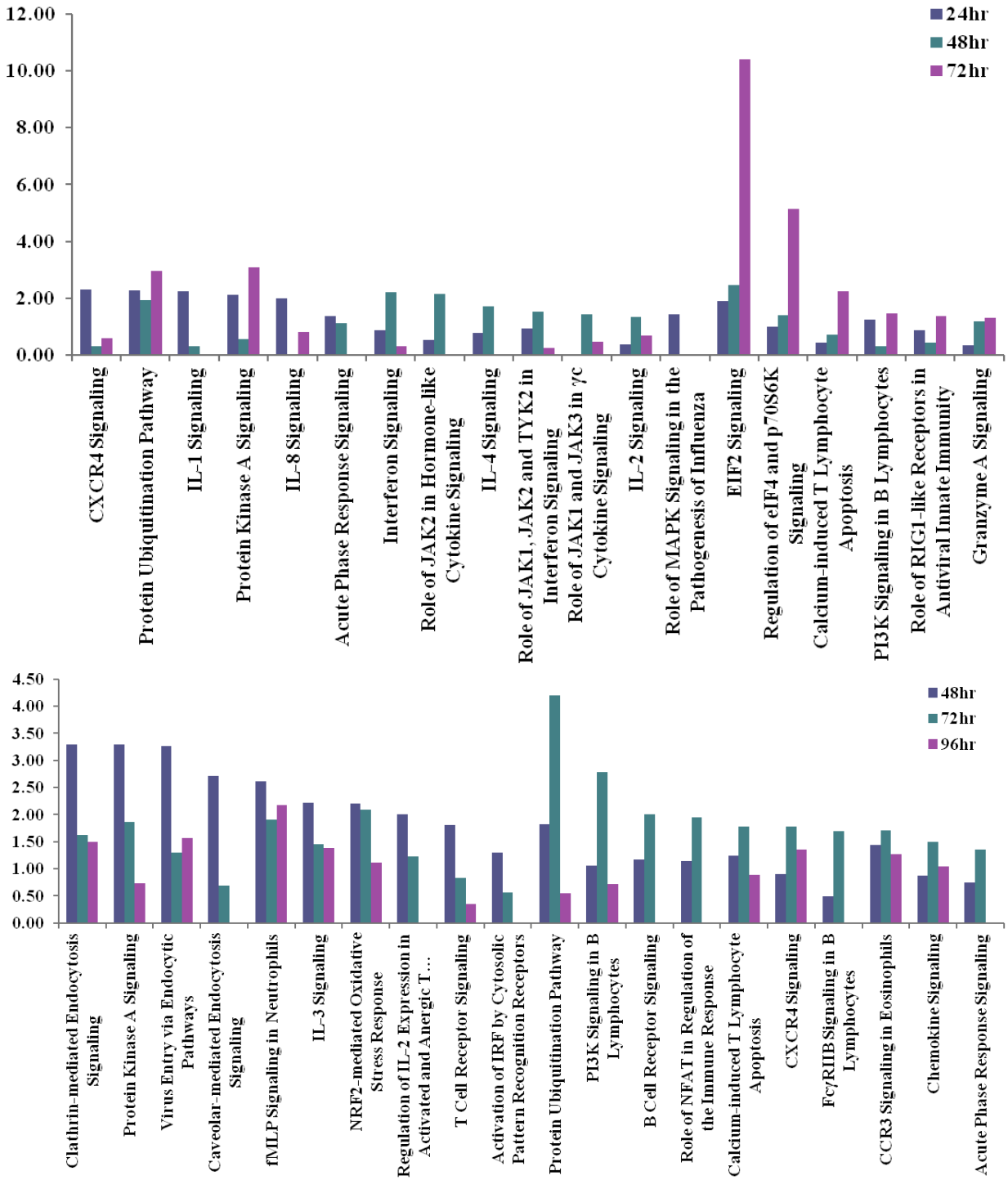


Figure-18 Host signaling responses associated with neurovirulent VEEV, V3000, infection in (a) spleen and (b) brain tissues:

Gene	UniGene	24hr		48hr		72hr	
		expression	SD	expression	SD	expression	SD
Igtp	Mm.33902	3.28797	0.02547				
Ifitm6	Mm.276440	2.83618	0.19191	2.43038	0.0147		
Ccl2	Mm.290320	2.76956	1.02438			2.71316	0.79159
Irf7	Mm.3233	1.83203	0.04438			0.60031	0.10231
Il2rg	Mm.2923	1.62002	0.56732	1.23249	0.32915	0.76775	0.47756
Xcl1	Mm.190	1.51028	0.07827	1.28419	0.12383		
Traf1	Mm.239514	1.19829	0.04253				
Cxcl1	Mm.21013	0.80593	0.05668			1.56533	0.35886
Traf7	Mm.275150	0.66669	0.20634				
Il23r	Mm.221227	0.62481	0.22653	0.6454	0.00066		
Tnfaip1	Mm.386774	-0.78804	0.1652	-0.75232	0.06858		
Il6ra	Mm.2856	-1.15108	0.17074			-1.03423	0.81209
Il17a	Mm.5419	-1.40762	0.01211			0.85961	0.46569
Sigirr	Mm.38017	-1.53704	0.18044	-0.80853	0.32915	-1.10592	0.10335
Ccr6	Mm.8007	-1.93518	0.56864			-1.62595	0.11101
Ilf3	Mm.440026	-4.72363	0.73538				
Socs1	Mm.130			1.47554	0.05211		
Ifit3	Mm.426079			1.04295	0.52239		
C1qtnf3	Mm.280158			0.96381	0.13436		
Tnfsf12	Mm.8983			0.85891	0.16846		
Ngfr	Mm.283893			0.7902	0.05793		
Il34	Mm.77697			0.72997	0.25791		
Cxcl16	Mm.425692			0.616	0.10205		
Ripk1	Mm.374799			-0.64026	0.0859	-0.59153	0.32532
Crlf3	Mm.272093			-1.36408	0.16937	-1.45022	0.44356
Ccl21a	Mm.458815			-1.55334	0.15223		
Ccl21c	Mm.407493			-1.58977	0.11986		
Ccl6	Mm.137			-2.83227	0.81105		
Ifi202b	Mm.218770					3.36668	0.51343
Il2	Mm.14190					1.04606	0.21236
Ilf2	Mm.227258					0.9749	0.19977
Ifna1	Mm.57127					0.7562	0.33352
Cklf	Mm.269219					0.70777	0.29345
Ngfrap1	Mm.90787					0.68374	0.11093
Il13ra1	Mm.24208					-1.01659	0.35267

Table-14 List of Inflammatory genes differentially modulated against V3000 infection in spleen.

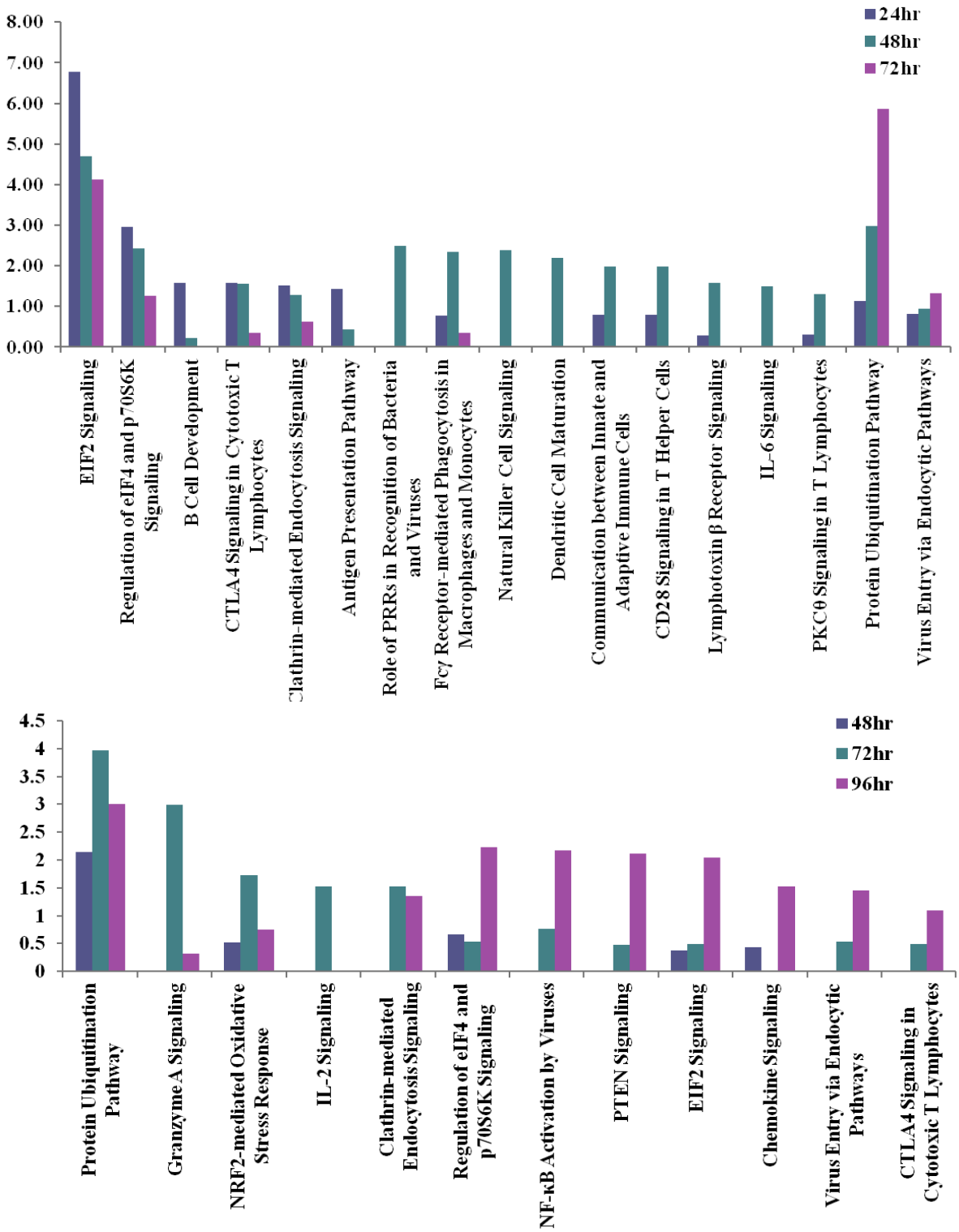
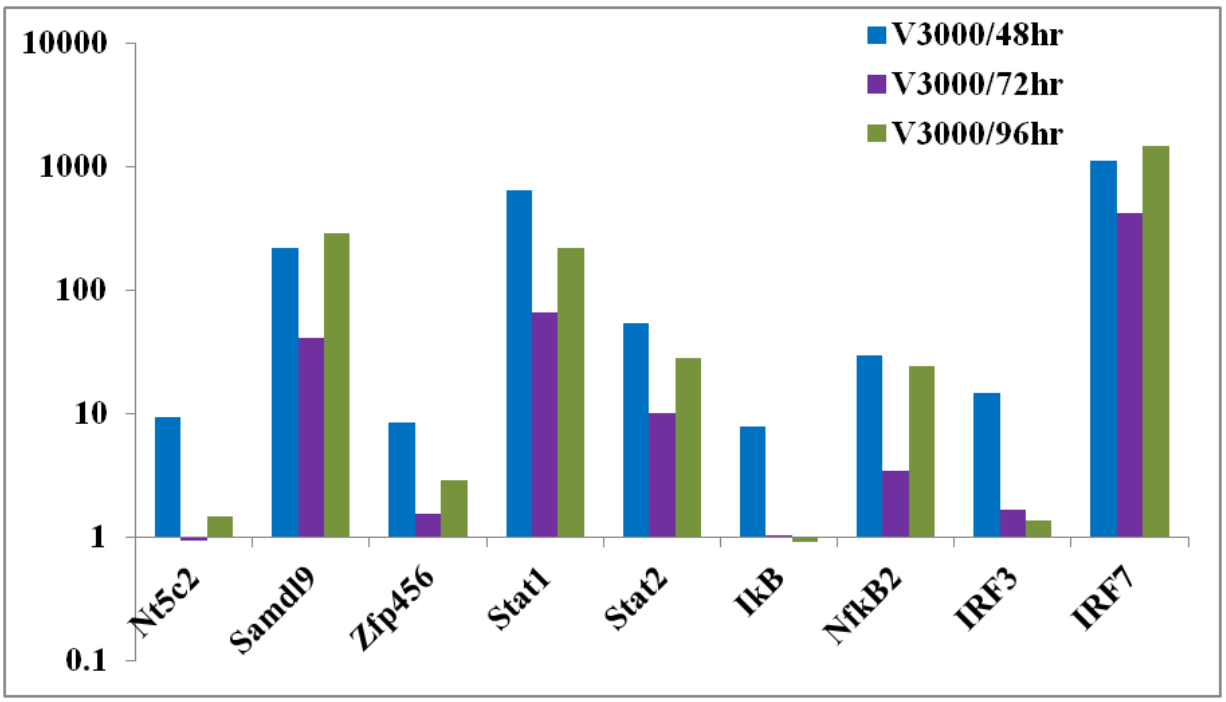


Figure-19 Host signaling responses associated with partially neurovirulent VEEV, V3034, infection in spleen and brain tissues.

Gene	Unigene	24hr		48hr		72hr	
		Expression	SD	Expression	SD	Expression	SD
Il10rb	Mm.4154	-0.949	0.259				
Irf1	Mm.105218			1.630	0.418	1.070	0.405
Il1b	Mm.222830			1.412	0.018		
Il18	Mm.1410			1.009	0.260	1.084	0.156
Ifi44	Mm.30756			0.958	0.060		
Ifih1	Mm.136224			0.884	0.003		
Il17f	Mm.222807			0.785	0.211		
Irf8	Mm.334861			0.745	0.119	0.828	0.276
Ccbp2	Mm.258105			0.607	0.231		
Ifna5	Mm.377089			0.586	0.043		
Ifnb1	Mm.1245			-0.596	0.109		
Tnfrsf1b	Mm.235328			-0.742	0.084		
Ccl5	Mm.284248					1.356	0.280

Table-15 List of Inflammatory genes differentially modulated against V3034 infection in spleen.



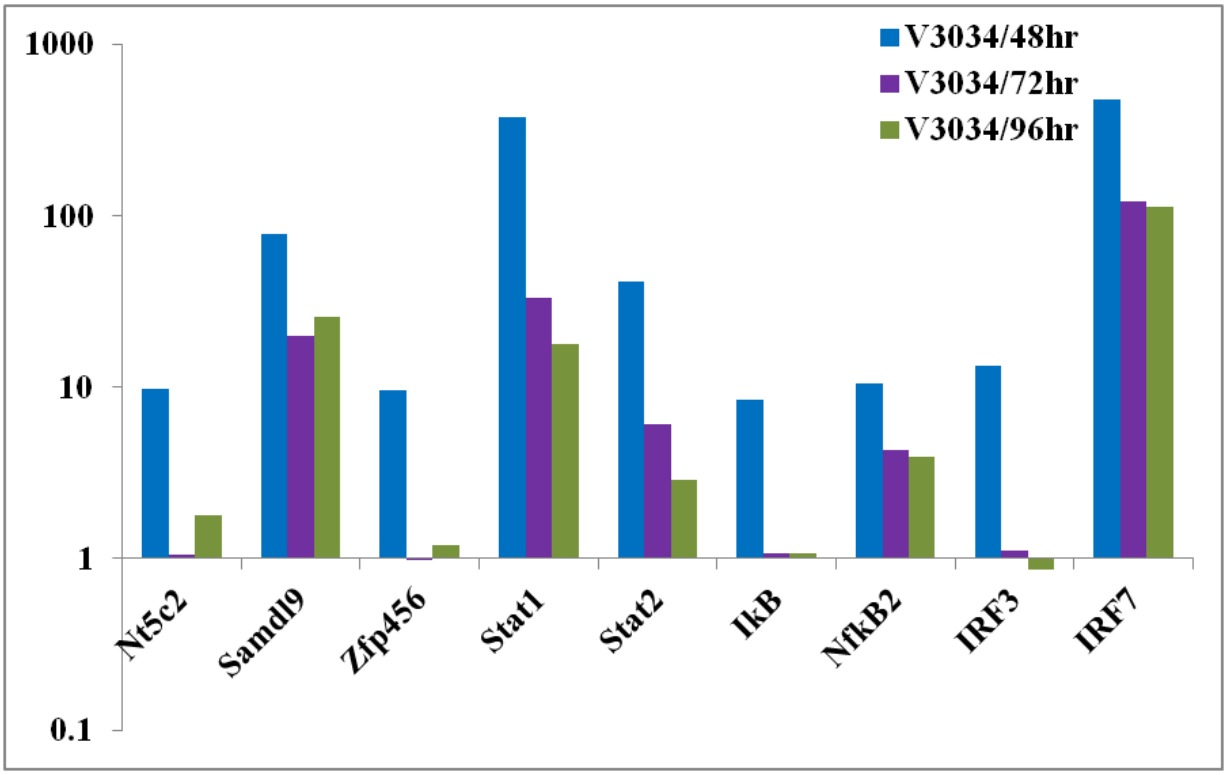


Figure-20 Quantitative RT-PCR based validation of some of the gens differentially modulated against (a) V3000 and (b) V3034 infection in brain tissue.

C. Studying the molecular mechanism(s) of enhanced pathogenesis of viruses in host brain by chemotherapeutic agents.

Abstract:

We have previously demonstrated that co-infection with malarial parasite and use of Chloroquine (CHL), Tunicamycin (TM) or exposure to environmental pollutants such as cadmium, manganese and lead can increase the pathogenesis and mortality associated with several viruses including Semliki Forest (SFV), Venezuelan equine encephalitis (VEEV) and encephalomyocarditis virus (EMCV) in mice. Increased mortality correlated with an early appearance as well as higher virus titer in the brain and enhanced brain pathology. To test if there is a common molecular mechanism(s) mediating the enhanced pathogenesis, we performed a comparative global gene expression analysis on TM–treated plus VEEV-infected mouse (VT-mice) brain with VEEV alone infected mouse (V-mice) brain samples. We observed that administration of TM enhanced the kinetics of VEEV induced encephalitis with an early expression of genes involved in VEEV pathogenesis. Functional analysis of TM treated VEEV infected brain gene expression data demonstrated an altered IFN signaling and upregulated antigen presentation pathway. A downregulation of IFN alpha receptor-1 (IFNAR1) gene expression was observed in TM treated VEEV infected brain samples. IFNAR1 expression was also down-regulated in the CHL treated VEEV infected and CHL, quinine, primaquine, sulfadoxine, TM or Cd treated SFV infected brains. Four miRNAs namely mmu-miR-15a, mmu-miR-18a, mmu-miR-199a-3p and mmu-miR-27b*, that are computationally predicted to

modulate IFNAR-1, were found significantly upregulated in VT-mice brains. Our data suggest that chemical agents and environmental pollutants may modulate the innate immune responses to virus infection by regulating the expression levels of IFN receptors. These findings are particularly important from a public health perspective, as it suggests that indiscriminate exposure/use of these agents especially antimalarials can impair the IFN regulated innate immune response and predispose the population to increased morbidity from viral infections. These studies are also very important, as VEEV is a biothreat pathogen, can be aerosolized, and is capable of causing highly virulent and fatal encephalitis in humans and equines and no vaccine or antiviral treatment is currently available.

Results:

TM treatment results in early modulation of antigen presentation and inflammatory genes induced by VEEV infection in mouse brain

TM treatment was previously shown to induce an early appearance of VEEV (by 48hr p.i.) in the VT-mice brain (Figure-25) (Steele et al 2006). Therefore, we compared the expression profile of VT-mice brain at 48hr p.i. with that of V-mice brains at 48, 72 and 96hr p.i., to identify the host determinants underlying the enhanced pathogenesis. The molecular function based Gene Ontology classification (GO) of the differentially expressed genes in the VT-mice brain at 48hr p.i. appeared very similar to the pattern that is representative of the V-mice at 96hr p.i. (**Figure-21)**. Interestingly, genes known to play a role in molecular functions like, leukocyte-mediated cytotoxicity, extracellular matrix (ECM) signaling, cell junction organization, activation of immune response and antigen presentation were significantly modulated in the VT-mice in

comparison to V-mice at 48hr p.i. Several pro-inflammatory chemokines like MIP1α, MIP1β, RANTES, and IP10 etc, known to up-regulate these processes, were found to be up-regulated in VT-mice brain samples. A canonical pathway analysis was also performed on these expression profiles (**Figure-22**). A significantly high representation of genes involved in CXCR4 pathway, fMLP signaling in neutrophils and NFAT pathway was observed. In addition, increased expression of infection-induced processes like calcium signaling, protein ubiquitination, i-proteasome and acute phase reactions were also observed. Most importantly, we observed a significantly higher representation of the 'IFN signaling' and the 'antigen presentation pathway' in the VT-mice brain samples. These observations possibly explain the enhanced immuno-pathology and cell death that was observed previously in the brain samples of these animals (Steele et al 2006). However, such a shift in the expression kinetics of these genes may also be a consequence of the increased level of virus in the brain.

TM alters the IFN response to VEEV infection

The IFN-signaling pathway is a critical innate immune response that is activated during any virus infection. Our initial analysis showed a significant upregulation of the IFN pathway in the VT-mice brain samples compared to V-mice. To characterize the IFN signaling pathway responses, the expression of IFN α/β and IFNγ in the brain of VT-mice was examined. Data in **Figure-23** shows a robust and enhanced expression of IFNs (Type I & II) in the VT-mice compared to the V-mice brain samples. Similarly, the classical pathogen recognizing receptors (PRR) e.g., MDA5, PKR and RIG1, which in turn trigger the expression of IFNs, were also found to be highly up-regulated (**Figure-23**).

Since, such a robust IFN production also failed to protect the host against viral infection; we further investigated if the observed increase in the expression of IFNs in these mice corresponded to the increase in the expression of IFN response genes. The antiviral genes like *Ifitm-1*, *2*, *3*, *5* and *7* (interferon induced trans-membrane protein), *Isg15*, *Isg20*, *Isg20lb* etc were not found to be highly up-regulated in the VT-mice brain samples. Similarly, the expression levels of *IRF9*, *Nfkb1* and *Stat1*, which are the transcriptional regulators of the IFN response genes and are known to play a role in the IFN signaling pathway, were also not found significantly up-regulated in VT-mice when compared to V-mice (**Figure-23**). However, a significantly higher expression of transcriptional repressor *IRF2* was observed.

TM, Cd, CHL and other antimalarials down-regulated IFNAR1 expression during VEEV infection

Since expression of several ISGs was not significantly modulated in VT-mice brain in spite of the significant upregulation of IFNs, the expression levels of IFNAR1 transcript was evaluated in VT-mice brain samples. There was an appreciable downregulation of the IFNAR1 expression in the VT-mice brain samples compared to V-mice (**Figure-24**). In order to evaluate if this was a common phenomenon resulting in the previously observed enhanced pathogenesis of SFV and VEEV infection during treatment with chemical compounds like Cd, TM, quinine, primaquine, sulfadoxine and CHL respectively, expression of IFNAR1 was also evaluated in these brain samples (Seth et al 2003 and 1999). A similar downregulation of IFNAR1 was observed in these samples as well (**Figure-24**). Thus, these results indicate that downregulation of IFNAR1 expression by TM, Cd, CHL and other antimalarials may attenuate the IFN signaling pathway that is a critical component of innate immune response to viruses.

TM treatment modulates expression of miRNAs involved in regulating IFN signaling

In order to identify the molecules regulating the expression of IFNAR1 in VT-mice, differential expression analysis of miRNAs in VT-mice and V-mice at 48hr p.i. was also carried out as described in methods. A correlation analysis between miRNA and mRNA expression values using IPA software identified 4 miRNAs namely miR-15a*, miR- 18a, 199a-3p and 27b* which may potentially interact with IFNAR1 and result in its downregulation (**Table 16**). These miRNAs were found upregulated in VT-mice brain samples in comparison to V-mice brain. Some of the other miRNAs were also identified which are suggested to interact with other members of IFN signaling (**Table-16**).

Discussion:

Virus-host interactions are complex and dynamic. A number of studies have underscored the importance of early host innate immune response in dictating the course, severity, and outcome of the infection (Ryman and Klimstra 2008, Konopka et al 2007). However, through our past studies, for the first time it was demonstrated that co-administration of prophylactic drugs such as TM, CHL, quinine, primaquine and other environmental toxins such as Cd, Mn and Pb with the virus as well as fungal infection alters the delicate balance of these immune responses and pathogen survival in favor of the pathogen thereby resulting in increased pathogenesis, morbidity and mortality (Maheshwari et al 1983, Singh et al 1987, Maheshwari et la 1988, Gupta et al 2002, Seth et al 2003 and Steele et al 2006). Recently, in a limited clinical trial, CHL treatment was shown to increase the severity of chikungunya virus induced symptoms

(De Lamballerie et al 2008). TM has also been shown to enhance mortality in mice infected with sublethal dose of *Neospora caninum*, which causes widespread abortions in cattle (Cao et al 2011). Thus, in order to understand the molecular mechanism of such an enhanced disease severity in presence of these drugs, we performed a whole genome transcript expression profile in the brain of VEEV infected mice in absence and presence of TM. TM was selected as chemical of choice as it has shown a significant and robust enhancement in disease severity of VEEV, EMCV and SFV infections (Steele et al 2006 and Maheshwari et al 1983). TM is also a functional substitute of corynetoxins produced by *Rathayibacter Toxicus*, which often contaminate the plants ingested by the cattle and sheep, and thus may lead to fatal hepatocerebral disorder in the affected animals (Takatsuki et al 1971, Vogel et al 1981).

Our results indicate that TM caused an enhanced and early expression of genes functionally classified as mediating the host immune responses. This early shift of the expression profile was concomitant with the early appearance, increased VEEV replication, and enhanced tissue pathology in brain, reported earlier from our laboratory (Steele et al 2006). Chemical agents like TM induce a systemic endoplasmic reticulum (ER) stress response resulting in an 'unfolded' or 'mis-folded' protein response (UPR) at the cellular level triggering the inflammatory response (Zhang and Kaufman 2008). Inflammation mediated secondary neuronal death and encephalitis has been shown to contribute to VEEV disease pathology and immuno-deficient mice have therefore been shown to survive longer than immuno-competent animals infected with VEEV (Sharma et al 2011 and Charles et al 2001, 1995). TM treatment was found to induce an early and increased expression of inflammatory response, antigen presentation and i-proteasome machinery genes during VEEV infection in the present study. Previously, we have also reported ultrastructural changes in the blood brain barrier with TM

treatment (Steele et al 2006) and recent studies suggest that BBB may play an important role in VEEV disease pathogenesis (Sharma et al 2011 and Schäfer et al 2011). Taken together, these observations suggest that increased inflammation at early time point due to mis-folded protein accumulation along with the BBB alterations caused by presence of TM might have resulted into increased viral load in the brain and worsening of the VEEV pathology.

IFN signaling is one of the most important primary host responses elicited against a virus infection. Exogenous IFN has been shown to be effective against different viral, fungal as well as plasmodium infection (Puri et al 1988, Maheshwari et al 1988 and 1990). However, we had observed that CHL or TM administration significantly impaired the protective antiviral effect of exogenously administered IFN or poly-I and poly-C against SFV or EMCV infections leading to increased lethality (Maheshwari et al 1983, 1991 and 1990). In the present study, we observed that TM treatment in presence of VEEV infection resulted in an altered IFN signaling within the host. Type-I and type-II IFN and PRRs such as RIG-1, MDA-5 and PKR were found to be highly upregulated in the brains of VT-mice. However, the IFN responsive genes like *Ifitm-1, 2, 3, 5* and *7*, *Isg15, Isg20* and *Isg20lb* as well as their transcriptional regulators like *IRF9*, *Nfkb1* and *Stat1* were not found to be similarly over expressed. Further analysis identified that this disruption in IFN signaling was due to downregulation of IFNAR-1 in these mice. MiRNA analysis also showed upregulation of miRNAs that are computationally predicted to modulate IFNAR-1, miR-15a*, miR- 18a, 199a-3p and 27b*, in VT-mice. Grieder et al (1999) had shown that IFN is critical for early protection against VEEV infection and mice deficient in IRF-1 and 2 as well as IFN$\alpha/\beta R^{-/-}$ mice were extremely susceptible to fatal VEEV infection along with altered VEEV tropism and disease progression. IFNAR1 is a very important component of IFN

pathway and thus modulation of its expression could possibly explain the incompetency of the IFN signaling to weaken the VEEV infection in presence of TM.

Other than TM, we had previously also observed similar worsening of viral disease in presence of several antimalarials like CHL, quinine, pyrimethamine, primaquine and sulfadoxine and environmental toxins like Cd, Mn and Pb as well (Seth et al 1999 and 2003). CHL has also been suggested to play an important role in the development of Burkitt's lymphoma by enhancing Epstein-Barr expression, intensify malaria infection in children in presence of Herpes zoster virus infection and cause dramatic increase in the trans-activation of Tat protein purified from HIV (Olweny et al 1977, Cook IF 1985, Frankel and Pabo 1988). Therefore, in the present study, we wanted to evaluate if a similar downexpression of IFNAR1 is also observed during treatment with antimalarials and environmental toxins and viral infections. We found that antimalarials like CHL, quinine, primaquine and sulfadoxine as well as environmental toxins e.g. Cd down-regulated the IFNAR-1 expression during viral infections. Similarly, cigarette smoke, rich in Cd levels has also been shown to exert a similar effect on IFNAR1 (Huang et al 2008). This may also explain the attenuation of prophylactic effect of IFN treatment against malaria parasite in combination with CHL administration as well as *Aspergillus fumigatus* infection in combination with TM during our previous studies (Maheshwari et al 1988 and 1991). Taken together, our results suggest that a downregulation of IFNAR1 by chemotherapeutic agents such as TM and CHL and environment pollutants such as Cd may lead to a transient state of immuno-suppression resulting in enhanced viral pathogenesis.

These results may suggest a possible connection between the increased spread of AIDS in malaria endemic areas where many of these antimalarials are widely used for the chemotherapy of malaria. The implication of the enhancement of virus replication by antimalarials is of

immense clinical relevance given the fact that the majority of the world population resides in areas endemic for malaria, where these antimalarials are widely used. In addition, downregulation of IFNAR1 levels among such individuals can in turn compromise the efficacy in clinical trials evaluating the antiviral capacity of IFN treatment. So, IFNAR1 expression can be used as a prognostic marker to screen whether or not a patient will be responsive to IFN therapy. Therefore implications of our findings are far reaching and add a new dimension to predisposition to viral illness which poses a major public health risk.

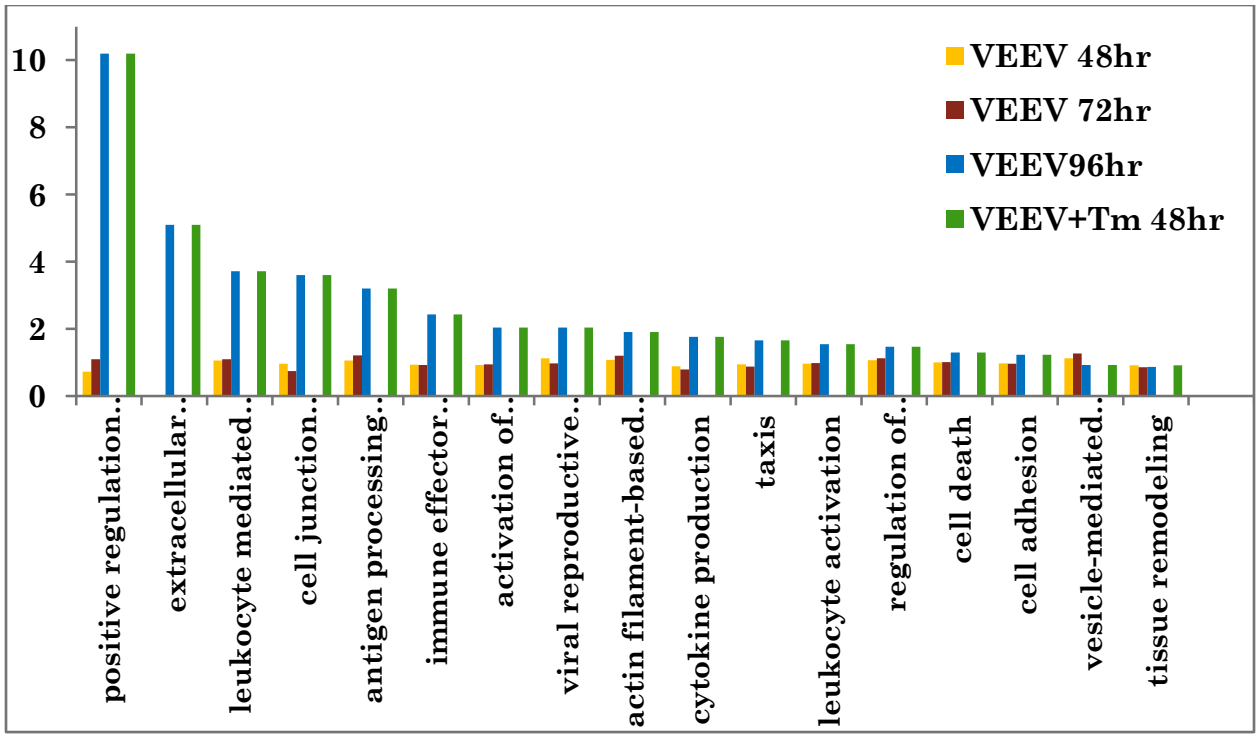


Figure-21 The functional annotation based on gene ontology classification of differential gene expression profiles against VEEV in presence and absence of TM: Molecular Function based gene ontology (GO) classification was performed for the differentially expressed genes against VEEVinfection alone at 48hr, 72hr and 96hr p.i. and against VEEV infection in presence of TM at 48hr p.i.

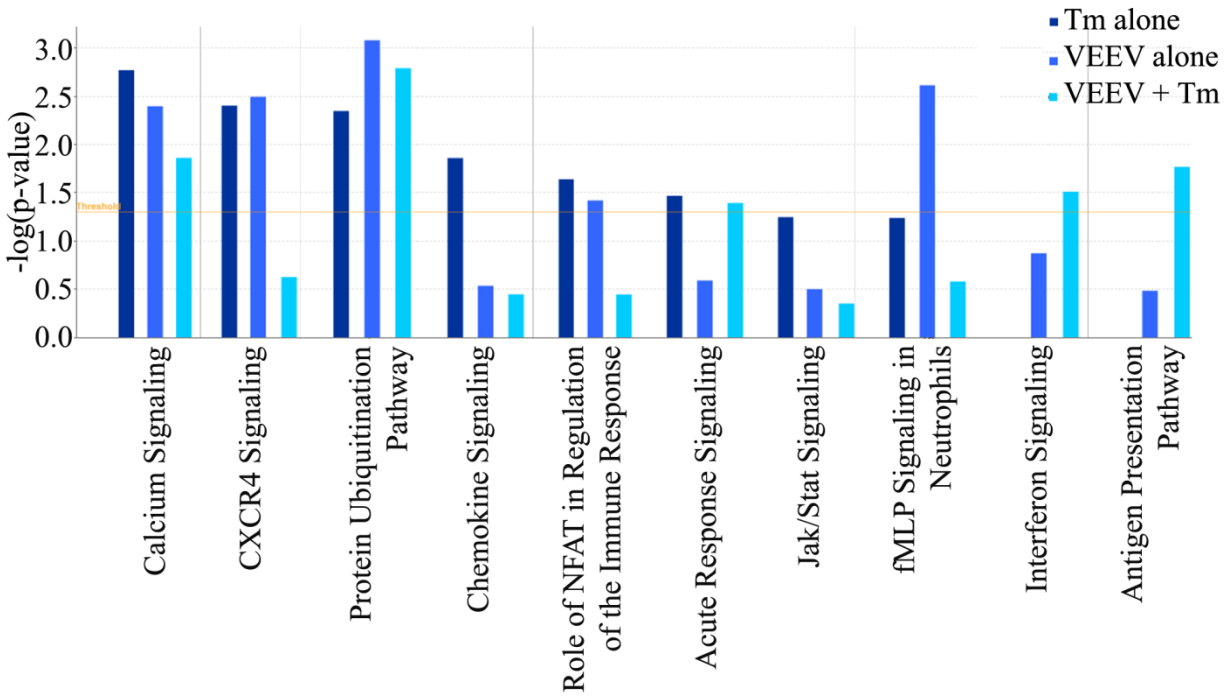
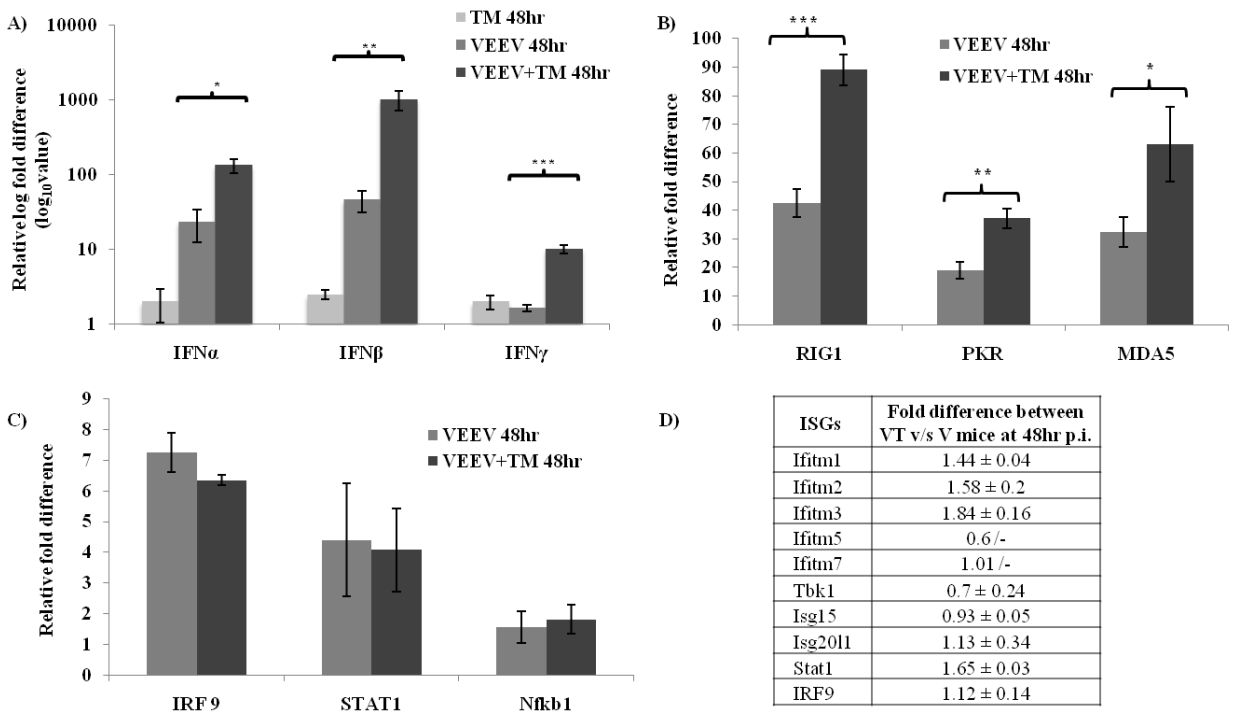


Figure-22 Comparison of the canonical pathways differentially modulated against TM alone, V-mice and VT-mice at 48hr p.i.: The canonical pathways that are involved in this analysis are displayed along the x-axis. As the default the y-axis displays the -log of p-value which is calculated by Fisher's exact test right-tailed. Taller bars are more significant than shorter bars. Functions are listed from most significant to least and the orange horizontal line denotes the cutoff for significance (p-value of 0.05)



D)

ISGs	Fold difference between VT v/s V mice at 48hr p.i.
Ifitm1	1.44 ± 0.04
Ifitm2	1.58 ± 0.2
Ifitm3	1.84 ± 0.16
Ifitm5	0.6/-
Ifitm7	1.01/-
Tbk1	0.7 ± 0.24
Isg15	0.93 ± 0.05
Isg20l1	1.13 ± 0.34
Stat1	1.65 ± 0.03
IRF9	1.12 ± 0.14

Figure-23 Differential expression of IFN and IFN response genes in V-mice and VT-mice: Total mRNA was isolated, and the amount of each transcript level was determined by real-time RT-PCR. Data were normalized to the amount of GAPDH mRNA and expressed as relative fold difference to control. Values expressed as means (±SD; * $p<0.05$, ** $p<0.005$ and *** $p<0.0005$): **A)** IFNα, IFNβ, IFNγ in mice treated with TM alone, V-mice and VT-mice. **B)** PKR, RIG1, MDA5 in V-mice and VT-mice. **C)** NFKB1, STAT1 and IRF9 in V-mice and VT-mice by quantitative RT-PCR. **D)** Microarray expression values for some of the IFN response genes in brain samples from VT-mice v/s V-mice at 48hr p.i. Two biological replicates were taken. Values are expressed as mean (n=2).

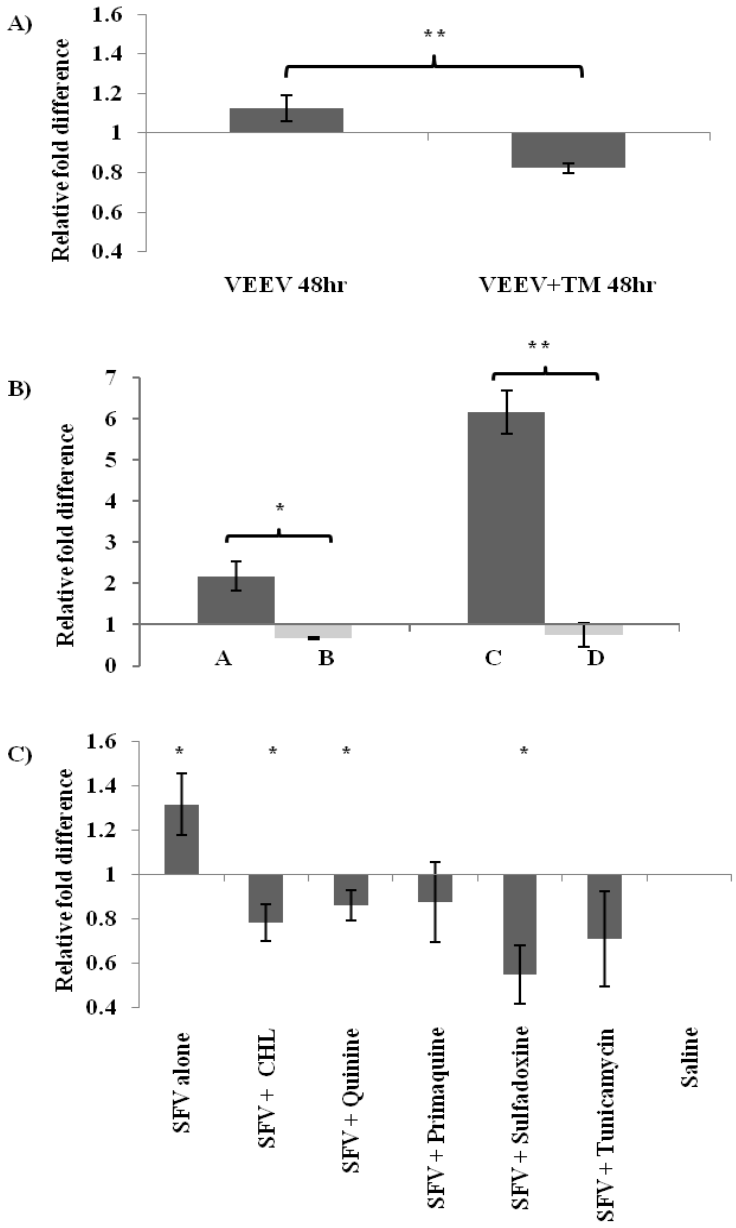


Figure-24 IFNAR-1 is down-regulated in presence of chemicals like TM, Cd, CHL and other antimalarials during virus infection: IFNAR1 expression levels were determined by real-time RT-PCR in the a) V-mice and VT-mice at 48hr p.i., b) A: SFV alone, B: SFV + CdCl2, C: VEEV alone, D: VEEV + CHL samples; c) SFV infected alone or treated with different anti-malarials or TM. Data was normalized against GAPDH and expressed as relative fold difference to control. Values expressed as means (±SD; * $p<0.05$ and ** $p<0.005$).

Gene name	Fold expression of the genes	MicroRNAs targeting different Interferon pathway genes	
		Up regulated	Down regulated
IFNAR1	-1.4	mmu-miR-15a* mmu-miR-18a mmu-miR-199a-3p mmu-miR-27b*	
OAS1	1.582		Mmu-miR-182, mmu-miR-411
PSMB8	1.673		mmu-miR-411, mmu-miR-125a-5p
STAT2	0.963		mmu-miR-188-5p mmu-miR-409-3p

Table-16 MicroRNAs targeting IFNAR-1 expression were up-regulated in VT-mice: Differential miRNA expression was evaluated in VT-mice and V-mice brain samples at 48hr post infection. The data obtained was analyzed using IPA software and correlated with the mRNA expression data. A correlation analysis identified several miRNA targeting the expression of different IFN signaling molecules. The fold expression of all the molecules in VT-mice in comparison to V-mice is shown in the table.

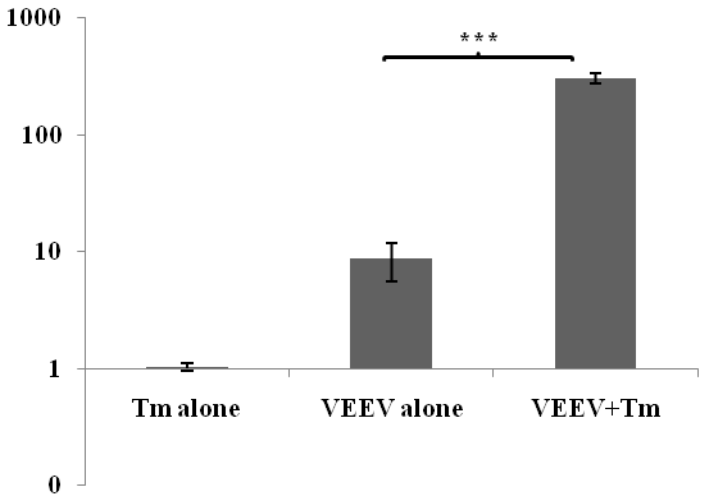


Figure-25 Presence of TM enhances the amount of virus replication in host: Relative quantitation of the VEEV nsP4 transcripts in animals treated with TM alone, V-mice and VT-mice at 48hr p.i. Total mRNA was isolated, and the amount of nsP4 transcript was determined by real-time RT-PCR. Data were normalized to the amount of GAPDH mRNA and expressed as log fold difference to control. Values expressed as means (±SD; *** $p<0.0005$)

Chapter-1.6: Conclusion and Future Directions

Neuroinvasion and neurovirulence play a very critical role during VEEV pathogenesis in the host. In this study, we have compared host responses against VEEV infection with varying degree of neuroinvasiveness and neurovirulence. Based on the observations from these studies, we have identified a VEEV specific gene signature which was significantly modulated in the blood during all the time points against all the three strains of VEEV. Since, these genes have never been correlated with any virus infection till now, upon further validation this gene signature can be developed as diagnostic biomarker signature against VEEV infection.

We also identified unique signaling pathways in the host against neuroinvasive and non-neuroinvasive VEEV infections (Figure-26). Significant modulation of these pathways can help in predicting the outcome of VEEV infection in a patient and thus help in better diagnosis and appropriate medical interventions. The neuroinvasion specific signaling responses can be further exploited to identify host derived-drug development targets against VEEV infection.

Similarly unique host signaling responses were also identified against V3000 and V3034 infection in the brain tissue. Some of the common pathways showing differential response patterns against the two strains of VEEV were also identified. Since, both these strains of VEEV replicate efficiently in the peripheral system but differ in their level of neurovirulence, the differential cytokine responses against V3000 and V3034 infection in the host spleen helped in identifying the differences at the molecular level against these strains. These results improve our current understanding of the host responses against a neurovirulent and a non-virulent VEEV infection.

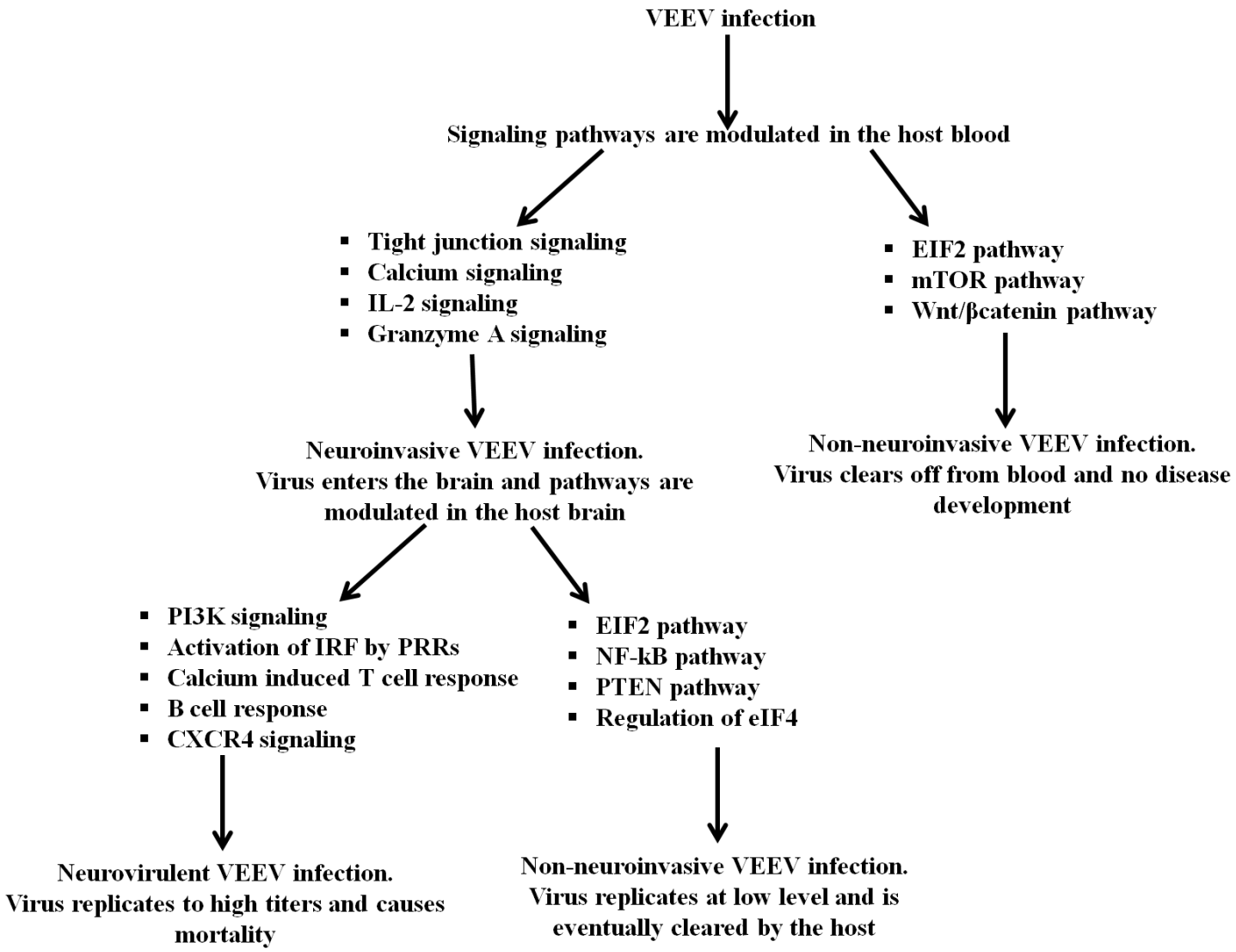


Figure-26 Proposed model of VEEV pathogenesis and host responses involved

In addition to identifying the host responses modulated against virulent and non-virulent virus infections, we also identified the host factors that are targeted during viral infections in presence of chemicals or environmental pollutants. Results from the VEEV+TM studies suggested that treatment with chemical drugs or contamination due to environmental pollutants can interfere with the IFN pathway by reducing the level of IFNAR1, which is the most important antiviral

response in our body (Figure-27). Thus, virus infection in presence of these compounds results in a more severe form of disease.

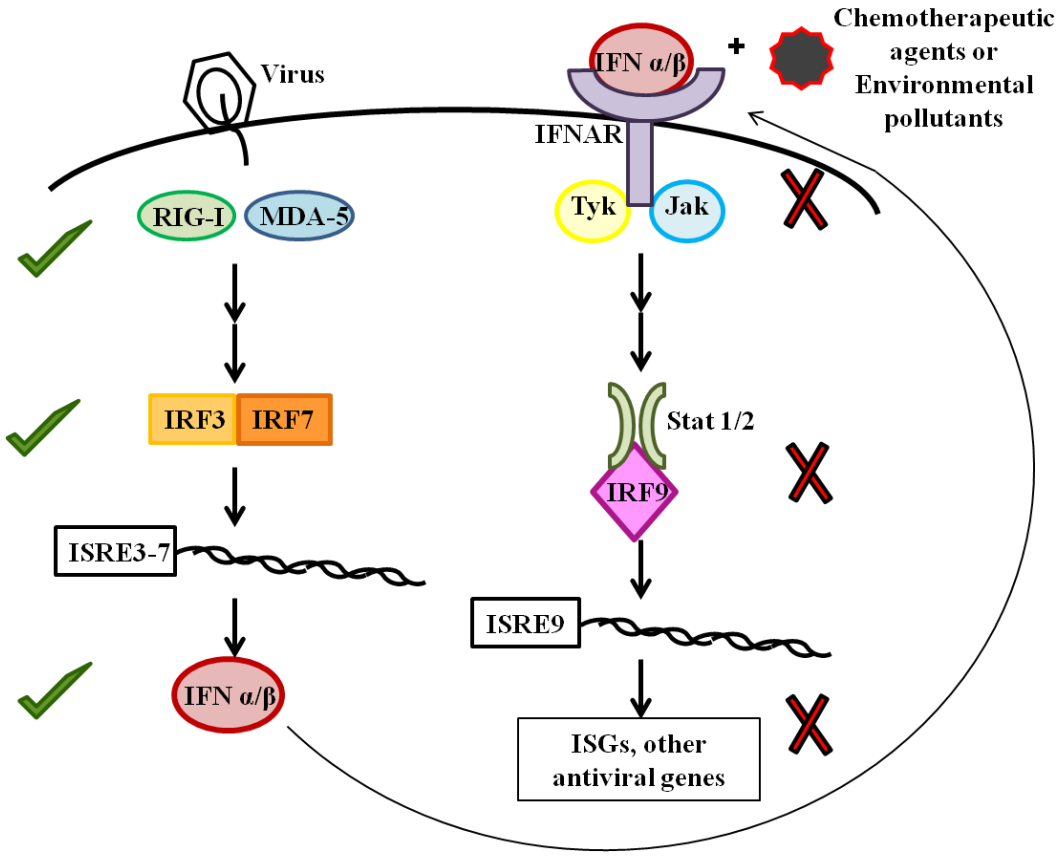


Figure-27 Proposed model of IFN pathway modulation in presence of chemotherapeutic agents like antimalarials, tunicamycin and environmental pollutants.

PART-2

Studying The Efficacy of Virus Inactivation Using Photoactive Hydrophobic Alkylating Azide, 1, 5 Iodonapthyl Azide and Their Evaluation as Vaccine Candidates

Chapter-2.1: Introduction

A. Types of Vaccines

Live attenuated vaccines

Live attenuated vaccines are prepared by growing the disease-causing organisms under special laboratory conditions that cause it to lose its virulence, or disease-causing properties, or by introducing genetic mutations. Upon administration in to the host, they replicate at a very low level and provide continuous antigenic stimulation over a long period of time. They produce both antibody-mediated and cell-mediated immunity and generally require only one boost, or additional dose. Live attenuated vaccines have been developed against several viruses including varicella, influenza, yellow fever, measles, rubella, and mumps. However, live attenuated vaccines require special handling and storage in order to maintain their potency and possess the inherent risk of inadequate attenuation or reverting back to the virulent form and thus cause disease upon immunization.

Inactivated Vaccines

One alternative to attenuated vaccines is inactivated vaccines which are prepared by using heat or chemicals such as formaldehyde or formalin. This destroys the pathogen's ability to replicate, but keeps it "intact" so that the immune system can still recognize it. Excessive treatment with chemicals can, however, destroy immunogenicity whereas insufficient treatment can leave infectious virus capable of causing disease.

Inactivated vaccines are relatively easy to store, stable and safer since they can't revert to a more virulent form but during several instances in past inadequate inactivation of vaccines with

formalin (e.g. poliovirus and VEEV) led to outbreak of disease in vaccines (reviewed by Brown F 1993). These vaccines also tend to provide a shorter length of protection than live vaccines, and are more likely to require boosters to create long-term immunity.

Virus like replicon particle (VRP) or DNA vaccines

Virus-like replicon particle (VRP) consists of a self-replicating RNA genome (replicon) which expresses the viral non structural genes (required for transcription and replication of viral genome) along with the other genes of interest (sequence for antigen of interest). Replicon RNA is transfected in to the host cell along with a helper plasmid containing the structural genes for generating a virus particle. Since, only the replicon RNA containing the packaging signal, the resulting virus particles only contain non-structural genes as its genome. Therefore, these virus-like particles cannot generate progeny virus particles when injected into the host yet trigger a strong immune response due to active replication of the gene of interest. VRPs have been used as a vaccine against SHIV, measles virus and ebola virus infections. This approach can also be used to express transgene antigens of interest using an efficiently replicating replicon particle and is known as a virus-vectored vaccine approach. This technique is of special importance due to its potential for generating multivalent vaccines (a vaccine targeting multiple viruses or strains of a virus). Some of the commonly used replicon particles include alphaviruses, vaccinia virus, sendai virus and lentiviruses.

Alternatively, the antigen specific genes can be incorporated into plasmid DNA sequences giving rise to DNA vaccines. DNA vaccines are relatively stable, easy to store and produce in large amounts via cost effective ways. However, it is only useful against protein based antigens and possesses a possibility of developing antibodies against the DNA itself.

Subunit vaccines

Subunit vaccines use only part of a target pathogen peptide sites encompassing the major antigenic sites of viral antigens. This method triggers an efficient immune response in the host, often with fewer side effects than might be caused by a vaccine made from the whole organism. However, coupling of the subunit vaccine to an immunogenic carrier protein or adjuvant is required to trigger a strong immune response. Purified subunit vaccines have been developed against *Streptococcus pneumoniae, Bacillus pertussis* and influenza virus.

B. V3526

Venezuelan equine encephalitis virus (VEEV) is a member of arbovirus group, family togaviridae in genus alphavirus. VEEV causes frequent outbreaks and is identified as an emerging pathogen. It is highly infectious in aerosol (Steele 1998) and was developed as a bioweapon and thus may likely be used in bioterrorism. VEEV is transmitted by mosquito in nature (Weaver et al 2004) and causes biphasic infection in horses and human (Grieder et al 1995, Charles et al 1995).

There is no licensed vaccine available for prophylaxis against VEEV. Live attenuated TC-83 vaccine for VEEV is under new- investigational drug status and is given to laboratory personal at –risk. Formaldehyde inactivated TC-83, known as C84, is used as a booster following immunization with live attenuated TC-83 vaccine (Pittman et l 1996). Another stably attenuated strain of VEEV is V3526 which has a deletion of the furine cleavage site between the E2 and E3 glycoprotein region present in the Trinidad donkey strain of VEEV. It has been tested for its efficacy as a vaccine candidate and shown to possess good immunogenicity (Pratt et al 2003; Rao et al 2004). Both TC-83 and V3526 have residual virulence in suckling mice.

C. Encephalomyocarditis virus (EMCV)

Encephalomyocarditis virus (EMCV) is a small (30 nm in diameter), non-enveloped particles with a single-stranded positive strand RNA genome, belonging to the *Picornaviridae* family of viruses. The picornaviruses constitute one of the largest and most important families of human and animal pathogens (Arnold et al 1988). Currently the family is divided into nine genera: *Enterovirus, Rhinovirus, Hepatovirus, Parechovirus, Cardiovirus, Aphthovirus, Erbovirus, Kobuvirus and Teschovirus*. The genus *Cardiovirus* comprises of two rodent pathogens, encephalomyocarditis virus (EMCV) and theiler's murine encephalomyocarditis virus (TMEV) (King et al 2000).

Genome:

EMCV genome consists of a single molecule of positive strand (infectious) RNA. The size of the genome is 7835 nucleotides (figure-28). A small virus encoded protein VPg, also called 3B, is covalently attached to the 5' end of the RNA. Interaction between RNA and capsid proteins is necessary for the stability of the virion structure.

5'-non coding region is long and highly structured and controls the genome replication and translation. It contains internal ribosome entry site (IRES) that facilitates direct translation of mRNA by internal ribosome binding. The entire genome is translated as a single polypeptide, which is proteolytically cleaved into precursor proteins P1, P2 and P3 (figure-28). These precursor proteins are further cleaved into the structural proteins VP1 to VP4 (from P1 precursor protein), and seven non-structural proteins 2A to 2C and 3A to 3D (from P2 and P3 precursor proteins respectively). Cardioviruses are acid stable (pH range 3-9) and therefore can pass through the low pH conditions in stomach before entering the intestine. It has a low buoyant

density (1.34) and a sedimentation coefficient of 160S (Vincent R. Racaniello, chapter 24, Picornaviridae: the viruses and their replication, Fields virology ed 5).

Capsid:

The structure of most picornaviruses except cardioviruses and aphthoviruses, have a canyon (a depression) on their surface which acts as a receptor for cell surface binding (Acharya et al., 1989). The capsid has an icosahedral symmetry and contains 12 pentagon-shaped pentamers consisting of 5 protomers each (giving 60 structural proteins). Each protomer is formed by one copy of the four structural proteins VP1 to VP4. The capsid surrounds a single-stranded infectious RNA genome (Arnold et al 1988). The amino-terminal (N-terminal) glycine of VP4 has a myristic acid covalently attached to it. In site-directed mutagenesis studies, myristoylation and myristate protein contacts were found to be necessary for pentamer formation, RNA encapsidation and virion stability. The VP4 protein has also been shown to be involved in uncoating and cell entry.

Pathogenesis:

EMCV was first isolated from non-human primates and then from pigs. Other animal species that can be infected with EMCV include pigs, rodents, cattle, elephants, raccoons, marsupials, and primates such as baboons, monkeys, chimpanzees, and humans. Rodents are the reservoir host and pigs are the primary domestic animal host. It is one of the major causes of fetal death and/or abortion in pregnant sows and acute necrotizing myocarditis in piglets etc (Yoon et al 2006).

Cardioviruses have been shown to induce demyelinating diseases, encephalitis, myocarditis, type 1 diabetes and multiple sclerosis-like symptoms after infection in rodents and

swine (Yoon et al 2006, Fauquet et al 2005). Different variants of EMCV have been isolated and characterized on the basis of disease symptoms in the host. These are: A-pregnancy related problems, B-non diabetogenic, D-diabetogenic, E-neurotropic, M-myocartitic.

Although, EMCV has rarely been recognized as the cause of human illness, studies indicate that EMCV can cause interspecies infections, making it an important zoonotic agent (Blinkova et al 2009, Pritchard et al 1992). Clinical signs in humans vary from mild febrile illness to severe encephalomyelitis. Recently, EMCV was isolated from febrile patients in Peru and 17% population was estimated to possess neutralizing antibodies against EMCV (Czechowicz et al 2011). Similarly, neutralizing antibodies against a new strain of cardioviruses, Saffold virus, were found in the human population of several countries in Europe, Africa, and Asia suggesting infection (Zoll et al 2009). Therefore, it is clearly evident that EMCV can successfully infect human beings. However, there is no treatment against EMCV at present; an inactivated vaccine is available in the United States. Other than this, the most effective method of controlling EMCV is generally to control the rodent population.

D. 1,5 iodonapthyl azide

1,5-iodonaphthyl-azide (INA), is a photo-inducible hydrophobic alkylating compound and can be radio-labeled with radioactive iodine (I_{125}) (Figure-29). When exposed to far UV radiations, INA binds to cysteine molecules of the peptides present in the hydrophobic pockets of the lipid bilayer (Hoppe et al 1983, Viard et al 2009, Raviv et al 1984, 1987, Kahane and Gitler 1978, Holowka et al 1981). INA was initially developed as a photolabel for lipid-embedded domains of membrane proteins in 1978. It strongly absorbs light at ≈310 nm, generating a highly reactive nitrene and binds to the lipids and proteins in the membrane bilayer (Gitler and Bercovici 1980). Irradiation is applied using UV-light at wavelengths that are not otherwise

harmful to the biological molecules (320-360 nm). Thus, the various components in the membrane bilayer are efficiently and selectively labeled with INA so that ectodomain of proteins or lipids outside the bilayer are not affected (Bercovici et al 1978, Raviv et al 1984).

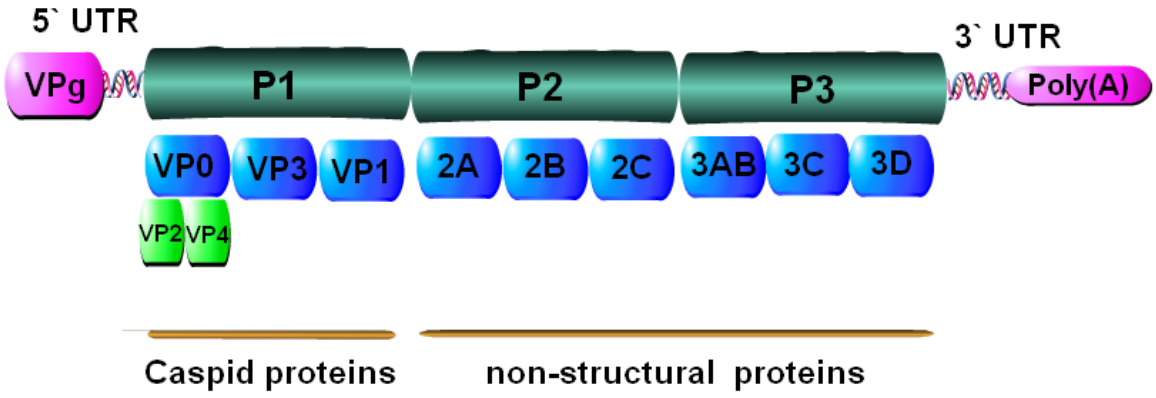


Figure-28 Genome structure of Picornaviruses

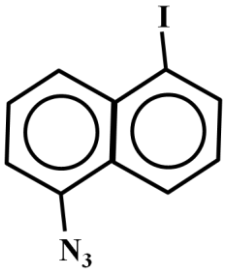


Figure-29 Structure of 1,5-iodonaphthyl-azide (INA).

Chapter-2.2: Review of Literature

A. Current vaccines against VEEV

Venezuelan equine encephalitis virus (VEEV) is a member of the arbovirus group, family *Togaviridae* in the genus alphavirus. VEEV causes frequent outbreaks and is identified as an emerging pathogen (Weaver et al 2004 a, b). VEEV is highly infectious in aerosol form (Steele et al 1998) and was developed as a bioweapon (Hawley et al 2001) and thus may likely be used in bioterrorism. VEEV is transmitted by mosquito in nature (Weaver et al 2004 a, b) and causes biphasic infection in horses and human (Bowen et al 1976, Grieder et al 1995, Charles et al 1995).

There is no licensed vaccine available for prophylaxis against VEEV. Live attenuated TC-83 vaccine for VEEV is under new- investigational drug status and is only given to laboratory personnel at –risk. Formaldehyde inactivated TC-83, known as C84, is used as a booster following immunization with live attenuated TC-83 vaccine (Pittman et al 1996). Another promising vaccine candidate for VEEV is an attenuated strain, V3526. V3526 has a deletion mutation at the furine cleavage site between the E2 and E3 glycoprotein region present in the Trinidad donkey strain of VEEV (Davis et al 1995). V3526 has an excellent immunogenic activity, but also causes febrile illness and low level neurotropism in non human primates (Pratt et al 2003, Reed et al 2005, Rao et al 2004, Fine et al 2007).

TC-83 demonstrates residual virulence in suckling mice (Paessler et al 2003) and V3526 has also been shown to have residual virulence in adult mice at high doses (Ludwig et al 2001). Infection of pregnant mice with TC-83 results in still birth, and decreased litter size and survival of new born (Spertzel et al 1972). TC-83 vaccine is also detrimental for the human fetus

(Casamassima et al 1987). Further limitations of TC-83 and up to an extent of C84 are short-lived immunity and several non-responders (Alevizatos et al 1967, Henderson et al 1971, Pittman et al 1996). Therefore, it is important to develop new safe vaccine candidates for VEEV.

B. Previous studies using INA:

1,5-iodonaphthyl-azide (INA), is a photo-inducible hydrophobic alkylating compound and when exposed to far UV radiations, INA binds to cysteine molecules of the peptides present in the hydrophobic pockets of the lipid bilayer (Hoppe et al 1983, Viard et al 2009). Previously, our laboratory and others have shown that treatment with INA in presence of UV-irradiation can successfully inactivate different enveloped viruses like Human Immunodeficiency Virus (HIV), Simian Immunodeficiency Virus (SIV), Venezuelan Equine Encephalitis Virus (VEEV), Ebola virus, and Influenza virus *in-vitro* and/or *in-vivo* and also acted as a good vaccine candidate (Raviv et al 2005, 2008, Sharma et al 2007, 2011, Warfield et al 2007).

Results from our previous study clearly showed that RNA isolated from INA-inactivated VEEV was non-infectious (Sharma et al 2011). Suggesting that INA-inactivation occurs by not just inactivating the viral envelop glycoproteins but somehow also by inactivating the viral genome. If this is true, then this inactivation strategy can be extended to all viruses irrespective of the presence or absence of the outer envelope. To date there have been no studies showing inactivation of non-enveloped viruses using INA.

In this study, we demonstrate that INA-inactivated VEEV is safe to administer in suckling mice and can induce a protective antibody response. Vaccination with INA inactivated-V3526 protected mice from virulent VEEV challenge through aerosol exposure. Use of an

adjuvant in the inactivated preparation improved protection from virulent VEEV challenge. We have also evaluated the extent of inactivation achieved in case of non-enveloped viruses like EMCV using this approach.

Chapter-2.3:
Research Gap, Hypothesis and Study Design

VEEV was first recognized in 1938 in Venezuela and even after more than 70 years of its discovery, there is no safe, licensed vaccine or antiviral therapy against VEEV infection. The current live attenuated TC-83 vaccine for VEEV is still under new- investigational drug status and is given to laboratory personal at –risk. Therefore, it is important to develop strategies and techniques to develop new vaccine candidates for VEEV.

V3526 is a vaccine strain of VEEV possessing good immunogenicity but has shown some adverse effects during human trials. Therefore, an additional layer inactivation of V3526 may help in producing a highly immunogenic and safe vaccine. Thus, in the present study we have evaluated the inactivation of V3526 using INA-inactivation method. Safety and protective efficacy of INA-inactivated V3526 was also evaluated in mouse model. Since VEEV is considered a bio-threat agent and can easily spread through aerosol, we have evaluated the protective efficacy of the vaccine candidate against aerosol challenge in the present study.

Additionally, during our previous studies we have shown that INA not only inactivates the virus particle but the NA isolated from INA-inactivated virus is no longer infectious. Thus, we propose that INA not only acts by binding to the surface proteins of the virus but also binds and inactivated the viral RNA genome. In order to evaluate this, we have inactivated a non-enveloped RNA virus, EMCV, using INA-inactivation method. We have also evaluated the safety and protective efficacy of INA-inactivated EMCV in mouse model.

Chapter-2.4: Material and Methods

Virus and animals:

Viruses: Molecularly cloned, virulent strain of VEEV, V3000 (Grider et al 1995) was used in the present study. V3526 strain of VEEV (Turell and Parker 2008) was prepared and purified at USAMRID, Frederick, MD. EMCV was originally obtained from C. Buckler (NIAID, NIH). L-11 cells (mouse fibroblast cells) were used to grow the virus and perform other assays. L-11 cells were grown in 1XMEM and infected with EMCV stock at a multiplicity of infection (MOI) of 1.0 in 37°C CO_2 incubator. After 1h virus suspension was removed and cells were washed with 1XDPBS. Fresh 1X Eagle's minimum essential medium (MEM) was added and the cells were incubated in 37°C CO_2 incubator for 24h. Supernatant was collected from the cells and cell debris was removed by centrifugation at 2000rpm for 10m at 4°C. The supernatant was passed through a 0.22µm filter and then centrifuged at 22000rpm for 3h at 4°C. The pellet obtained was carefully re-suspended in 1X Dulbecco's PBS (DPBS) and stored at -80°C. Virus titration was done on L-11 cells by standard plaque assay. Protein concentration of the stocks was determined by using the BCA protein assay kit by Thermo scientific Inc. according to the manufacturer's protocol.

Animals: 5-6 week old Swiss CD-1 mice and 1 day old CD-1 pups with mother were purchased from Charles River Laboratories, Wilmington, MA. Mice were housed in micro isolator cages and were provided with food and water *ad libitum* with a 12 hr light/dark cycle. All experiments with V3000 virus strain were carried out in bio-safety level 3 (BSL-3) facility at Uniformed Services University of The Health Sciences (USUHS, Bethesda, MD). All experiments were

conducted in accordance with the Guide for the Care and Use of Laboratory Animals (Committee on Care And Use of Laboratory Animals of The Institute of Laboratory Animal Resources, National Research Council, NIH Publication No. 86-23, revised 1996).

Inactivation of VEEV and EMCV with 1, 5 iodonaphthylazide (INA):

INA was kindly provided by Drs. Robert Blumenthal and Yossef Raviv, National Cancer Research Institute, NIH Frederick, MD under a material transfer agreement. VEEV was inactivated with INA as described earlier (Sharma et al 2007). Briefly, purified VEEV or EMCV stocks with known virus titer were resuspended in 1X DPBS at a protein concentration of 0.5mg/ml. The preparations were then passed through 30 gauge needle syringe in order to break any virus clogs in the suspension. Under reduced light conditions, INA was added to the virus suspension in less than 2.5µl volumes along the wall of an ultraclear 1.5 ml micro-centrifuge tube (GeneMate, Catalog No. C-3269-1, ISC Bioexpress, Kaysville, UT) to obtain final concentrations of 10µM, 30µM. 50µM, 100µM and 200µM. Preparations were immediately vortexed after each addition of INA. Samples were incubated for 30 min in the dark at room temperature and then centrifuged at 1000 rpm for 1 min to remove any precipitated INA. Supernatant was transferred to new tube and glutathione was added to V3526 preparations to obtain a final concentration of 20µM in order to quench any unbound INA present in the solution. Virus suspension was vortexed and irradiated using 100 W mercury UV lamp as described before (Sharma et al 2007). Briefly, a clear glass plate was placed immediately in front of the lamp (to filter lower UV wavelengths of light) and water jacket (used as a heat filter) was placed approximately 5cm apart from the glass plate (Figure-30). Finally, samples were placed approximately 10cm away from the UV lamp in such a way that samples are completely

illuminated with the light passing through the water jacket. Virus suspension was irradiated twice for 90sec and once for 120sec followed by vortexing each time. Thereafter, full light conditions were used and samples were stored at −80°C. Virus titers were back calculated and dilutions were made accordingly for testing the infectivity of INA-inactivated VEEV *in vitro* and *in vivo*.

Treatment groups and controls :

Based on the previous study with INA-inactivation of the virulent strain of VEEV (Sharma et al 2007), V3000, the following control and test groups were taken. For V3526 experiments 1.) Uninfected saline only control; 2.) V3526; and 3.) V3526 + INA (100 μM) + irradiation (INA-inactivated V3526). Other controls such as Virus + irradiation, Virus + (dimethyl sulphoxide) DMSO, Virus + DMSO + irradiation, and Virus + INA have been shown to have no effect on VEEV infectivity (Sharma et al 2007).

To determine the effect of INA treatment procedure and irradiation alone on EMCV, following test groups and controls were prepared and studied: PBS only **(UN),** EMCV only **(E),** EMCV plus irradiation **(Ei),** EMCV plus 1% DMSO **(ED),** EMCV plus DMSO plus irradiation **(EDi),** EMCV plus INA (200 μM) only **(EI_{200}),** EMCV plus INA (200 μM) plus irradiation **($EI_{200}i$),** EMCV plus INA (100 μM) only **(EI_{100}),** EMCV plus INA (100 μM) plus irradiation **($EI_{100}i$),** EMCV plus INA (50 μM) only **(EI_{50}),** EMCV plus INA (50 μM) plus irradiation **($EI_{50}i$),** EMCV plus INA (30 μM) only **(EI_{30}),** EMCV plus INA (30 μM) plus irradiation **($EI_{30}i$),** EMCV plus INA (10 μM) only **(EI_{10})** and EMCV plus INA (10 μM) plus irradiation **($EI_{10}i$).** Since INA was dissolved in DMSO and highest concentration of DMSO achieved was 1.0% in the 200 μM INA treated sample, so 1.0% DMSO was used in controls.

Determination of cytopathic effect (CPE assay) :

Vero cells were plated in 24-well tissue culture plates and infected with V3526 or INA-inactivated V3526 at a multiplicity of infection (MOI) of 1.0 and incubated at 37°C and 5% CO_2 incubator. Virus was removed after 1hr incubation and fresh media was added to the cells after washing once with 1X Dulbecco's Phosphate Buffered Saline (DPBS) (GIBCO, Invitrogen Corporation, Carlsbad, CA). Cells were observed for cytopathic effect such as rounding and sloughing off from the surface. At 72hr post-infection (p.i.) cell supernatants were collected from the wells and cells were fixed and stained with 0.1% crystal violet (CV) and 2% neutral buffered formaline (NBF) solution, for 10 min at room temperature (RT). L-cells were used for EMCV studies using the protocol described above.

Virus titeration by plaque assay:

Virus titers in the brain of suckling mice were determined by standard plaque assay. 20% (weight/volume) brain samples were prepared by homogenizing brain tissue in 1X PBS supplemented with 0.1% bovine serum. Nearly confluent monolayer of Vero cells in 60mm culture dishes were incubated with serial dilutions of the samples for 1hr at 37°C and 5% CO_2 incubator and then were rinsed once with 1X DPBS. Agarose overlay (1% agarose, 1X MEM, 6% NCS and 0.5% penicillin-streptomycin) was poured over the cells and plates were incubated at 37°C and 5% CO_2 incubator. Plaques were visualized and counted at 96hr p.i by fixing and staining the cells with 2% NBF and 0.1% CV solution for 10min at RT.

50% Tissue Culture Infectivity Dose ($TCID_{50}$) determination:

Virus titer was determined in the cell supernatant collected from infectivity assay as a 50% tissue culture infective dose ($TCID_{50}$) for all samples. L-11 cells were plated in 96-well plates and infected with several serial dilutions of the cell supernatant. After 72hr plates were stained with 0.1% crystal violet and calculations were done based on the observations.

Total RNA isolation and RT-PCR:

RNA was isolated from the cells infected with control and test inactivated virus groups by using the Viral RNA/DNA purification kit from Invitrogen Inc. RNA was also isolated from cells 12-18 hours post infection with control and test virus groups. RNA isolation from cells was done using the TriZol kit (Invitrogen) according to the manufacturer's protocol. Similarly total RNA was also isolated from the brains of V3526 and INA-inactivated V3526 infected mice using the TriZol reagent (Invitrogen) according to the manufacturer's protocol.

cDNA was made from the total RNA by using the SuperScript II First-Strand Synthesis kit (Invitrogen Inc., Carlsbad, CA) according to manufacturer's protocol. Briefly, 1ug of total RNA in nuclease free water was incubated with oligodT primers and dNTPs at 65°C for 5 min and then transferred to ice for 2min. Thereafter, 50units of enzyme, 2µl of 0.1M DTT, 4µl of 25mM $MgCl_2$, 1ul of RNase out and 2ul of 10x buffer were added to make 20µl of final reaction volume. Reaction mixture was incubated at 42°C for 50 min and 75°C for 15min. Samples were kept on ice for 2min and 1µl of RNaseH was added followed by an incubation at 37°C for 20 min.

V3526 specific PCR for nsP4 gene (virus RNA dependent RNA polymerase) and EMCV specific PCR for 3D gene (virus RNA dependent RNA polymerase) were done to evaluate the virus infection. GAPDH served as a house keeping gene. Primer sequences used for nsP4 gene

were: Forward- 5'GCTAACAGAAGCAGATACCAG3'; Reverse- 5'GCAGCCGAATCCAATACGGGC3'. Primer sequences used for 3D gene were: Forward- 5' TCCCGTTTGCGGCAGAAAGATT 3'; Reverse- 5' AAGCGGAACATTGCCACCGAAT 3'. Primer sequences used for GAPDH gene were: Forward- 5'CCATCACCATCTTCCAGGAGCGAG3'; Reverse- 5'CACAGTCTTCTGGGTGGCAGTGAT3'.

Following PCR cycle was used: initial denaturation at 95^0C for 5min, followed by 25 cycles of denaturation at 95^0C for 30sec, annealing at 60^0C for 45 sec, and extension at 72^0C for 30sec. A final extension was done at 72^0C/5 min.

Immunofluorescence for VEEV antigen:

VEEV antigen specific immunofluorescence was done as described before (Sharma et al, 2007). Briefly, slides were rinsed thrice with 1X PBS for 5 min each and non specific binding was blocked using 1% BSA for 1hr at room temperature. Excess BSA was removed and slides were incubated with 1:1000 diluted polyclonal rabbit anti-VEEV antibody (kindly provided by Dr Franziska B Grieder, USUHS, Bethesda, MD) for 1 hr at 37^0C. Slides were then rinsed thrice with 1X PBS for 5 min each and incubated with FITC conjugated goat anti-rabbit IgG (1:1000) for 30min at 37^0C. Slides were washed thrice with 1X PBS for 5 min each and mounted with vectashield mounting medium containing DAPI (Vector laboratories, Inc. Burlingame, CA) and were observed under fluorescence microscope.

Electron microscopy evaluation of virus structural integrity after inactivation:

Two hundred mesh gold grids (Electron Microscopy Sciences, Hatfield, PA) were glow discharged for 15 min in a vacuum evaporator and samples were placed on the grids for 10 min at RT and grids were fixed in 4% electron microscopy grade paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 10 min at RT. Grids were then rinsed with distilled water and negative staining was done with 10 µl of 1% uranyl acetate (UA) for 90 s. Observations were made under Philips CM100 electron microscope operating at 80 kV.

Evaluation of virus genome for infectivity by transfection:

RNA genome was isolated from INA-inactivated and virulent virus preparations using PureLink viral RNA/DNA mini kit (Invitrogen, Carlsbad, CA) as per manufacturer's protocol. RNA was eluted into a final volume of 20µl. RNA from two samples was quantitated using Beckman 640 spectrophotometer (Beckman instruments Inc., Columbia, MD, USA). 100ng of RNA was mixed with siPORT Amine transfection reagent (Applied Biosystems/Ambion, Austin, TX) and transfection was done as per manufacture's protocol. Briefly, RNA and transfection reagent mixture was incubated at room temperature for 20 min. BHK cells were resuspended in fresh MEM medium and mixed with the RNA containing transfection reagent and plated in 8-well chamber slides. After 12 hr, transfection reagent mix was removed and replaced with fresh MEM media. Cells were incubated for 48 hr and then fixed with chilled Acetone:Methanol solution in the ratio of 1:1 for 10 min. Slides were then stored at -20^0C until stained for VEEV antigen. This experiment was repeated with three biological replicates. For EMCV studies, due to absence of suitable antibodies against EMCV, active replication of EMCV was evaluated by performing EMCV specific RT-PCR on cellular RNA and virus titer determination in cell supernatant by plaque assay as described.

Residual virulence evaluation in suckling mice:

For the entire *in-vivo* study, stock virus suspension was diluted to desired working concentration in 1X DPBS. Groups of 8 and 10 mice (3 days old) were infected i.c. with 10,000 pfu of V3526 and INA-inactivated V3526 respectively in a final volume of 20µl. Control mice (n=2) were similarly injected with 1X Phosphate Buffered Saline (PBS). Animals were observed for a period of 2 weeks for developing any clinical signs of disease and infection. Brain tissues were collected from all mice at the end of two weeks observation period and were stored at -80°C.

Enzyme-Linked ImmunoSorbent Assay for Detecting Anti-EMCV Antibody in Serum:

Blood was allowed to stand on ice for 30 min and was centrifuged at high speed for 30 min. Clarified top aqueous layer of serum was then collected and immediately stored at -80^{0}C. For Enzyme-Linked ImmunoSorbent Assay (ELISA), 96 well Immulon 4HBX ultra- high binding polystyrene microtiter plates (Thermo Electron Corp., Milford, MA) were coated with EMCV at a protein concentration of 5ug each well in 50µl volume at 4^{0}C for overnight. Virus suspension was then removed and plates were blocked with 50µl of 1% Bovine Serum Albumin (BSA) per well at 4^{0}C for overnight. Blocking agent was then removed and 50µl serially diluted serum (1:50, 1: 250, 1:625 and 1:1250) was added to each well in triplicates. Plates were then incubated for 4 hr at 37^{0}C followed by washing twice, first with 0.05% Tween 20 in 1X PBS and then with distilled water. 50ul of alkaline phosphatase conjugated goat anti-mouse IgG (secondary antibody) (1:1000) was added to each well and incubated at 37^{0}C for 1hr. Plates were again washed twice with 0.05% Tween 20 in 1X PBS first and then with distilled water. 50µl of

5mg/ml of phosphatase substrate was then added in each well and color was allowed to develop for 10min at 37^0C. Plates were read at 405nm wavelength using ELISA reader (Bio-Rad Laboratories, Inc. Hercules, CA 94547).

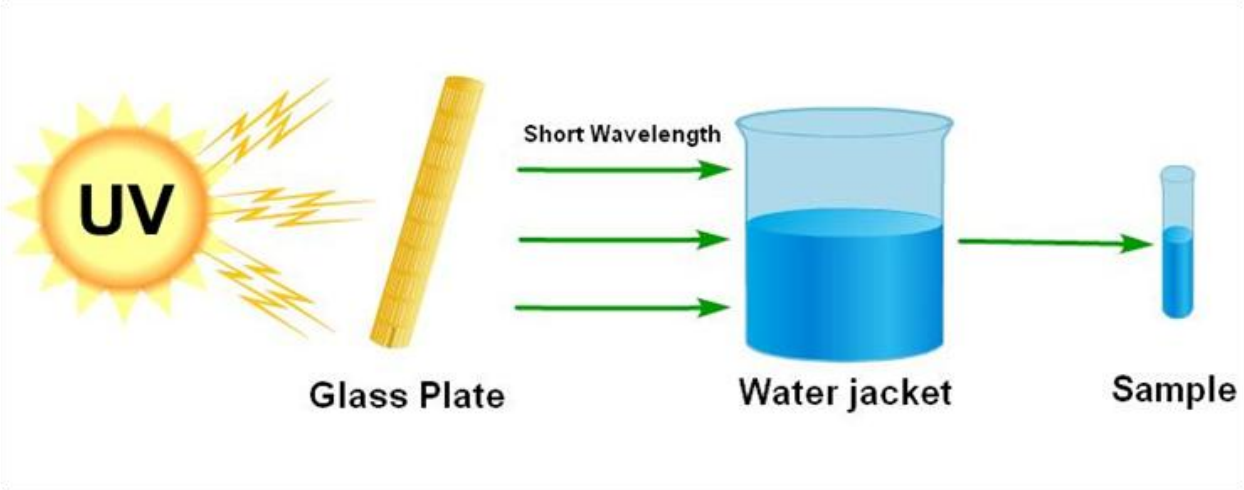


Figure-30 Experimental set up for INA-inactivation using UV-irradiation.

Chapter-2.5: Results and Discussions

A. Inactivation of V3526, an enveloped virus

Abstract:

VEEV is a human pathogen causing mortality in almost 5% cases. But no licensed vaccine or therapeutics are available against VEEV infection at present. The vaccine strains of VEEV, TC-83 and V3526, have inherent residual virulence. These strains cause mortality in the suckling mice and also replicate in the brains of adult mice although to lower levels as compared to the wild type VEEV. INA has been utilized to successfully inactivate several enveloped viruses in past including the wild type VEEV (V3000). In this study, the INA-inactivation strategy was utilized to inactivate V3526, an attenuated vaccine strain of VEEV. V3526 in spite of being an attenuated strain of VEEV displays some residual virulence and was withdrawn from phase-II clinical trials due to adverse effects. Thus, in the present study we add another level of protection to V3526 by completely inactivating it using INA but still protect its immunogenic epitopes for a strong antibody response. In this study we show that inactivated V3526 can successfully protect animals against an aerosol challenge with the virulent VEEV with no clinical signs of disease. We also show a strong antibody response against different doses of immunization with INA-inactivated V3526.

Results:

INA is not toxic to cell culture up to 200µM concentration

In order to evaluate INA-toxicity *in-vitro*, cells were treated with different concentrations of INA (10-500µM). The amount of cell proliferation was evaluated using MTT assay. In this assay, a tetrazolium compound MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) is added to the cells which is reduced by metabolically active cells to insoluble purple formazan dye crystals. These crystals are then solubilized and the absorbance is measured at 570nm. The rate of tetrazolium reduction by cells is directly proportional to the rate of cell proliferation. We found that cells treated with up to 200µM dose of INA did not have any effect on their proliferation (Figure-31). A marginal difference in the rate of cell proliferation was seen when cells were treated with higher doses of INA. Thus, INA is non-toxic to cells at the dose used for subsequent *in-vitro* analysis which is almost 1000 fold less than doses used for this assay.

V3526 is non-infectious *in-vitro* upon INA treatment and UV-irradiation

V3526 was inactivated by using 100µM INA and UV-irradiation for 5 minutes based on the observations from our previous studies (Sharma et al 2007). Cytopathic effect evaluation was performed using virulent and INA-inactivated V3526 in Vero cells to determine the extent of virus inactivation achieved. INA-inactivated V3526 did not induce cytopathic effect (CPE) and cell death in Vero cells (Figure-32). However, cells infected with virulent V3526 rounded up and sloughed off the surface at 72hr p.i.

In order to further confirm any residual level of virus replication *in-vitro*, cell supernatant was collected from cells infected with virulent and INA-inactivated V3526 for virus titration by

plaque assay and RNA was isolated for virus specific RT-PCR analysis. No virus replication was detected in the supernatant from the cells infected with INA-inactivated V3526 where as V3526 replicated as expected in these cells (Figure-33). Similarly, no virus specific amplification was detected by NsP-4 specific RT-PCR amplification in the cellular RNA. Both these results further confirm complete inactivation of V3526 upon INA treatment and UV-irradiation.

Iimmuno-fluorescence staining in the Vero cells infected with the INA-inactivated V3526 also did not show any VEEV specific immuno-fluorescence (Figure-34). Cells were infected with virulent and INA-inactivated V3526 and fixed at 36hr and 48hr p.i. The cells were then stained with a VEEV specific antibody. The cells infected with virulent V3526 were stained whereas no virus specific staining was observed in INA-inactivated V3526 infected cells. All the cells were also stained with DAPI to confirm the presence of live cells.

INA treatment and UV-irradiation of V3526 also inactivates the V3526 RNA genome

The positive sense RNA genome of alphavirus is infectious and when transfected into sensitive cell lines results in the generation of live virus particles (Griffin 1999, Smith et al 1997, Guzman et al 2005, Kolykhalov et al. 1992, Pratt et al 2003). In order to confirm inactivation of RNA genome by INA, RNA was isolated from INA-inactivated V3526 and transfected in to fresh cells. At 72hr p.i., cells were stained with a VEEV specific antibody. Cells transfected with RNA from INA-inactivated V3526 did not show any positive staining whereas cells transfected with RNA from virulent V3526 were found positive for VEEV specific immuno-florescence staining. This indicates that RNA from INA-inactivated V3526 failed to cause infection in cells (Figure-35).

Antigenic epitopes of V3526 are protected after INA-inactivation

Western blot analysis was performed using an antibody specific to the PE2 epitopes of V3526, 13D4-1, to evaluate the antigenicity of the INA-inactivated V3526. Our results indicate that antigenic epitopes of V3526 are protected upon INA-inactivation; however, there is some loss of antigenicity in comparison to the virulent V3526 (Figure-36).

INA-inactivated V3526 is safe in suckling mice

Suckling mice are immunologically immature and either fail to or induce inadequate antibody response to infection. Also the immature neurons are more susceptible to virus induced apoptosis (Griffin et al 1994). Therefore, these mice are sensitive to any residual virulence that may be associated with inactivated preparations of virus. INA-inactivated V3526 also did not induce any disease symptoms in suckling mice and all the mice survived the two weeks observational period p.i. and developed normally. All the suckling mice injected with V3526 developed the disease and either succumbed to the infection with in 48-96 hr p.i. or were humanely euthanized at 96 hr p.i. due to disease morbidity such as stunted growth, slow breathing rate and little to no movement (Table-17, Figure-37). No virus was detected in the brain of INA-inactivated V3526 infected mice whereas brains of V3526 infected mice were tested positive for V3526 by plaque assay and nsP4 specific PCR (Figure-38).

No sign of virus induced histological changes was detected in INA-inactivated infected mice.

In the brain sections of suckling mice infected with V3526, over 90% of the neurons within the caudal cerebrum, caudal to the hippocampus region, especially those is the superficial

layers, were either necrotic or apoptotic. The necrosis extended into the purkinje cells and inner granular layer multifocally. There was karryorectic debris throughout and low numbers of neutrophils and lymphocytes. Endothelial hypertrophy was also observed but there was no perivascular cuffing. Multifocal vacuolation of the neuropil was also observed. The thalamus was similarly but less severely affected. A low number of necrotic/apoptotic cells were observed in the periventricular area. The choroid plexus and ependymal cells appeared to be unaffected. The neurons within the hippocampus were negative for VEEV specific staining. A diffused and strong cytoplasmic reactivity to VEEV antibody was noted in all the affected areas on H&E. Additionally, approximately 30% of the neurons throughout the section were found positive for VEEV (Figure-39).

However, in case of mice infected with INA-inactivated V3526 and saline controls, all corresponding areas (caudal cerebrum, thalamus, hippocampus, cerebellum, lateral ventricle/periventricular area) were found within normal limits (Figure-40). The outer granular layer of the cerebellum was much less pronounced and is only 1-2 cell layers thick, due to the older age of this mouse. There was no histological evidence of viral infection. None of the neurons stained positive for VEEV. To summarize, INA inactivation of V3526 was successful and none of the suckling mice showed evidence of viral infection histologically.

INA-inactivated V3526 protected mice against aerosol challenge with virulent VEEV.

Mice were immunized with two different doses of INA-inactivated V3526 (1µg or 3µg protein) through different routes (intranasal (IN), intramuscular (IM) and subcutaneous (SC)). Post immunization the animals were challenged with 1000pfu of the virulent VEEV strain (V3000) through aerosol exposure. Animals were monitored for any disease symptoms. While

the animals immunized the investigation vaccine for VEEV, C84 showed only 30% protection, INA-inactivated V3526 immunized mice showed up to 100% protection against aerosol challenge (Figure-41 and 42). Highest protection was achieved in case of IM immunization with both 1µg and 3µg doses. SC immunization resulted in 90% protection with both the doses however IN immunization provided only 10% protection at 3µg dose. All the saline treated animals succumbed to VEEV infection. These results, thus, indicate that INA-inactivated V3526 can successfully protect mice against aerosol challenge with a single immunization at 1µg dose through IM rout even without any adjuvant.

Discussion:

VEEV is endemic in the Central and South America, and the Texas region of North America. VEEV is a human pathogen and highly infectious through aerosol route. Also likelihood of VEEV to be used as the bioweapon and bioterror agent is higher since VEEV has been developed as a bioweapon in past. Absence of any licensed vaccine against VEEV, thus presents an urgent need for an efficient vaccine for immunization against VEEV. Thus, in the present study we have evaluated a novel method of virus inactivation using INA and UV-irradiation. V3526 which is already attenuated at genomic level was further inactivated using this technique. Our results show complete inactivation of V3526 with INA treatment and UV-irradiation. These observations were confirmed both *in-vitro* as well as *in-vivo*. Histological evaluation also confirmed complete inactivation of INA-inactivated V3526. These results corroborated with our previous findings with V3000 strain of VEEV (Sharma et al 2007) and also with the other reports of inactivation of influenza, Ebola, HIV and SIV viruses by INA (Raviv et al 2005 and 2008, Warfeild et al 2007).

Most importantly, we have shown that the RNA isolated from INA-inactivated V3526 is non-infectious and fails to cause infection when transfected in cells. INA-inactivated VEEV is safe with no residual virulence and INA-inactivation also addresses the issue of infectious positive sense RNA genome of alphaviruses. We have also shown that INA-inactivated V3526 does not lose its antigenicity due to INA treatment or UV-irradiation. Thus, the inactivation of VEEV by INA is a dual-inactivation strategy, one targeting the virus envelope protein without damaging the antigenic epitopes and second targeting the infectious RNA genome.

Finally, we also showed that INA-inactivated V3526 successfully protects mice against aerosol challenge with the virulent VEEV. A single immunization at a dose of 1µg through intramuscular route provided 100% protection against aerosol challenge with virulent VEEV whereas the current investigational vaccine candidate, C84, could provide only 30% protection. The protection efficiency through other routes of immunization can be further improved by using suitable adjuvants. These results thus prove that INA-inactivated V3526 is a promising vaccine candidate against VEEV infection.

These results show that INA-inactivated VEEV is safe and can protect against virulent VEEV challenge in mice. These results have significant implications as INA-inactivated VEEV overcomes various limitation of the currently available vaccine strains for VEEV i.e. TC-83, V3526 and formalin inactivated VEEV. Therefore, this method of inactivation may prove helpful in generating multivalent vaccines and reducing the interference phenomena among the vaccine constituents.

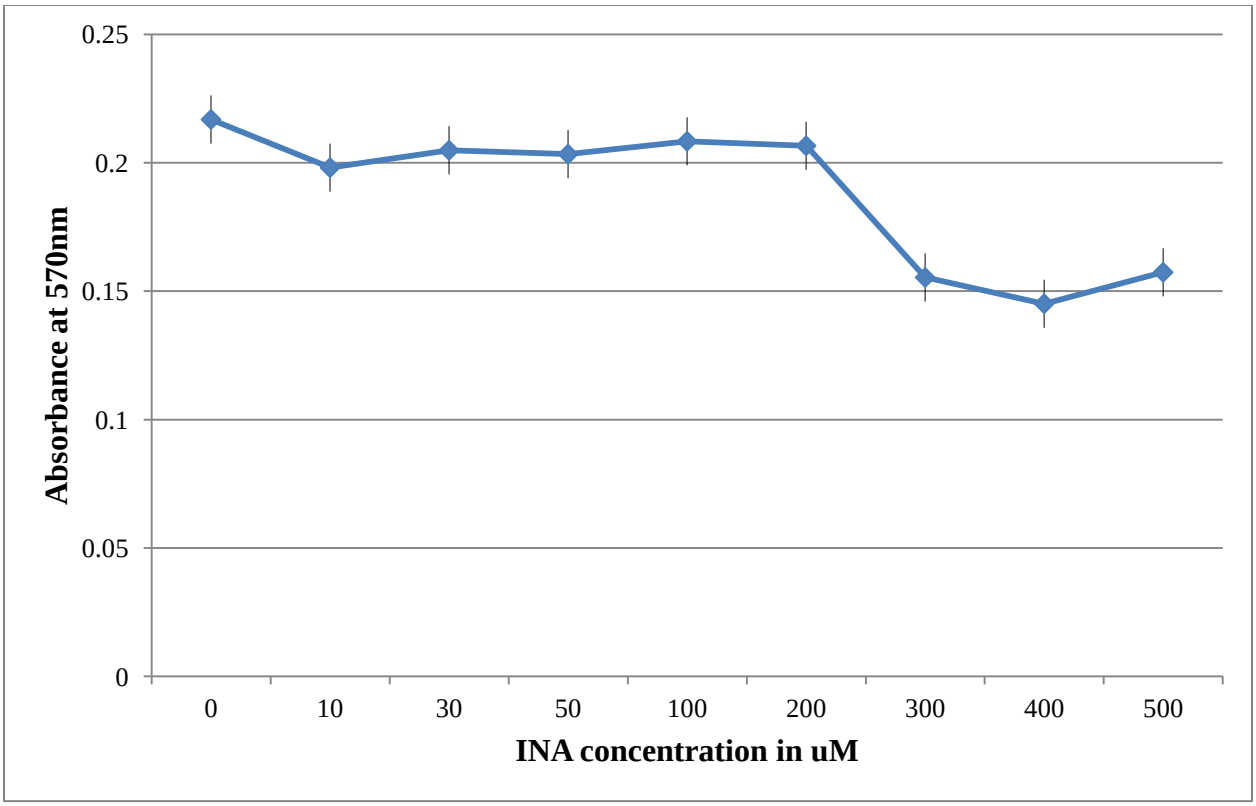


Figure-31 INA toxicity evaluation *in-vitro*: Cells were treated with different concentrations of INA and cell proliferation was measured by using MTT assay as described in methods. Decreased absorbance value indicates lesser number of proliferating cells. Thus, INA was found to be safe up to a dose of 200uM in cell culture, which is a much higher dose than the dose actually given to the cells during *in-vitro* assays.

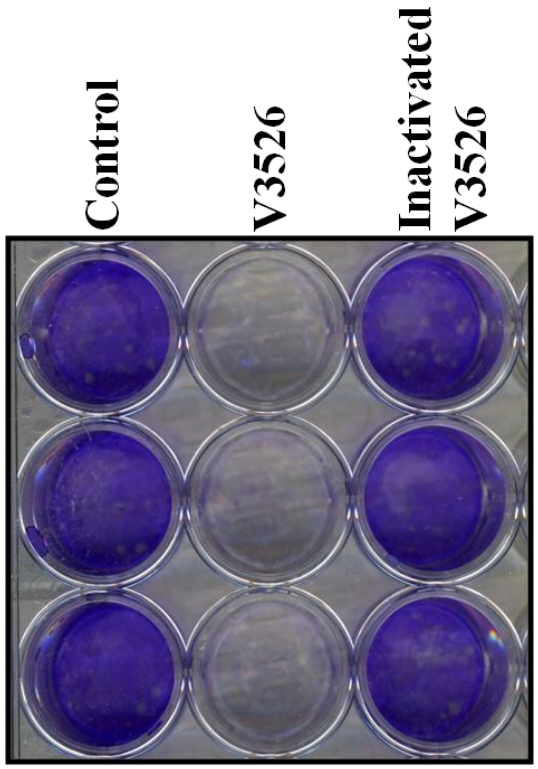


Figure-32 V3526 is inactivated with 100µM INA and UV-irradiation: Vero cells were treated with INA-inactivated V3526 to evaluate complete inactivation of V3526 with 100µM INA and UV-irradiation by crystal violet assay. At 72hr p.i., cells infected with V3526 rounded up and sloughed off the surface. Cells infected with INA-inactivated V3526 grew normally and formed a monolayer similar to that of the saline treated cells. Live cells were stained blue with 0.1 % CV solution and absence of the blue color indicates loss of cells due to virus infection.

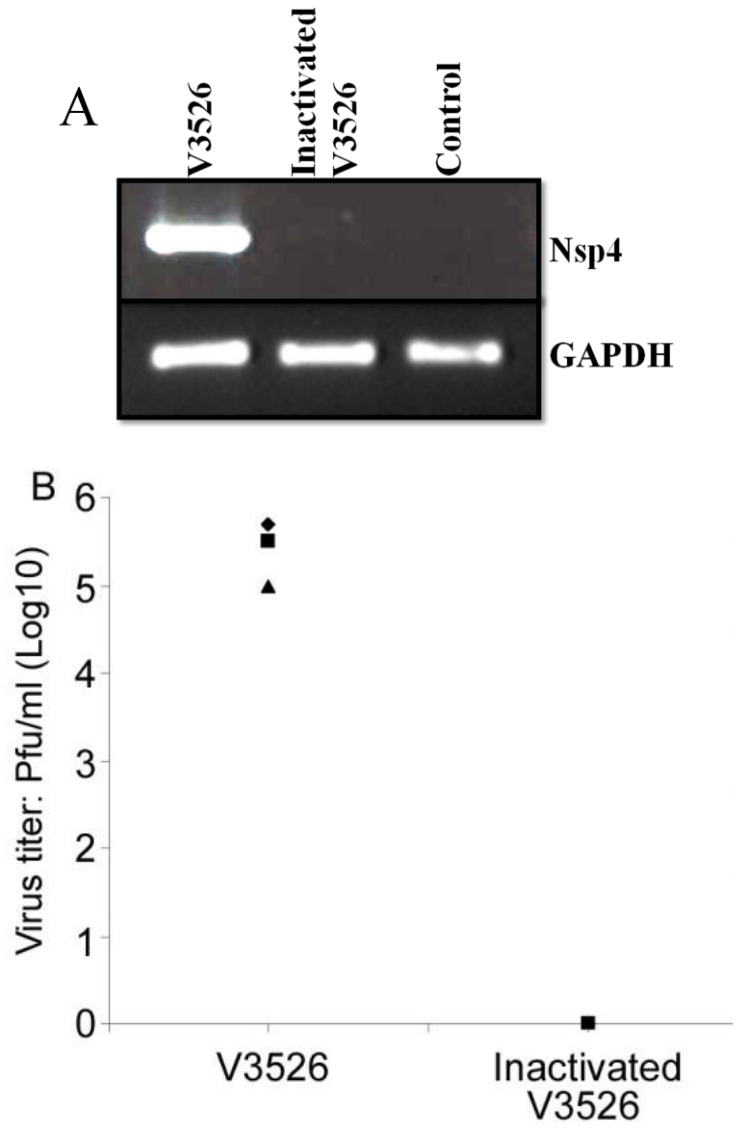


Figure-33 VEEV specific PCR and virus titer confirm V3526 inactivation by INA: A) RNA was isolated from cells infected with virulent and INA-inactivated V3526. No virus specific amplification (NsP4 gene of virus genome) was observed in cells infected with INA-infected V3526 by RT-PCR. B) Virus titer in the cell supernatant of Vero cells infected with V3526 or INA-inactivated V3526 was determined by plaque assay to determine any residual virus replication in the cells infected with INA-inactivated V3526. No virus was detected in the supernatant of cells infected with INA-inactivated V3526 infected cells.

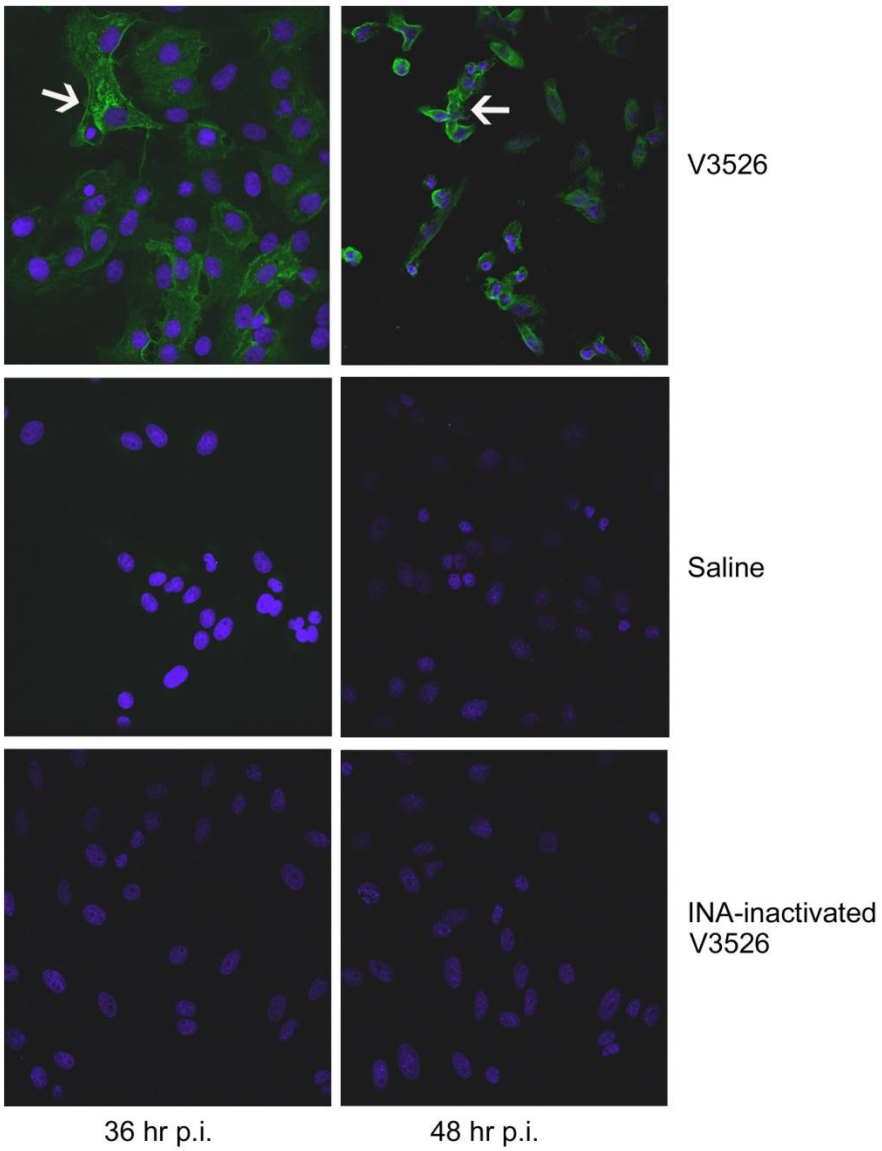


Figure-34 Localization of V3526 infection *in-vitro* by Immuno-florescence: Vero cells were infected with V3526 or INA-inactivated V3526 with an MOI of 10. Cells were fixed and stained for VEEV antigen as described in methods. VEEV specific antigen (green fluorescence) was localized in the cells infected with V3526 as indicated by arrows. No VEEV specific staining was observed in the cells infected with INA-inactivated V3526 suggesting failure of INA-inactivated V3526 to replicate in the cells.

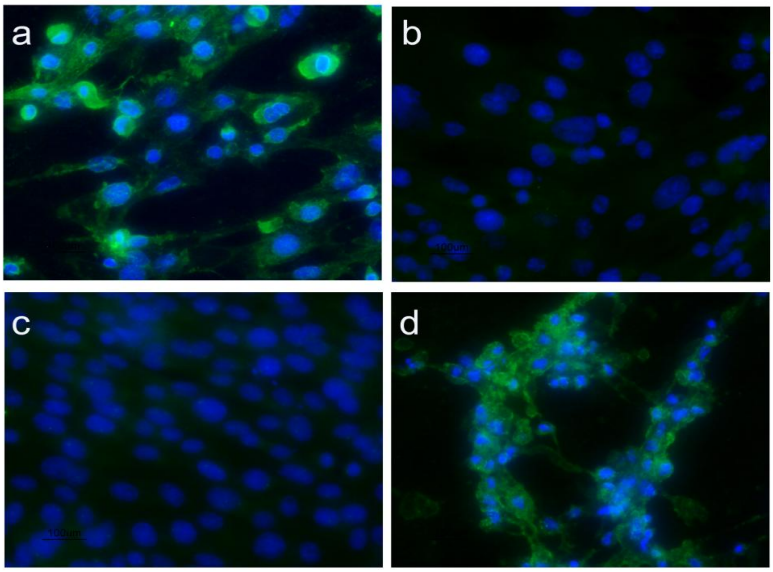


Figure-35 RNA isolated from INA-inactivated V3526 is inactivated *in-vitro*: Cells were fixed at 72hr post transfection. a) V3526 RNA transfected cells; b) INA-inactivated V3526 RNA transfected cells; c) saline treated cells; d) V3526 virus infected cells. No virus specific staining (green) was observed in the cells transfected with INA-inactivated V3526 RNA. Cells were also stained with DAPI to confirm the presence of live cells.

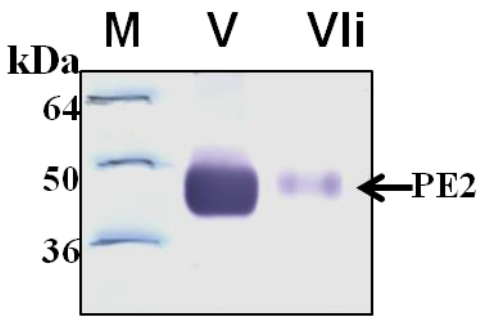


Figure-36 Antigenic epitopes are protected after INA-inactivation of V3526: Western blot analysis was performed to evaluate the structural integrity of the viral epitopes after inactivation with INA and UV-irradiation. Monoclonal antibody (13D4-1) specific to E3 epitope of the V3526 strain was used. Both V3526 and V3526+INA preparations reacted with the antibody confirming that the structural integrity of V3526 epitopes is maintained after INA inactivation.

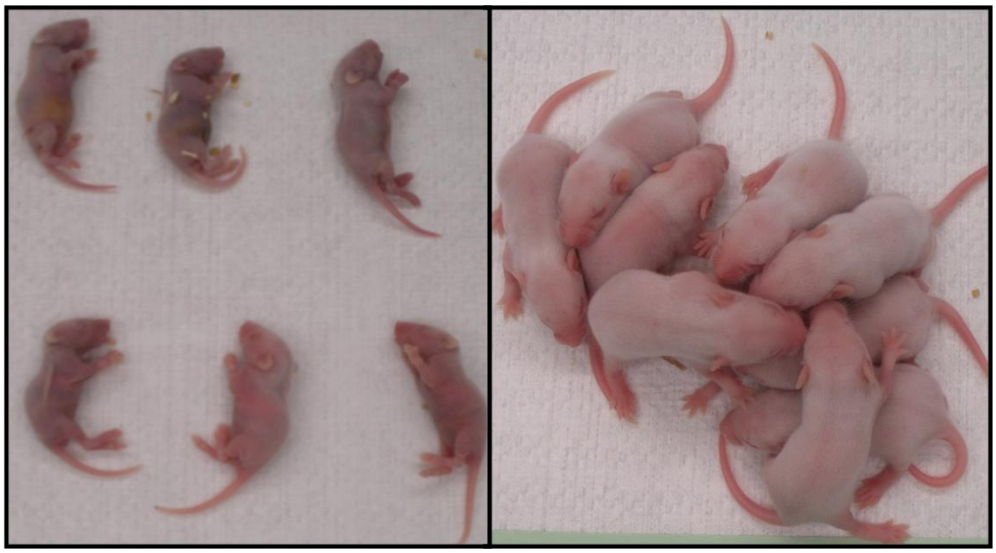


Figure-37 INA-inactivated V3526 infection of suckling mice: a) 3 day old suckling mice infected i.c. with V3526; b) 3 day old suckling mice infected i.p. with INA-inactivated V3526. All the mice injected with V3526 succumbed to infection or because moribund, however, the mice injected with INA-inactivated V3526 survived and developed normally.

Samples	No. of animals	Mice died / Total mice	Percent Survival	Mean weight of the animal groups	
				Day 3- p.i.	Day 13- p.i.
V3526	**8**	**8/8**	**0**	**2.61 gm ±0.08***	**-**
V3526+INA +Irradiation	**10**	**0/10**	**100**	**4.90 gm ±0.04**	**9.73 gm ±0.1**
Saline	**2**	**0/2**	**100**	**4.43 gm ±0.005**	**11.0 gm ±0.2**

Table-13 Safety evaluation of INA-inactivated V3526 in suckling mice. Suckling mice were infected with virulent and INA-inactivated V3526 as described in methods. All the animals that received INA-inactivated V3526 survived and developed normally. All the animals that received virulent V3526 developed the disease. Animals either succumbed to the infection or were euthanized due to the severe disease morbidity at 96 hr p.i. None of the mice infected with INA inactivated V3526 showed any clinical signs of disease or infection like hunched back, paralysis, loss of weight and excitability. These mice survived and developed normally like the uninfected mice (n=2) for 13 days p.i. after which animals were sacrificed. However, all the mice infected with virulent V3526 developed infection and 8 of them died within 72hr p.i. * Mean weight of seven mice.

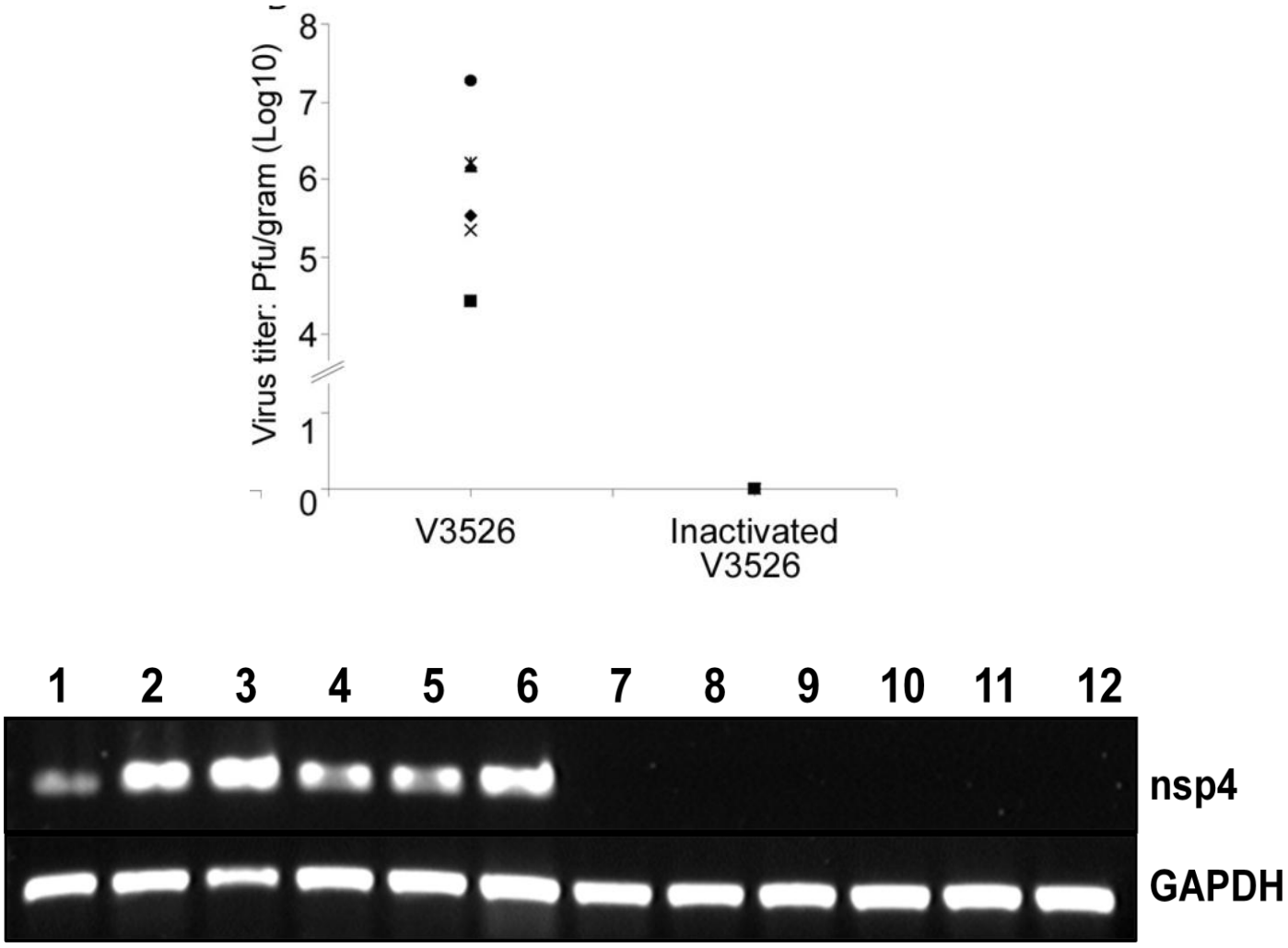


Figure-38 No virus was detected in the brain of suckling mice infected with INA-inactivated V3526: All the mice infected with INA-inactivated V3526 survived the 13 day p.i. period and did not show any virus their brain, whereas all the mice infected with the V3526 succumbed to the infection and showed positive virus replication. A) Virus titer was determined in 20% brain tissue homogenate (w/v) of suckling mice that succumbed to V3526 infection and the mice infected with INA-inactivated V3526. B) Nsp4 specific PCR amplification was evaluated in the RNA isolated from brain samples of mice infected with V3526 or INA-inactivated V3526 (1 through 6- V3526 infected mice, 7 through 11- INA inactivated V3526 infected mice and 12- saline treated mice).

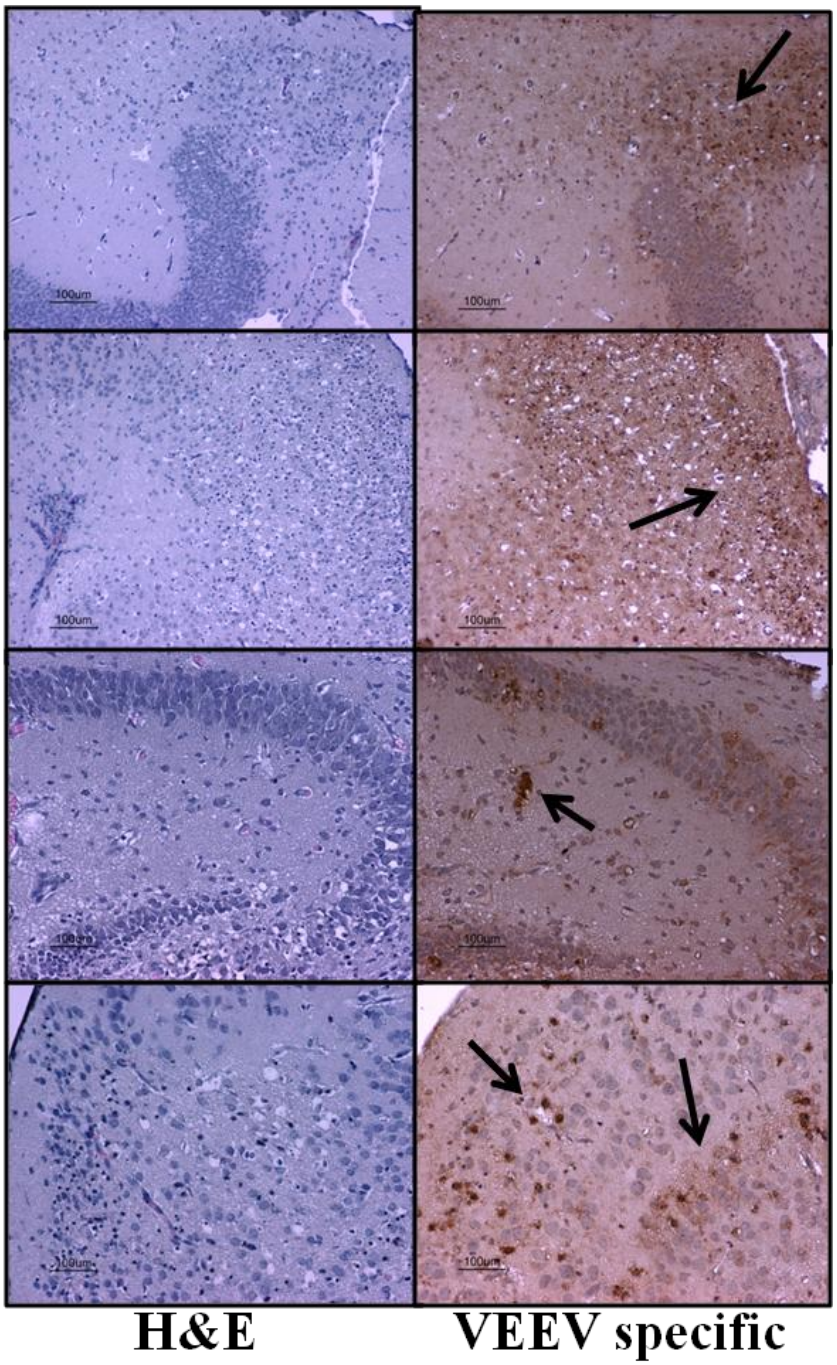


Figure-39 Histopathology of V3526 infected mice brain: Brain section of V3526 infected suckling mice were stained with Hematoxylin and eosin stain and a VEEV specific antibody. Cell death and inflammation was detected by H&E stain and V3526 antigen was detected at multifocal points in the brain sections at 96hr p.i.

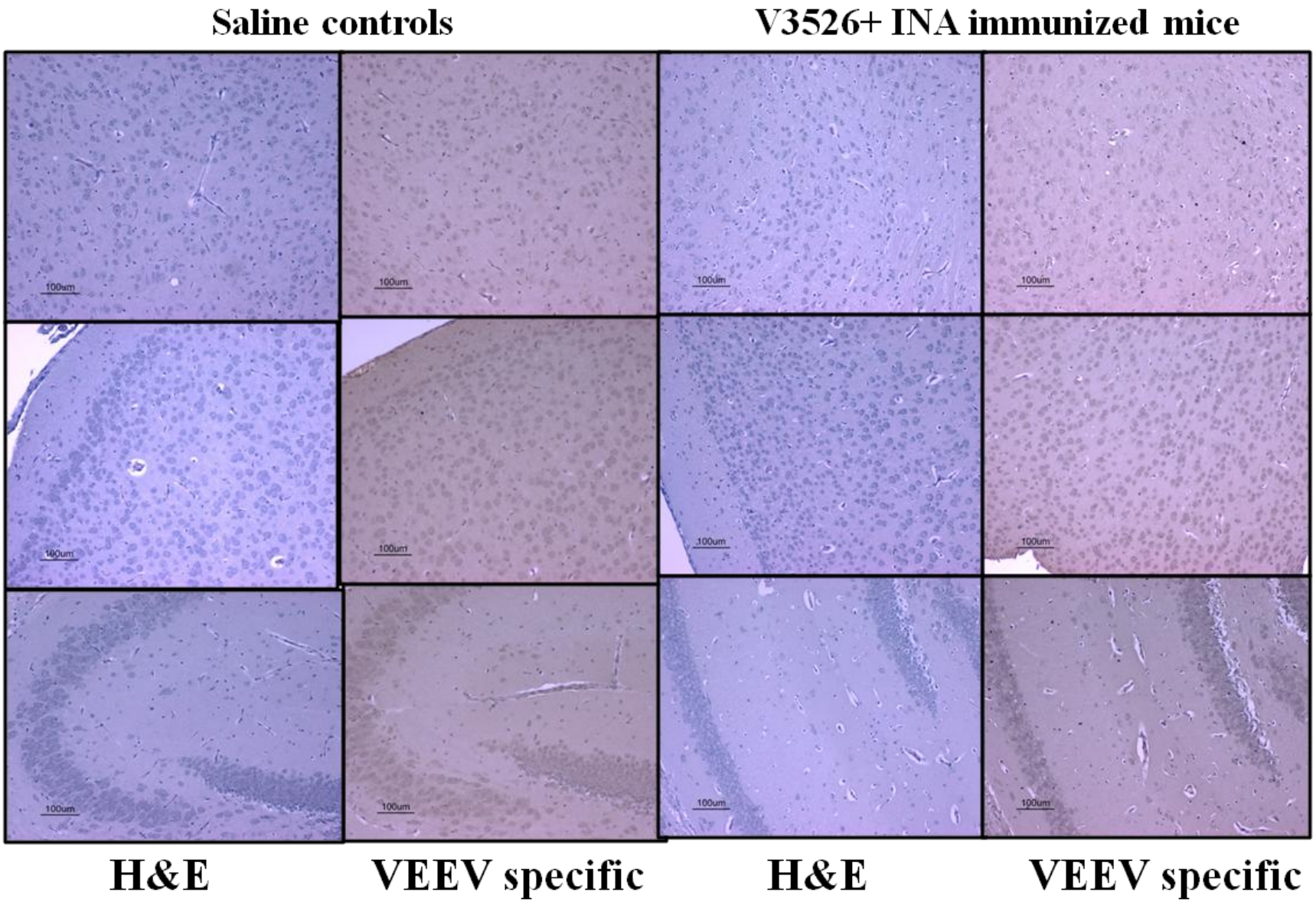

Saline controls
V3526+ INA immunized mice
H&E
VEEV specific
H&E
VEEV specific

Figure-40 Histopathology of INA-inactivated V3526 infected and control mice brain: Brain sections of Control and INA-inactivated V3526 infected suckling mice were stained with Hematoxylin and eosin stain and VEEV specific antibody. No signs of inflammation, cell death, or virus presence were detected.

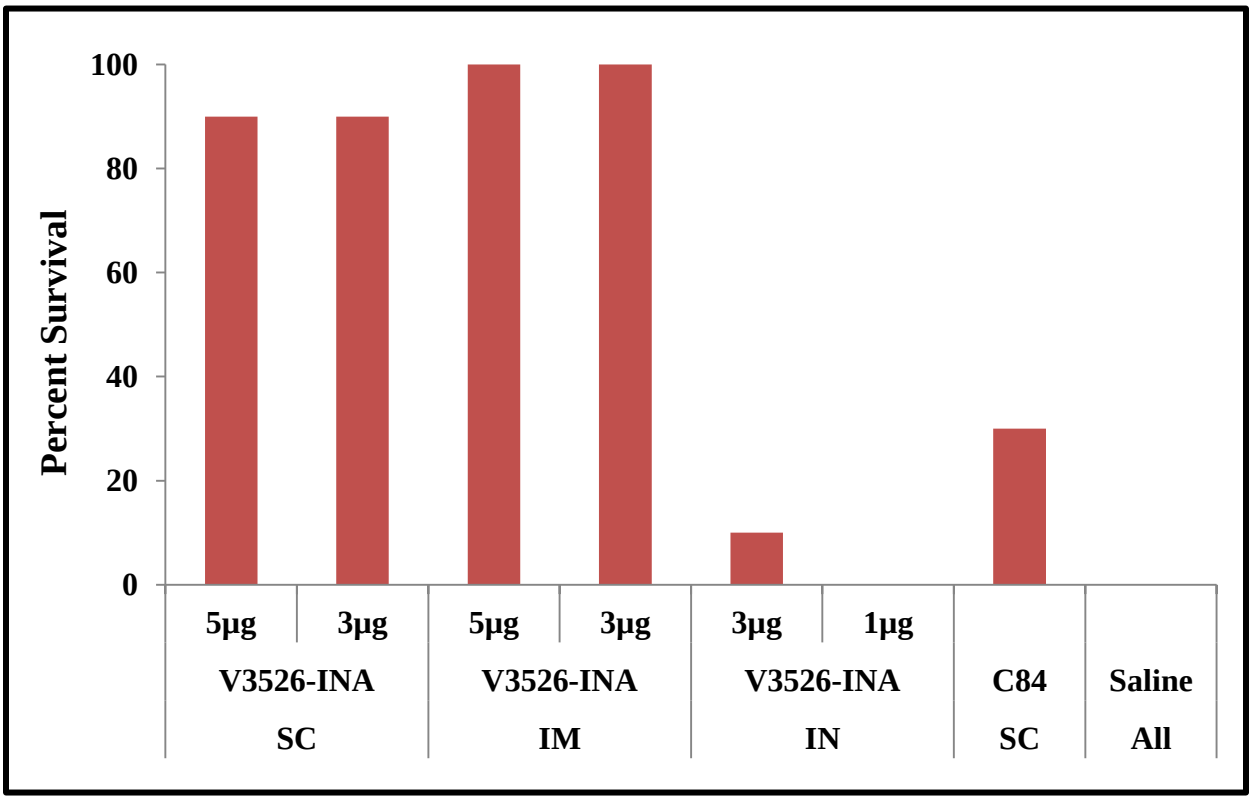


Figure-41 Protective efficacy of INA-inactivated V3526: Immunization with INA-inactivated V3526 protected animals from virulent VEEV challenge. Highest protection was observed through intra-muscular route of immunization.

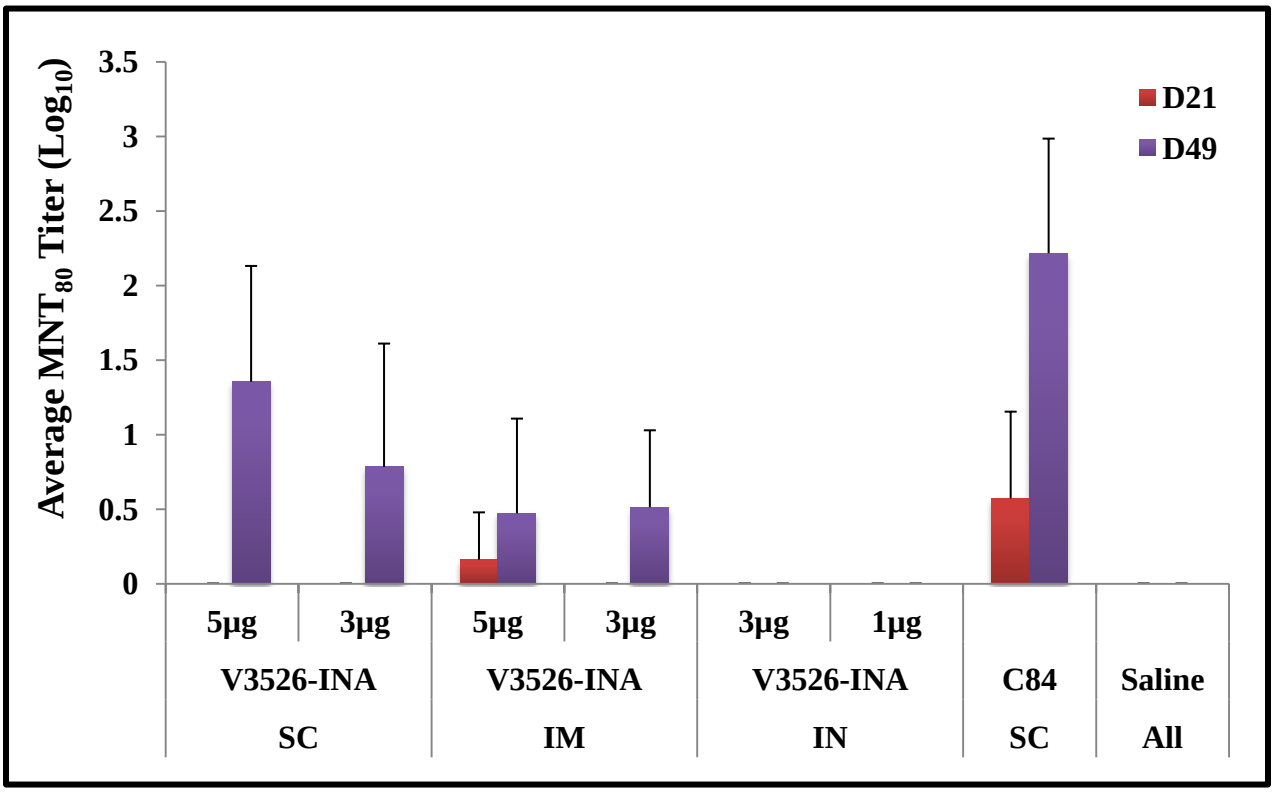


Figure-42 Neutralizing antibody titers after vaccination with INA-inactivated V3526: Immunization with INA-inactivated V3526 protected animals from virulent VEEV challenge. Highest protection was observed through intra-muscular route of immunization.

B. Inactivation of Encephalomyocarditis virus, a non-enveloped virus

Abstract:

A novel approach of using a hydrophobic photoactive compound 1,5-iodonaphthyl-azide (INA) has been shown to successfully inactivate several enveloped viruses by us and others. In our earlier studies with VEEV inactivation, we demonstrated that the viral RNA was also inactivated after INA treatment and UV-irradiation. Therefore, in the present study, we used Encephalomyocarditis virus (EMCV), a non-enveloped RNA virus, to evaluate inactivation efficacy of INA. EMCV inactivation with INA treatment followed by UV irradiation was achieved in a dose dependent manner. Inactivated EMCV was structurally intact as determined by electron microscopy and western blot analysis however it failed to actively replicate in cell culture. Furthermore, RNA isolated from INA-inactivated EMCV was non-infectious when transfected into cell culture. Studies with [(125)I]INA-inactivated EMCV suggested INA incorporation into the viral RNA upon UV-irradiation. No residual infectivity was observed in mice. A strong total antibody response was also observed however INA-inactivated EMCV failed to protect mice against a lethal EMCV challenge.

This is the first study to show that non-enveloped viruses like EMCV can be completely inactivated with INA. However, INA inactivation may interfere with the protective epitopes of the virus resulting in loss of protective efficacy of the inactivated vaccine. These results for the first time demonstrate that INA can efficiently inactivate non-enveloped viruses like EMCV and can further be explored for developing vaccine candidates of livestock importance.

Results:

EMCV is inactivated *in-vitro* by INA treatment and UV-irradiation:

In order to determine inactivation of virus after treatment with INA and irradiation with UV light qualitatively, cytopathic effect in cell culture was evaluated by performing a crystal violet assay. L-11 cells were infected with the inactivated virus and controls. Observations were recorded at 72hr post infection by staining the cells with crystal violet dye. EMCV inactivated with 10 μM dose of INA and UV-irradiation ($EI_{10}i$) was as infectious as the EMCV alone and EMCV treated with DMSO controls (E, Ei, ED and EDi respectively). There was some protection at 30 μM dose of INA after UV-irradiation but no significant protection was observed without irradiation. However, at 50 μM, 100 μM and 200 μM doses of INA and UV-irradiation, cells were completely protected against EMCV infection (**Figure-43A**). The results were consistently reproduced over more than 4 separate experiments. INA treatment inactivated EMCV at 50 μM, 100 μM and 200 μM doses even without irradiation.

Results from the crystal violet assay were confirmed by determining the amount of virus present in the cell supernatant at different time points after infection with inactivated virus and control groups. Virus titer was calculated as 50% tissue culture infectivity dose ($TCID_{50}$) in supernatants collected from crystal violet assay at 12hr, 24hr, 48hr and 72hr pi. There was no detectable virus replication in supernatants from 50μM, 100μM and 200μM doses at any time points after UV-irradiation whereas very low amount of virus was detected at later time points in INA treated samples without UV-irradiation (**Table-18**). Therefore, although no virus induced cytopathic effect was observed by crystal violet assay, virus titer in cell supernatant indicated residual level of virulence in samples treated with INA alone.

Virus replication was also confirmed by performing EMCV specific PCR on RNA collected from infected cells. RNA was isolated from L-11 cells infected with inactivated and control group of viruses after 12-14 hours of infection. EMCV specific amplification was carried out on the cDNA from this RNA and amplicon was run on a 1% agarose gel. No virus was detected in cells infected with the $EI_{50}i$ and $EI_{100}i$ **(Figure-43B)**. This observation confirmed again that the virus treated with INA and irradiated with UV light is completely inactivated and does not undergo any replication at all. However, some amplification was observed in non-irradiated samples at the same doses. RNA from uninfected cells and cells infected with the control group of viruses were used as negative and positive controls respectively. Virus specific amplification was observed in RNA samples from cells infected with virus inactivated with other doses of INA. This suggests that complete inactivation was achieved only after INA treatment (50µM and 100µM doses) and UV-irradiation.

RNA from inactivated EMCV is not infectious *in-vitro*:

One of the aims of the study was to determine the inactivation of EMCV RNA genome with INA treatment and UV-irradiation. In order to confirm this, RNA was isolated from virulent or INA-inactivated ($EI_{100}i$) EMCV and transfected in to fresh cells. At 48hr p.i., RNA was isolated from these cells and virus specific (3D gene encoding viral polymerase) PCR amplification was performed. Cells transfected with INA-inactivated EMCV did not show any virus specific amplification whereas positive amplification was detected in cells transfected with RNA from virulent EMCV (Figure-44).

Cell supernatant was also isolated from the cells at 72hr p.i. to detect any residual level of virus replication in cells by plaque assay. Similar observations were recorded by plaque assay and no virus was detected in the cell supernatant of cells transfected with RNA from INA-

inactivated EMCV (Figure-44). This indicates that RNA from INA-inactivated EMCV failed to cause infection in cells and is inactivated due to INA treatment and UV-irradiation.

INA gets incorporated in viral RNA after irradiation:

In order to confirm the interaction of virus RNA with INA, EMCV was inactivated with radioactive iodine labeled INA. RNA was isolated from the inactivated virus and northern blot was performed to detect the presence of INA in the RNA. INA specific band was obtained only in case of EMCV treatment with INA and UV-irradiation. No similar band was detected in samples only treated with INA and no UV-irradiation. This indicates that INA gets incorporated in the virus RNA upon UV-irradiation. To confirm that the bands obtained corresponded to RNA, viral RNA was treated with RNAse or proteinase before running on the gel. Northern blot was performed as before. Upon RNAse treatment, the INA specific band fades away whereas no such effect is observed after proteinase treatment indicating that the INA gets incorporated into viral RNA (Figure-45).

INA-inactivation does not change structural integrity of EMCV:

In order to evaluate any effect of INA-inactivation on the virus structural integrity, electron microscopy and western blot analysis using polyclonal antibody against EMCV were performed. Equal amount of protein for INA-inactivated EMCV and control samples were ran on tris-glycine gel and the bands were transferred onto a nitrocellulose membrane. The membrane was then stained with a polyclonal antibody against EMCV. Four major bands corresponding to EMCV structural proteins were identified in all the samples, however, there was a slight but non-significant difference in the intensity of bands obtained for the INA-treated EMCV samples with and without UV-irradiation (Figure-46A). This suggested that there was no significant difference

in the antigenicity of EMCV structural epitopes upon INA-inactivation and all the structural proteins were intact. Virulent and INA-inactivated EMCV was negative stained and analyzed by electron microscopy. Overall structure, integrity and density of virus particles looked similar in both the samples, suggesting no effect of INA-inactivation on virus particles (Figure-46B).

Evaluation of safety and protective efficacy of INA-inactivated EMCV *in-vivo*:

Since INA-inactivated EMCV was found completely inactivated *in-vitro* and the safety evaluation was also performed *in-vivo* in adult mouse model. Two different studies were performed with 2 and 3 immunizations respectively followed by challenge with virulent EMCV to evaluate the protective efficacy of INA-inactivated EMCV (Figure-47A and B). Each immunization consisted of $1x10^8$ PFU of INA-inactivated EMCV intraperitoneally with or without adjuvant (Alum, during study-2).

All the mice developed normally upon immunizations. There was no sign of disease or morbidity after immunization with INA-inactivated EMCV. All the mice gained weight normally like the saline treated control mice (Figure-47C and D). This suggests that INA-inactivated EMCV is safe to administer in adult mouse and did not cause any disease. In the study-2, even the animals that were injected with adjuvant developed normally and gained weight steadily over the period of 9 weeks before challenge.

Animals were bled before each immunization and total IgG titers against EMCV were calculated using ELISA. A robust antibody response was obtained post immunizations with a significant increase after the booster doses (Figure-47E and F). Use of adjuvant resulted in a more uniform and consistent antibody response between the animals; however, there was no significant difference between the mean titers with or without adjuvant. During the study-2,

animals were given an extended period post last immunization to develop a stronger and better antibody response. Comparing the antibody response obtained in study-1 & 2 we do not see a significant difference in the mean antibody titers post last immunization. Thus, it seems that extended period after immunization does not help in obtaining an improved antibody response.

INA-inactivation may block the neutralizing epitopes on EMCV surface:

Since, the INA-inactivated EMCV failed to protect mice against virulent EMCV challenge, western blot analysis was performed using neutralizing antibody against EMCV to evaluate the integrity of neutralizing epitopes on EMCV surface after INA-inactivation. We found that the neutralizing antibody could not bind to INA-treated EMCV both with and without UV-irradiation (Figure-48). However, DMSO treatment or UV-irradiation alone did not affect the neutralizing epitopes of EMCV. These results thus suggest that INA-inactivation may interfere with the neutralizing epitopes on the surface of inactivated EMCV.

Discussion:

INA-inactivation has been shown to successfully inactivate several enveloped RNA viruses in past. But the ability of INA to inactivate non-enveloped viruses has not been tested till now. So, in the present study we wanted to evaluate whether INA-inactivation strategy can be expanded to non-enveloped viruses or not. Also, past studies from our lab have indicated that INA may also bind to the viral genome. Due to the presence of an azide group, it has been suggested that INA can react with the sulfhydryl group present on the cystein molecules in the RNA or DNA molecules and thus bind to the viral genomes. In order to test both these

hypothesis, we have inactivated EMCV, a non-enveloped RNA virus using INA inactivation strategy in the present study.

INA-inactivation was tested using different doses of INA. Results from our *in-vitro* studies indicate that EMCV gets inactivated with INA treatment to some extent without UV-irradiation and completely after UV-irradiation at 50µM, 100µM and 200µM doses. Detailed evaluation using virus specific PCR on cellular RNA, virus titration in cell supernatant and immune-florescence staining of infected cells confirmed complete inactivation of EMCV at 50µM and 100µM doses.

This was further confirmed by immunizing adult mice with the inactivated EMCV ($EI_{100}i$). INA-inactivated EMCV was found to be completely safe in mice during two separate studies with 2 and 3 immunizations respectively. These mice developed normally like the saline treated control mice as indicated by the overall appearance and weight gain over a period of up to 9 weeks (69 days). Thus, we can say that INA treatment and UV-irradiation inactivated EMCV completely. Electron microscopic evaluation and western blot using polyclonal antibody against EMCV confirmed no gross structural changes in the EMCV particles after INA-inactivation. INA-inactivated EMCV also triggered a robust total antibody response in the immunized mice as shown by ELISA. Thus, we can say that EMCV is completely inactivated with INA treatment and UV-irradiation without damaging the structure integrity of the virion.

RNA from INA-inactivated EMCV was also found to be completely inactive as shown by transfection studies. Using radio labeled INA, we also showed that INA goes and binds to the viral RNA genome. These results confirm the hypothesis that INA inactivation also acts by binding to the viral RNA. This is of greater importance for the viruses with positive sense single

stranded RNA genome since RNA from these viruses is fully capable of infecting the host cells and generating infectious virus particles. Thus, the inactivation by INA and UV-irradiation acts at dual-level, one by targeting the virus envelope protein without damaging the antigenic epitopes and second by targeting the infectious RNA genome.

However, INA-inactivated EMCV immunization failed to confer protection against virulent EMCV challenge in mice. Western blot analysis using neutralizing antibody suggests that INA although INA does not affect the overall structural integrity of the virus particle but it may interfere with the neutralizing epitopes in case of EMCV. Taken together, these results suggest that INA-inactivation strategy can successfully inactivate non-enveloped viruses and INA-inactivation has a dual mechanism of virus inactivation. However, the extent of protection provided by the inactivated-virus may vary between different viruses.

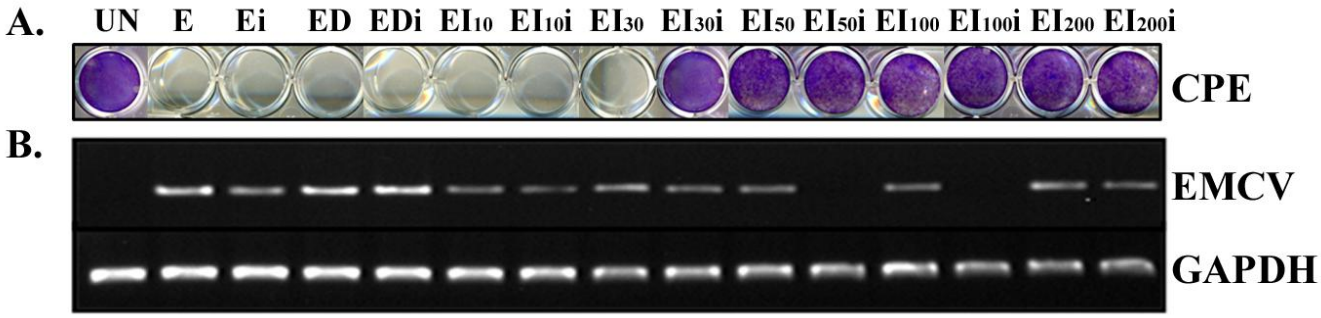


Figure-43 Cytopathic effect evaluation of INA-treated EMCV with and without UV-irradiation *in-vitro*: A) L-cells were infected with virus preparations at an MOI=10 for 72h after which cells were stained using crystal violet. The wells with live cells are stained in blue. Clear wells indicate cell death due to virus-induced cytopathic effect. B) RNA was isolated from cells infected with control and inactivated EMCV samples. No virus specific amplification (3D gene of virus genome) was observed in cells infected with $EI_{50}i$ and $EI_{100}i$ by RT-PCR. GAPDH was used as the reference housekeeping gene.

	12 hr		24hr		48hr		72 hr	
	Sample1	**Sample2**	**Sample1**	**Sample2**	**Sample1**	**Sample2**	**Sample1**	**Sample2**
E	**3.16×10^4**	**4.6×10^4**	**4.3×10^4**	**2.7×10^4**	**3.16×10^4**	**4.6×10^5**	**6.3×10^4**	**1.0×10^4**
Ei	**1.95×10^4**	**2.7×10^4**	**5.13×10^4**	**4.6×10^4**	**6.8×10^4**	**2.6×10^5**	**5.2×10^4**	**3.7×10^4**
ED	**2.7×10^4**	**1.95×10^4**	**1.95×10^4**	**3.9×10^4**	**3.9×10^4**	**3.8×10^5**	**3.7×10^4**	**1.6×10^4**
EDi	**5.6×10^4**	**2.6×10^4**	**3.9×10^4**	**2.0×10^4**	**1.0×10^4**	**1.0×10^5**	**5.6×10^4**	**2.6×10^4**
EI50	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
EI50i	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
EI100	**0**	**0**	**0**	**0**	**0**	**0**	**2.15×10^3**	**1.9×10^2**
EI100i	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
EI200	**0**	**0**	**0**	**0**	**0**	**0**	**6.3×10^3**	**1.6×10^3**
EI200i	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**

Table-18 $TCID_{50}$ values showing virus titer in the cell supernatant after infection. Virus titer in the cell supernatant of L-cells infected with control and inactivated EMCV was determined by $TCID_{50}$ titration to determine any residual virus replication in the cells upon virus infection. The results are representative of at least 4 technical replicates.

A.

Samples	Cytopathic effect	Virus titer in supernatant (PFU/ml)
EMCV	Yes	1.45×10^8
EMCV-RNA	Yes	7.75×10^5
EIi-RNA	No	0
Saline	No	0

B.

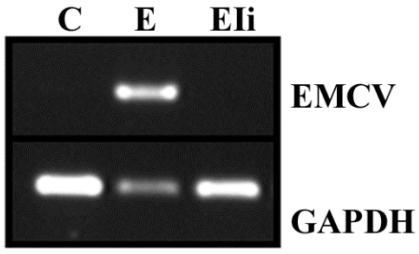


Figure-44 RNA isolated from INA-inactivated EMCV is not infectious *in-vitro*: RNA was isolated from INA-inactivated EMCV at a 100 µM dose and L-cells were transfected with this RNA. A) No virus-induced cytopathic effect or infectious virus particles were detected in cell supernatant by plaque assay. Whereas RNA from virulent EMCV successfully infected cells and virus was detected in cell supernatant by plaque assay. B) RNA was isolated from cells transfected with RNA from INA-inactivated EMCV after 48h and no virus specific amplification was observed by RT-PCR. Virus specific amplification was detected in cells transfected with RNA from virulent EMCV.

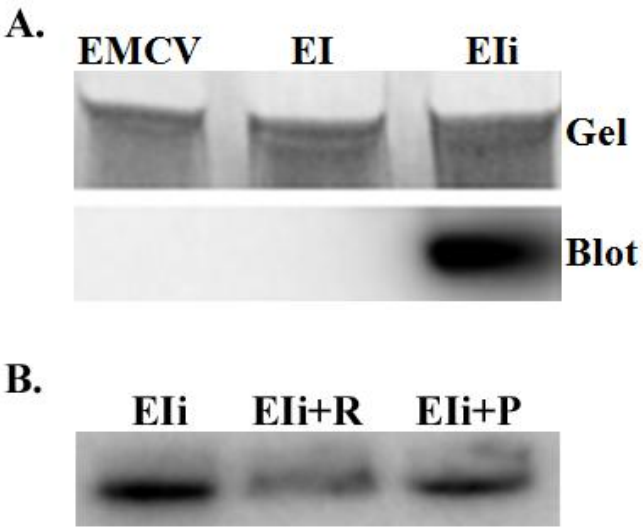


Figure-435 INA gets incorporated into viral RNA after UV-irradiation: RNA was isolated from EMCV inactivated using radio labeled INA (I_{125}). A) Northern blot was performed using this RNA. INA specific band was obtained only in case of EMCV treated with INA and UV-irradiated. B) The RNA was treated with RNase (R) or Proteinase (P) before running on the gel to confirm the presence of RNA.

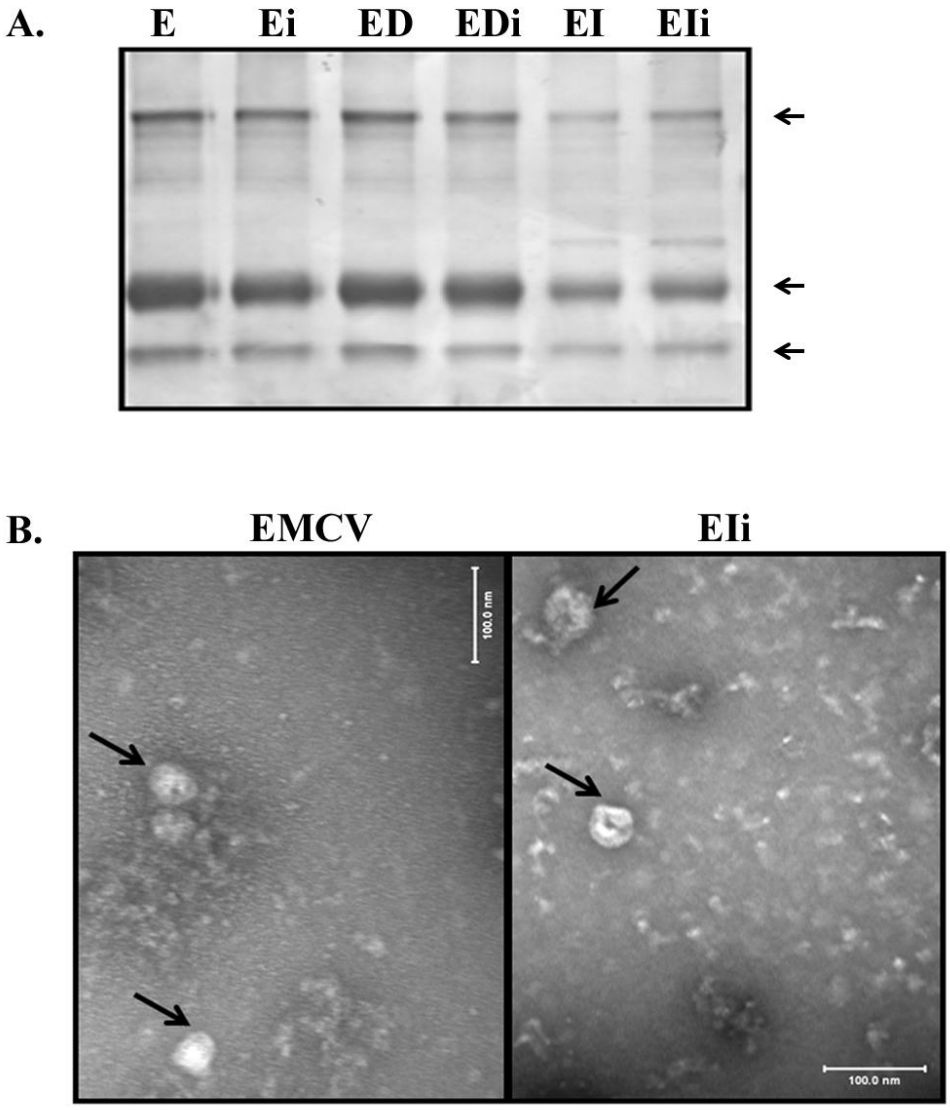


Figure-46 Structural integrity of INA-inactivated EMCV is maintained even after inactivation: A) INA-inactivated EMCV and other controls were run on 4-20% tris-glycine gel. The western blot was stained using a polyclonal antibody against EMCV. No significant difference was observed in the antigenicity of INA-inactivated EMCV in comparison to different controls. B) Electron microscopic evaluation shows that structural integrity of the INA-inactivated EMCV is maintained after UV-irradiation and the virions looked similar to those in the control sample. This indicates that inactivation using INA and UV-irradiation does not affect the overall structural integrity of EMCV particles.

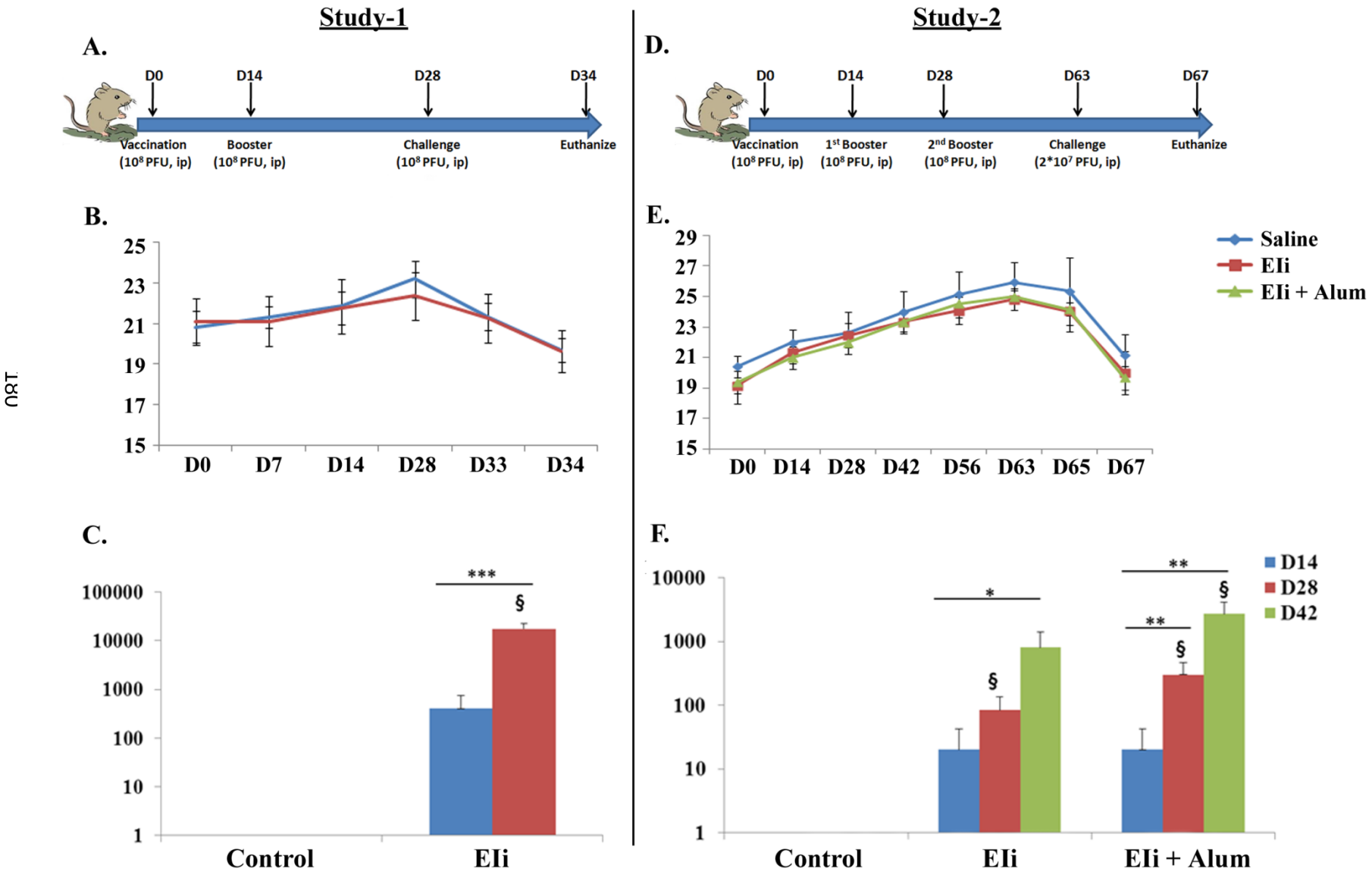

Study-1
A.
D0
D14
D28
D34
Vaccination
(10⁸ PFU, ip)
Booster
(10⁸ PFU, ip)
Challenge
(10⁸ PFU, ip)
Euthanize
B.
25
23
21
19
17
15
D0
D7
D14
D28
D33
D34
C.
***
§
100000
10000
1000
100
10
1
Control
EIi
Study-2
D.
D0
D14
D28
D63
D67
Vaccination
(10⁸ PFU, ip)
1st Booster
(10⁸ PFU, ip)
2nd Booster
(10⁸ PFU, ip)
Challenge
(2*10⁷ PFU, ip)
Euthanize
E.
29
27
25
23
21
19
17
15
D0
D14
D28
D42
D56
D63
D65
D67
Saline
EIi
EIi + Alum
F.
10000
1000
100
10
1
*
**
**
§
§
§
D14
D28
D42
Control
EIi
EIi + Alum

Figure-47 Body weight and total antibody response of mice immunized with INA-inactivated EMCV: A & B) Mice immunized with INA-inactivated EMCV with or without adjuvant (alum) developed normally similar to control mice as evident from the similar amount of body weight gain over the time. Body weight dropped rapidly after challenge during both the studies. **C & D)** Total IgG response against EMCV was evaluated in serum collected after each immunization and before challenge by end point dilution method. Significant increase in the total antibody was observed after booster immunization during both the studies. Total antibody response was more consistent between animals when alum was used however no significant increase in the total antibody was observed due to adjuvant. (C= saline controls, T=EIi immunized and AT= EIi + Alum immunized)

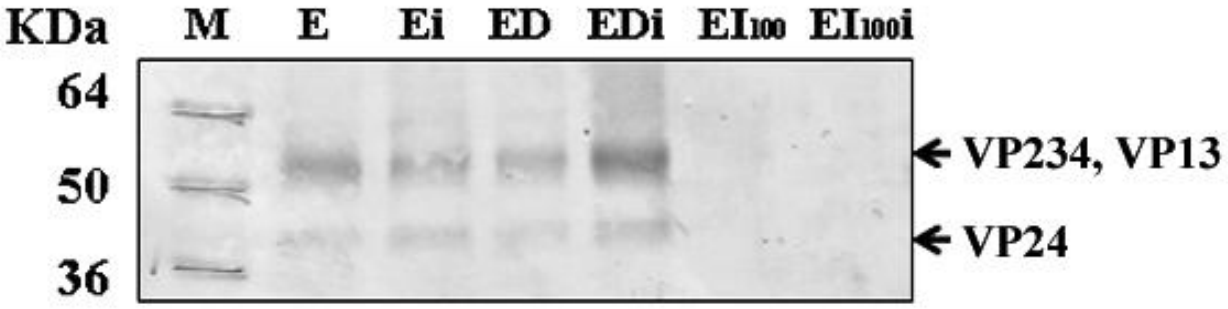


Figure-48 Western blot analysis using neutralizing antibody against EMCV: INA-inactivated EMCV and other controls were run on 4-20% tris-glycine gel. EMCV specific neutralizing antibody was used to perform western blot. No EMCV specific band were observed in INA treated samples suggesting that INA may bind to the neutralizing epitopes present on EMCV surface.

Chapter-2.6: Conclusion and Future Directions

The results from these studies show that INA-inactivation with UV irradiation can be successfully used for inactivation of both enveloped as well non-enveloped viruses. INA-inactivated V3526 as well as EMCV could not infect cell cultures as evaluated by different techniques including crystal violet assay, virus specific PCR, immune-florescence and virus titration in cell supernatants etc. INA-inactivation did not alter the gross structures of the viral epitopes as shown by electron microscopy and western blots. The inactivated virus preparations were found safe in animal models. The inactivated preparations triggered strong total antibody responses. The extent of neutralizing antibody response may vary based on the different viruses. INA-inactivated V3526 could successfully protect animals against aerosol challenge with virulent VEEV. However, INA-inactivated EMCV failed to confer protection to mice against lethal challenge with virulent virus. This can be a virus specific response but requires evaluation by further studies using different doses of immunization along with better adjuvant. These studies also show that INA not only binds to on the viral surface as hypothesized but also gets incorporated in the viral genome as indicated by radio-labeled INA and transfection studies. Although further studies would be needed to identify how INA binding to the viral RNA results in loss of infectivity of the viral RNA.

In nut shell, we can say that INA seems to inactivate viruses in two ways: by binding to the surface proteins and by binding to the genome (Figure-49). This is of more importance in case of RNA viruses with positive sense genome which is fully capable of generating infectious virus particles in the host cell. Thus, INA-inactivation presents as a promising way of virus inactivation and generating effective vaccine candidates. However, the extent of protection

conferred by immunization with INA-inactivated virus preparations may vary among different viruses.

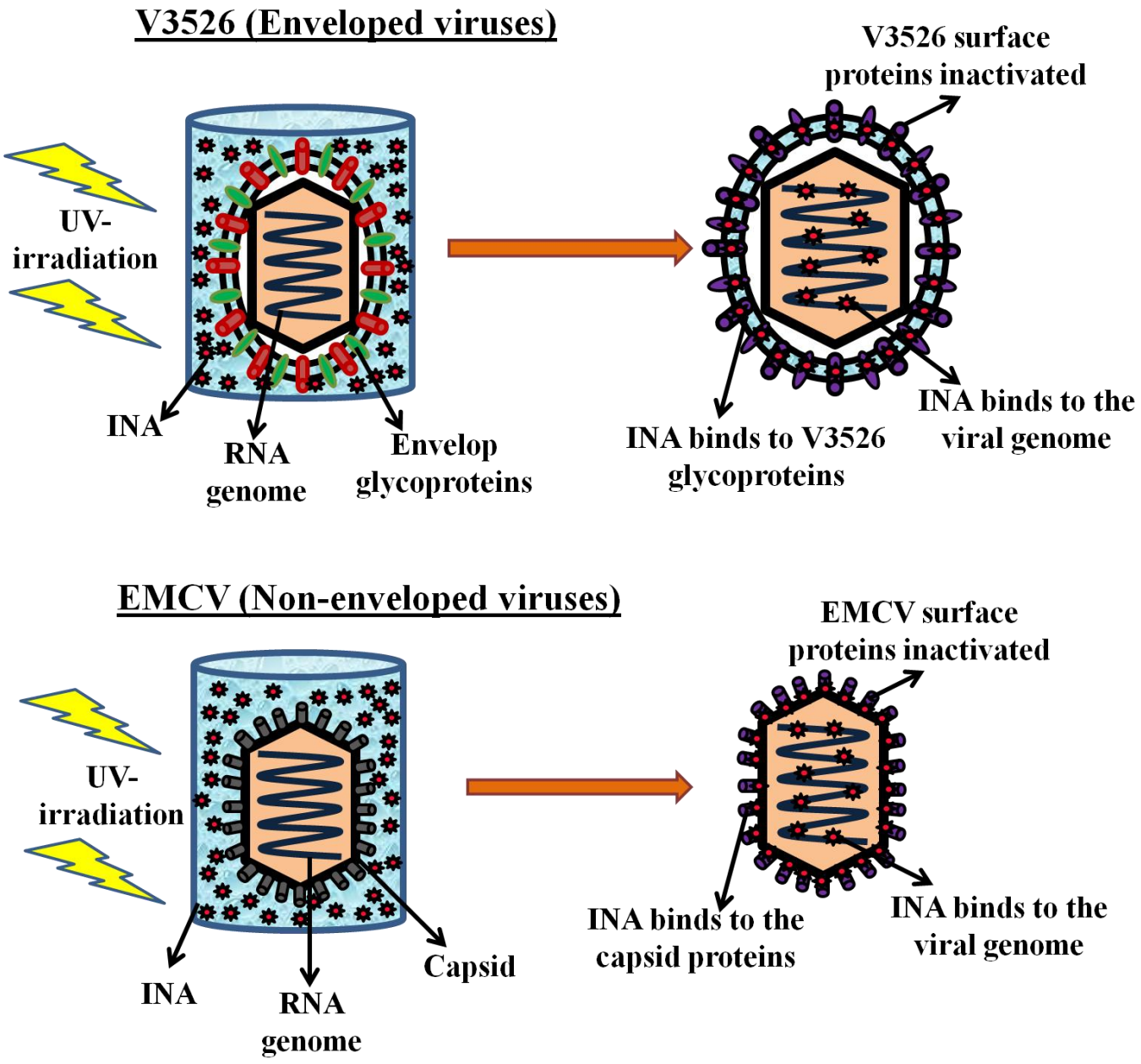


Figure-49 Proposed model for the dual-mechanism of INA-inactivation

References

1. Achan, J., Talisuna, A.O., Erhart, A., Yeka, A., Tibenderana, J.K., Baliraine, F.N., Rosenthal, P.J., D'Alessandro, U., 2011. Quinine, an old anti-malarial drug in a modern world: role in the treatment of malaria. Malaria journal 10, 144.

2. Aguilar, P.V., Greene, I.P., Coffey, L.L., Medina, G., Moncayo, A.C., Anishchenko, M., Ludwig, G.V., Turell, M.J., O'Guinn, M.L., Lee, J., Tesh, R.B., Watts, D.M., Russell, K.L., Hice, C., Yanoviak, S., Morrison, A.C., Klein, T.A., Dohm, D.J., Guzman, H., Travassos da Rosa, A.P., Guevara, C., Kochel, T., Olson, J., Cabezas, C., Weaver, S.C., 2004. Endemic Venezuelan equine encephalitis in northern Peru. Emerging infectious diseases 10, 880-888.

3. Alevizatos, A.C., McKinney, R.W., Feigin, R.D., 1967. Live, attenuated Venezuelan equine encephalomyelitis virus vaccine. I. Clinical effects in man. The American journal of tropical medicine and hygiene 16, 762-768.

4. Anishchenko, M., Bowen, R.A., Paessler, S., Austgen, L., Greene, I.P., Weaver, S.C., 2006. Venezuelan encephalitis emergence mediated by a phylogenetically predicted viral mutation. Proceedings of the National Academy of Sciences of the United States of America 103, 4994-4999.

5. Arnold E.,Rossmann M. G. The use of molecular-replacement phases for the refinement of the human rhinovirus 14 structure. Acta Crystallogr A. 1988, 44 (Pt 3):270-82

6. Atasheva, S., Garmashova, N., Frolov, I., Frolova, E., 2008. Venezuelan equine encephalitis virus capsid protein inhibits nuclear import in Mammalian but not in mosquito cells. Journal of virology 82, 4028-4041.

7. Beck, C.E., Wyckoff, R.W., 1938. VENEZUELAN EQUINE ENCEPHALOMYELITIS. Science (New York, N.Y.) 88, 530.

8. Bercovici, T., Gitler, C., 1978. 5-[125I]Iodonaphthyl azide, a reagent to determine the penetration of proteins into the lipid bilayer of biological membranes. Biochemistry 17, 1484-1489.

9. Bernard, K.A., Klimstra, W.B., Johnston, R.E., 2000. Mutations in the E2 glycoprotein of Venezuelan equine encephalitis virus confer heparan sulfate interaction, low morbidity, and rapid clearance from blood of mice. Virology 276, 93-103.

10. Bhomia, M., Balakathiresan, N., Sharma, A., Gupta, P., Biswas, R., Maheshwari, R., 2010. Analysis of microRNAs induced by Venezuelan equine encephalitis virus infection in mouse brain. Biochemical and biophysical research communications 395, 11-16.

11. Bigler, W.J., Lewis, A.L., Wellings, F.M., 1974. Experimental infection of the cotton mouse (Peromyscus gossypinus) with Venezuelan equine encephalomyelitis virus. The American journal of tropical medicine and hygiene 23, 1185-1188.

12. Blinkova, O., Kapoor, A., Victoria, J., Jones, M., Wolfe, N., Naeem, A., Shaukat, S., Sharif, S., Alam, M.M., Angez, M., Zaidi, S., Delwart, E.L., 2009. Cardioviruses are genetically diverse and cause common enteric infections in South Asian children. Journal of virology 83, 4631-4641.

13. Bowen, G.S., Fashinell, T.R., Dean, P.B., Gregg, M.B., 1976. Clinical aspects of human Venezuelan equine encephalitis in Texas. Bulletin of the Pan American Health Organization 10, 46-57.
14. Briceno Rossi, A.L., 1965. [Study of dengue and interferon]. Revista venezolana de sanidad y asistencia social 30, 41-46.
15. Brooke, C.B., Schafer, A., Matsushima, G.K., White, L.J., Johnston, R.E., 2012. Early activation of the host complement system is required to restrict central nervous system invasion and limit neuropathology during Venezuelan equine encephalitis virus infection. The Journal of general virology 93, 797-806.
16. Brown, D.T., Hernandez, R., 2012. Infection of cells by alphaviruses. Advances in experimental medicine and biology 726, 181-199.
17. Brown, F., 1993. Review of accidents caused by incomplete inactivation of viruses. Developments in biological standardization 81, 103-107.
18. Caley, I.J., Betts, M.R., Irlbeck, D.M., Davis, N.L., Swanstrom, R., Frelinger, J.A., Johnston, R.E., 1997. Humoral, mucosal, and cellular immunity in response to a human immunodeficiency virus type 1 immunogen expressed by a Venezuelan equine encephalitis virus vaccine vector. Journal of virology 71, 3031-3038.
19. Calisher, C.H., Maness, K.C., 1974. Virulence of Venezuelan equine encephalomyelitis virus subtypes for various laboratory hosts. Applied microbiology 28, 881-884.
20. Calisher, C.H., Monath, T.P., Mitchell, C.J., Sabattini, M.S., Cropp, C.B., Kerschner, J., Hunt, A.R., Lazuick, J.S., 1985. Arbovirus investigations in Argentina, 1977-1980. III. Identification and characterization of viruses isolated, including new subtypes of western and Venezuelan equine encephalitis viruses and four new bunyaviruses (Las Maloyas, Resistencia, Barranqueras, and Antequera). The American journal of tropical medicine and hygiene 34, 956-965.
21. Cao, L., Zhang, X., Tuo, W., 2011. Tunicamycins, a class of nucleoside antibiotics similar to corynetoxins of the Rathayibacter toxicus, increase susceptibility of mice to Neospora caninum. Veterinary parasitology 177, 13-19.
22. Casals, J., Curnen, E.C., Thomas, L., 1943. VENEZUELAN EQUINE ENCEPHALOMYELITIS IN MAN. The Journal of experimental medicine 77, 521-530.
23. Casamassima, A.C., Hess, L.W., Marty, A., 1987. TC-83 Venezuelan equine encephalitis vaccine exposure during pregnancy. Teratology 36, 287-289.
24. Charles, P.C., Brown, K.W., Davis, N.L., Hart, M.K., Johnston, R.E., 1997. Mucosal immunity induced by parenteral immunization with a live attenuated Venezuelan equine encephalitis virus vaccine candidate. Virology 228, 153-160.
25. Charles, P.C., Trgovcich, J., Davis, N.L., Johnston, R.E., 2001. Immunopathogenesis and immune modulation of Venezuelan equine encephalitis virus-induced disease in the mouse. Virology 284, 190-202.
26. Charles, P.C., Walters, E., Margolis, F., Johnston, R.E., 1995. Mechanism of neuroinvasion of Venezuelan equine encephalitis virus in the mouse. Virology 208, 662-671.
27. Cook, I.F., 1985. Herpes zoster in children following malaria. The Journal of tropical medicine and hygiene 88, 261-264.
28. Cooper, R.G., Magwere, T., 2008. Chloroquine: novel uses & manifestations. The Indian journal of medical research 127, 305-316.
29. Czechowicz, J., Huaman, J.L., Forshey, B.M., Morrison, A.C., Castillo, R., Huaman, A., Caceda, R., Eza, D., Rocha, C., Blair, P.J., Olson, J.G., Kochel, T.J., 2011. Prevalence and

risk factors for encephalomyocarditis virus infection in Peru. Vector borne and zoonotic diseases (Larchmont, N.Y.) 11, 367-374.

30. Davis, N.L., Brown, K.W., Greenwald, G.F., Zajac, A.J., Zacny, V.L., Smith, J.F., Johnston, R.E., 1995. Attenuated mutants of Venezuelan equine encephalitis virus containing lethal mutations in the PE2 cleavage signal combined with a second-site suppressor mutation in E1. Virology 212, 102-110.

31. Davis, N.L., Brown, K.W., Johnston, R.E., 1996. A viral vaccine vector that expresses foreign genes in lymph nodes and protects against mucosal challenge. Journal of virology 70, 3781-3787.

32. Davis, N.L., Caley, I.J., Brown, K.W., Betts, M.R., Irlbeck, D.M., McGrath, K.M., Connell, M.J., Montefiori, D.C., Frelinger, J.A., Swanstrom, R., Johnson, P.R., Johnston, R.E., 2000. Vaccination of macaques against pathogenic simian immunodeficiency virus with Venezuelan equine encephalitis virus replicon particles. Journal of virology 74, 371-378.

33. Davis, N.L., Grieder, F.B., Smith, J.F., Greenwald, G.F., Valenski, M.L., Sellon, D.C., Charles, P.C., Johnston, R.E., 1994. A molecular genetic approach to the study of Venezuelan equine encephalitis virus pathogenesis. Archives of virology. Supplementum 9, 99-109.

34. Davis, N.L., Powell, N., Greenwald, G.F., Willis, L.V., Johnson, B.J., Smith, J.F., Johnston, R.E., 1991. Attenuating mutations in the E2 glycoprotein gene of Venezuelan equine encephalitis virus: construction of single and multiple mutants in a full-length cDNA clone. Virology 183, 20-31.

35. de la Monte, S., Castro, F., Bonilla, N.J., Gaskin de Urdaneta, A., Hutchins, G.M., 1985. The systemic pathology of Venezuelan equine encephalitis virus infection in humans. The American journal of tropical medicine and hygiene 34, 194-202.

36. De Lamballerie, X., Boisson, V., Reynier, J.C., Enault, S., Charrel, R.N., Flahault, A., Roques, P., Le Grand, R., 2008. On chikungunya acute infection and chloroquine treatment. Vector borne and zoonotic diseases (Larchmont, N.Y.) 8, 837-839.

37. Delogu, I., de Lamballerie, X., 2011. Chikungunya disease and chloroquine treatment. Journal of medical virology 83, 1058-1059.

38. DeTulleo, L., Kirchhausen, T., 1998. The clathrin endocytic pathway in viral infection. The EMBO journal 17, 4585-4593.

39. Esen, N., Blakely, P.K., Rainey-Barger, E.K., Irani, D.N., 2012. Complexity of the microglial activation pathways that drive innate host responses during lethal alphavirus encephalitis in mice. ASN neuro.

40. Estrada-Franco, J.G., Navarro-Lopez, R., Freier, J.E., Cordova, D., Clements, T., Moncayo, A., Kang, W., Gomez-Hernandez, C., Rodriguez-Dominguez, G., Ludwig, G.V., Weaver, S.C., 2004. Venezuelan equine encephalitis virus, southern Mexico. Emerging infectious diseases 10, 2113-2121.

41. Estrada-Franco, J.G., Navarro-Lopez, R., Freier, J.E., Cordova, D., Clements, T., Moncayo, A., Kang, W., Gomez-Hernandez, C., Rodriguez-Dominguez, G., Ludwig, G.V., Weaver, S.C., 2004. Venezuelan equine encephalitis virus, southern Mexico. Emerging infectious diseases 10, 2113-2121.

42. Fauquet, C. M., Mayo M. A., Maniloff J., Desselberger U., and Ball L. A., *Virus taxonomy. 8th reports of the International Committee on Taxonomy of Viruses Academic Press*, 2005.

43. Fine, D.L., Roberts, B.A., Teehee, M.L., Terpening, S.J., Kelly, C.L., Raetz, J.L., Baker, D.C., Powers, A.M., Bowen, R.A., 2007. Venezuelan equine encephalitis virus vaccine candidate (V3526) safety, immunogenicity and efficacy in horses. Vaccine 25, 1868-1876.
44. Frankel, A.D., Pabo, C.O., 1988. Cellular uptake of the tat protein from human immunodeficiency virus. Cell 55, 1189-1193.
45. Freiberg, A.N., Worthy, M.N., Lee, B., Holbrook, M.R., 2010. Combined chloroquine and ribavirin treatment does not prevent death in a hamster model of Nipah and Hendra virus infection. The Journal of general virology 91, 765-772.
46. Galappaththy, G.N., Omari, A.A., Tharyan, P., 2007. Primaquine for preventing relapses in people with Plasmodium vivax malaria. Cochrane database of systematic reviews (Online), CD004389.
47. Garmashova, N., Atasheva, S., Kang, W., Weaver, S.C., Frolova, E., Frolov, I., 2007. Analysis of Venezuelan equine encephalitis virus capsid protein function in the inhibition of cellular transcription. Journal of virology 81, 13552-13565.
48. Garmashova, N., Gorchakov, R., Volkova, E., Paessler, S., Frolova, E., Frolov, I., 2007. The Old World and New World alphaviruses use different virus-specific proteins for induction of transcriptional shutoff. Journal of virology 81, 2472-2484.
49. Gitler, C., Bercovici, T., 1980. Use of lipophilic photoactivatable reagents to identify the lipid-embedded domains of membrane proteins. Annals of the New York Academy of Sciences 346, 199-211.
50. Gleiser, C.A., Gochenour, W.S., Jr., Berge, T.O., Tigertt, W.D., 1962. The comparative pathology of experimental Venezuelan equine encephalomyelitis infection in different animal hosts. The Journal of infectious diseases 110, 80-97.
51. Grieder, F.B., Davis, B.K., Zhou, X.D., Chen, S.J., Finkelman, F.D., Gause, W.C., 1997. Kinetics of cytokine expression and regulation of host protection following infection with molecularly cloned Venezuelan equine encephalitis virus. Virology 233, 302-312.
52. Grieder, F.B., Davis, N.L., Aronson, J.F., Charles, P.C., Sellon, D.C., Suzuki, K., Johnston, R.E., 1995. Specific restrictions in the progression of Venezuelan equine encephalitis virus-induced disease resulting from single amino acid changes in the glycoproteins. Virology 206, 994-1006.
53. Grieder, F.B., Vogel, S.N., 1999. Role of interferon and interferon regulatory factors in early protection against Venezuelan equine encephalitis virus infection. Virology 257, 106-118.
54. Griffin, D.E., Levine, B., Ubol, S., Hardwick, J.M., 1994. The effects of alphavirus infection on neurons. Annals of neurology 35 Suppl, S23-27.
55. Gupta, P., Husain, M.M., Shankar, R., Maheshwari, R.K., 2002. Lead exposure enhances virus multiplication and pathogenesis in mice. Veterinary and human toxicology 44, 205-210.
56. Gupta, P., Seth, P., Husain, M.M., Puri, S.K., Maheshwari, R.K., 2006. Co-infection by Semliki forest virus and malarial parasite modulates viral multipucation, pathogenesis and cytokines in mice. Parasite (Paris, France) 13, 251-255.
57. Guzman, H., Ding, X., Xiao, S.Y., Tesh, R.B., 2005. Duration of infectivity and RNA of Venezuelan equine encephalitis, West Nile, and yellow fever viruses dried on filter paper and maintained at room temperature. The American journal of tropical medicine and hygiene 72, 474-477.

58. Hammamieh, R., Barmada, M., Ludwig, G., Peel, S., Koterski, N., Jett, M., 2007. Blood genomic profiles of exposures to Venezuelan equine encephalitis in Cynomolgus macaques (Macaca fascicularis). Virology journal 4, 82.
59. Hanson, R.P., Sulkin, S.E., Beuscher, E.L., Hammon, W.M., McKinney, R.W., Work, T.H., 1967. Arbovirus infections of laboratory workers. Extent of problem emphasizes the need for more effective measures to reduce hazards. Science (New York, N.Y.) 158, 1283-1286.
60. Hardy, W.R., Hahn, Y.S., de Groot, R.J., Strauss, E.G., Strauss, J.H., 1990. Synthesis and processing of the nonstructural polyproteins of several temperature-sensitive mutants of Sindbis virus. Virology 177, 199-208.
61. Hart, M.K., Caswell-Stephan, K., Bakken, R., Tammariello, R., Pratt, W., Davis, N., Johnston, R.E., Smith, J., Steele, K., 2000. Improved mucosal protection against Venezuelan equine encephalitis virus is induced by the molecularly defined, live-attenuated V3526 vaccine candidate. Vaccine 18, 3067-3075.
62. Hart, M.K., Pratt, W., Panelo, F., Tammariello, R., Dertzbaugh, M., 1997. Venezuelan equine encephalitis virus vaccines induce mucosal IgA responses and protection from airborne infection in BALB/c, but not C3H/HeN mice. Vaccine 15, 363-369.
63. Hawley, R.J., Eitzen, E.M., Jr., 2001. Biological weapons--a primer for microbiologists. Annual review of microbiology 55, 235-253.
64. Hayashi, T., Chaichoune, K., Patchimasiri, T., Hiromoto, Y., Kawasaki, Y., Wiriyarat, W., Chakritbudsabong, W., Prayoonwong, N., Chaisilp, N., Parchariyanon, S., Ratanakorn, P., Uchida, Y., Tsuda, T., Saito, T., 2011. Differential host gene responses in mice infected with two highly pathogenic avian influenza viruses of subtype H5N1 isolated from wild birds in Thailand. Virology 412, 9-18.
65. Helenius, A., Kartenbeck, J., Simons, K., Fries, E., 1980. On the entry of Semliki forest virus into BHK-21 cells. The Journal of cell biology 84, 404-420.
66. Henderson, B.E., Chappell, W.A., Johnston, J.G., Jr., Sudia, W.D., 1971. Experimental infection of horses with three strains of Venezuelan equine encephalomyelitis virus. I. Clinical and virological studies. American journal of epidemiology 93, 194-205.
67. Holowka, D., Gitler, C., Bercovici, T., Metzger, H., 1981. Reaction of 5-iodonaphthyl-1-nitrene with the IgE receptor on normal and tumour mast cells. Nature 289, 806-808.
68. Hoppe, J., Friedl, P., Jorgensen, B.B., 1983. [125I]Iodonaphtylazide labeling selectively a cysteine residue in the F0 of the ATP-synthase from E. coli is unsuitable for topographic studies of membrane proteins. FEBS letters 160, 239-242.
69. Huang, I.C., Bailey, C.C., Weyer, J.L., Radoshitzky, S.R., Becker, M.M., Chiang, J.J., Brass, A.L., Ahmed, A.A., Chi, X., Dong, L., Longobardi, L.E., Boltz, D., Kuhn, J.H., Elledge, S.J., Bavari, S., Denison, M.R., Choe, H., Farzan, M., 2011. Distinct patterns of IFITM-mediated restriction of filoviruses, SARS coronavirus, and influenza A virus. PLoS pathogens 7, e1001258.
70. HuangFu, W.C., Liu, J., Harty, R.N., Fuchs, S.Y., 2008. Cigarette smoking products suppress anti-viral effects of Type I interferon via phosphorylation-dependent downregulation of its receptor. FEBS letters 582, 3206-3210.
71. Irani, D.N., Prow, N.A., 2007. Neuroprotective interventions targeting detrimental host immune responses protect mice from fatal alphavirus encephalitis. Journal of neuropathology and experimental neurology 66, 533-544.

72. Jackson, A.C., Rossiter, J.P., 1997. Apoptotic cell death is an important cause of neuronal injury in experimental Venezuelan equine encephalitis virus infection of mice. Acta neuropathologica 93, 349-353.
73. Jackson, A.C., SenGupta, S.K., Smith, J.F., 1991. Pathogenesis of Venezuelan equine encephalitis virus infection in mice and hamsters. Veterinary pathology 28, 410-418.
74. Johnson, K.M., Martin, D.H., 1974. Venezuelan equine encephalitis. Advances in veterinary science and comparative medicine 18, 79-116.
75. Junn, E., Mouradian, M.M., 2012. MicroRNAs in neurodegenerative diseases and their therapeutic potential. Pharmacology & therapeutics 133, 142-150.
76. Kahane, I., Gitler, C., 1978. Red cell membrane glycophorin labeling from within the lipid bilayer. Science (New York, N.Y.) 201, 351-352.
77. Kaufmann, A.F., Meltzer, M.I., Schmid, G.P., 1997. The economic impact of a bioterrorist attack: are prevention and postattack intervention programs justifiable? Emerging infectious diseases 3, 83-94.
78. Keyaerts, E., Li, S., Vijgen, L., Rysman, E., Verbeeck, J., Van Ranst, M., Maes, P., 2009. Antiviral activity of chloroquine against human coronavirus OC43 infection in newborn mice. Antimicrobial agents and chemotherapy 53, 3416-3421.
79. Kielian, M., 2006. Class II virus membrane fusion proteins. Virology 344, 38-47.
80. Kielian, M., Chanel-Vos, C., Liao, M., 2010. Alphavirus Entry and Membrane Fusion. Viruses 2, 796-825.
81. King, A. M. Q., Brown, F., Christian, P., Hovi, T., Hyypiä, T., Knowles, N.J., Lemon, S.M., Minor, P.D., Palmenberg, A.C., Skern, T. and Stanway, G.. Picornaviridae. In *Virus Taxonomy. Seventh Report of the International Committee for the Taxonomy of Viruses*, M. H. V. Van Regenmortel, Fauquet, C.M., Bishop, D.H.L., Calisher, C.H., Carsten, E.B., Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R. and Wickner, R.B (Ed.), Academic Press New-York, San Diego 2000, 657-673.
82. Kinney, R.M., Pfeffer, M., Tsuchiya, K.R., Chang, G.J., Roehrig, J.T., 1998. Nucleotide sequences of the 26S mRNAs of the viruses defining the Venezuelan equine encephalitis antigenic complex. The American journal of tropical medicine and hygiene 59, 952-964.
83. Kolykhalov, A.A., Frolov, I.V., Agapov, E.V., Netesov, S.V., Sandakhchiev, L.S., 1992. [Obtaining infectious Venezuelan equine encephalomyelitis virus based on a full length DNA copy of its genome]. Doklady Akademii nauk / [Rossiiskaia akademii nauk] 327, 160-164.
84. Konopka, J.L., Penalva, L.O., Thompson, J.M., White, L.J., Beard, C.W., Keene, J.D., Johnston, R.E., 2007. A two-phase innate host response to alphavirus infection identified by mRNP-tagging in vivo. PLoS pathogens 3, e199.
85. Koterski, J., Twenhafel, N., Porter, A., Reed, D.S., Martino-Catt, S., Sobral, B., Crasta, O., Downey, T., DaSilva, L., 2007. Gene expression profiling of nonhuman primates exposed to aerosolized Venezuelan equine encephalitis virus. FEMS immunology and medical microbiology 51, 462-472.
86. Kubes, V., Rios, F.A., 1939. THE CAUSATIVE AGENT OF INFECTIOUS EQUINE ENCEPHALOMYELITIS IN VENEZUELA. Science (New York, N.Y.) 90, 20-21.
87. Kublin, J.G., Patnaik, P., Jere, C.S., Miller, W.C., Hoffman, I.F., Chimbiya, N., Pendame, R., Taylor, T.E., Molyneux, M.E., 2005. Effect of Plasmodium falciparum malaria on concentration of HIV-1-RNA in the blood of adults in rural Malawi: a prospective cohort study. Lancet 365, 233-240.

88. Lederer, S., Favre, D., Walters, K.A., Proll, S., Kanwar, B., Kasakow, Z., Baskin, C.R., Palermo, R., McCune, J.M., Katze, M.G., 2009. Transcriptional profiling in pathogenic and non-pathogenic SIV infections reveals significant distinctions in kinetics and tissue compartmentalization. PLoS pathogens 5, e1000296.
89. Lee, R.C., Feinbaum, R.L., Ambros, V., 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75, 843-854.
90. Li, Q., Means, R., Lang, S., Jung, J.U., 2007. Downregulation of gamma interferon receptor 1 by Kaposi's sarcoma-associated herpesvirus K3 and K5. Journal of virology 81, 2117-2127.
91. Lobigs, M., Zhao, H.X., Garoff, H., 1990. Function of Semliki Forest virus E3 peptide in virus assembly: replacement of E3 with an artificial signal peptide abolishes spike heterodimerization and surface expression of E1. Journal of virology 64, 4346-4355.
92. Lu, J., Pan, Q., Rong, L., He, W., Liu, S.L., Liang, C., 2011. The IFITM proteins inhibit HIV-1 infection. Journal of virology 85, 2126-2137.
93. Ludwig, G.V., Kondig, J.P., Smith, J.F., 1996. A putative receptor for Venezuelan equine encephalitis virus from mosquito cells. Journal of virology 70, 5592-5599.
94. Ludwig, G.V., Turell, M.J., Vogel, P., Kondig, J.P., Kell, W.K., Smith, J.F., Pratt, W.D., 2001. Comparative neurovirulence of attenuated and non-attenuated strains of Venezuelan equine encephalitis virus in mice. The American journal of tropical medicine and hygiene 64, 49-55.
95. Maheshwari, R.K., Husain, M.M., Attallah, A.M., Friedman, R.M., 1983. Tunicamycin treatment inhibits the antiviral activity of interferon in mice. Infection and immunity 41, 61-66.
96. Maheshwari, R.K., Srikantan, V., Bhartiya, D., 1991. Chloroquine enhances replication of Semliki Forest virus and encephalomyocarditis virus in mice. Journal of virology 65, 992-995.
97. Maheshwari, R.K., Srikantan, V., Bhartiya, D., Puri, S.K., Dutta, G.P., Dhawan, B.N., 1990. Effects of interferon in malaria infection. Immunology letters 25, 53-57.
98. Maheshwari, R.K., Tandon, R.N., Feuillette, A.R., Mahouy, G., Badillet, G., Friedman, R.M., 1988. Interferon inhibits Aspergillus fumigatus growth in mice: an activity against an extracellular infection. Journal of interferon research 8, 35-44.
99. Monlux, W.S., Luedke, A.J., 1973. Brain and spinal cord lesions in horses inoculated with Venezuelan equine encephalomyelitis virus (epidemic American and Trinidad strains). American journal of veterinary research 34, 465-473.
100. Mori, I., Goshima, F., Ito, H., Koide, N., Yoshida, T., Yokochi, T., Kimura, Y., Nishiyama, Y., 2005. The vomeronasal chemosensory system as a route of neuroinvasion by herpes simplex virus. Virology 334, 51-58.
101. Morrison, A.C., Forshey, B.M., Notyce, D., Astete, H., Lopez, V., Rocha, C., Carrion, R., Carey, C., Eza, D., Montgomery, J.M., Kochel, T.J., 2008. Venezuelan equine encephalitis virus in Iquitos, Peru: urban transmission of a sylvatic strain. PLoS neglected tropical diseases 2, e349.
102. Morrison, A.C., Forshey, B.M., Notyce, D., Astete, H., Lopez, V., Rocha, C., Carrion, R., Carey, C., Eza, D., Montgomery, J.M., Kochel, T.J., 2008. Venezuelan equine encephalitis virus in Iquitos, Peru: urban transmission of a sylvatic strain. PLoS neglected tropical diseases 2, e349.

103. Navarro, J.C., Medina, G., Vasquez, C., Coffey, L.L., Wang, E., Suarez, A., Biord, H., Salas, M., Weaver, S.C., 2005. Postepizootic persistence of Venezuelan equine encephalitis virus, Venezuela. Emerging infectious diseases 11, 1907-1915.
104. Navarro, J.C., Medina, G., Vasquez, C., Coffey, L.L., Wang, E., Suarez, A., Biord, H., Salas, M., Weaver, S.C., 2005. Postepizootic persistence of Venezuelan equine encephalitis virus, Venezuela. Emerging infectious diseases 11, 1907-1915.
105. Oberste, M.S., Fraire, M., Navarro, R., Zepeda, C., Zarate, M.L., Ludwig, G.V., Kondig, J.F., Weaver, S.C., Smith, J.F., Rico-Hesse, R., 1998. Association of Venezuelan equine encephalitis virus subtype IE with two equine epizootics in Mexico. The American journal of tropical medicine and hygiene 59, 100-107.
106. O'Carroll, D., Schaefer, A., 2012. General Principals of miRNA Biogenesis and Regulation in the Brain. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology.
107. Olweny, C.L., Atine, I., Kaddu-Mukasa, A., Owor, R., Andersson-Anvret, M., Klein, G., Henle, W., de-The, G., 1977. Epstein-Barr virus genome studies in Burkitt's and non-Burkitt's lymphomas in Uganda. Journal of the National Cancer Institute 58, 1191-1196.
108. Ooi, E.E., Chew, J.S., Loh, J.P., Chua, R.C., 2006. In vitro inhibition of human influenza A virus replication by chloroquine. Virology journal 3, 39.
109. Paessler, S., Fayzulin, R.Z., Anishchenko, M., Greene, I.P., Weaver, S.C., Frolov, I., 2003. Recombinant sindbis/Venezuelan equine encephalitis virus is highly attenuated and immunogenic. Journal of virology 77, 9278-9286.
110. Pallister, J., Middleton, D., Crameri, G., Yamada, M., Klein, R., Hancock, T.J., Foord, A., Shiell, B., Michalski, W., Broder, C.C., Wang, L.F., 2009. Chloroquine administration does not prevent Nipah virus infection and disease in ferrets. Journal of virology 83, 11979-11982.
111. Paredes, A., Alwell-Warda, K., Weaver, S.C., Chiu, W., Watowich, S.J., 2001. Venezuelan equine encephalomyelitis virus structure and its divergence from old world alphaviruses. Journal of virology 75, 9532-9537.
112. Paredes, A., Alwell-Warda, K., Weaver, S.C., Chiu, W., Watowich, S.J., 2003. Structure of isolated nucleocapsids from venezuelan equine encephalitis virus and implications for assembly and disassembly of enveloped virus. Journal of virology 77, 659-664.
113. Paton, N.I., Lee, L., Xu, Y., Ooi, E.E., Cheung, Y.B., Archuleta, S., Wong, G., Wilder-Smith, A., 2011. Chloroquine for influenza prevention: a randomised, double-blind, placebo controlled trial. The Lancet infectious diseases 11, 677-683.
114. Pittman, P.R., Makuch, R.S., Mangiafico, J.A., Cannon, T.L., Gibbs, P.H., Peters, C.J., 1996. Long-term duration of detectable neutralizing antibodies after administration of live-attenuated VEE vaccine and following booster vaccination with inactivated VEE vaccine. Vaccine 14, 337-343.
115. Powers, A.M., Brault, A.C., Shirako, Y., Strauss, E.G., Kang, W., Strauss, J.H., Weaver, S.C., 2001. Evolutionary relationships and systematics of the alphaviruses. Journal of virology 75, 10118-10131.
116. Pratt, W.D., Davis, N.L., Johnston, R.E., Smith, J.F., 2003. Genetically engineered, live attenuated vaccines for Venezuelan equine encephalitis: testing in animal models. Vaccine 21, 3854-3862.
117. Pritchard, A.E., Strom, T., Lipton, H.L., 1992. Nucleotide sequence identifies Vilyuisk virus as a divergent Theiler's virus. Virology 191, 469-472.

118. Puri, S.K., Maheshwari, R.K., Dutta, G.P., Friedman, R.M., Dhar, M.M., 1988. Human interferon-gamma protects rhesus monkeys against sporozoite-induced Plasmodium cynomolgi malaria infection. Journal of interferon research 8, 201-206.
119. Quiroz, E., Aguilar, P.V., Cisneros, J., Tesh, R.B., Weaver, S.C., 2009. Venezuelan equine encephalitis in Panama: fatal endemic disease and genetic diversity of etiologic viral strains. PLoS neglected tropical diseases 3, e472.
120. Rao, V., Hinz, M.E., Roberts, B.A., Fine, D., 2004. Environmental hazard assessment of Venezuelan equine encephalitis virus vaccine candidate strain V3526. Vaccine 22, 2667-2673.
121. Raviv, Y., Bercovici, T., Gitler, C., Salomon, Y., 1984. Selective photoinduced uncoupling of the response of adenylate cyclase to gonadotropins by 5-iodonaphthyl 1-azide. Biochemistry 23, 503-508.
122. Raviv, Y., Blumenthal, R., Tompkins, S.M., Humberd, J., Hogan, R.J., Viard, M., 2008. Hydrophobic inactivation of influenza viruses confers preservation of viral structure with enhanced immunogenicity. Journal of virology 82, 4612-4619.
123. Raviv, Y., Salomon, Y., Gitler, C., Bercovici, T., 1987. Selective labeling of proteins in biological systems by photosensitization of 5-iodonaphthalene-1-azide. Proceedings of the National Academy of Sciences of the United States of America 84, 6103-6107.
124. Raviv, Y., Viard, M., Bess, J.W., Jr., Chertova, E., Blumenthal, R., 2005. Inactivation of retroviruses with preservation of structural integrity by targeting the hydrophobic domain of the viral envelope. Journal of virology 79, 12394-12400.
125. Reed, D.S., Lind, C.M., Lackemeyer, M.G., Sullivan, L.J., Pratt, W.D., Parker, M.D., 2005. Genetically engineered, live, attenuated vaccines protect nonhuman primates against aerosol challenge with a virulent IE strain of Venezuelan equine encephalitis virus. Vaccine 23, 3139-3147.
126. Rico-Hesse, R., Weaver, S.C., de Siger, J., Medina, G., Salas, R.A., 1995. Emergence of a new epidemic/epizootic Venezuelan equine encephalitis virus in South America. Proceedings of the National Academy of Sciences of the United States of America 92, 5278-5281.
127. Rintahaka, J., Wiik, D., Kovanen, P.E., Alenius, H., Matikainen, S., 2008. Cytosolic antiviral RNA recognition pathway activates caspases 1 and 3. Journal of immunology (Baltimore, Md. : 1950) 180, 1749-1757.
128. Rivas, F., Diaz, L.A., Cardenas, V.M., Daza, E., Bruzon, L., Alcala, A., De la Hoz, O., Caceres, F.M., Aristizabal, G., Martinez, J.W., Revelo, D., De la Hoz, F., Boshell, J., Camacho, T., Calderon, L., Olano, V.A., Villarreal, L.I., Roselli, D., Alvarez, G., Ludwig, G., Tsai, T., 1997. Epidemic Venezuelan equine encephalitis in La Guajira, Colombia, 1995. The Journal of infectious diseases 175, 828-832.
129. Ryman, K.D., Klimstra, W.B., 2008. Host responses to alphavirus infection. Immunological reviews 225, 27-45.
130. Ryman, K.D., Meier, K.C., Nangle, E.M., Ragsdale, S.L., Korneeva, N.L., Rhoads, R.E., MacDonald, M.R., Klimstra, W.B., 2005. Sindbis virus translation is inhibited by a PKR/RNase L-independent effector induced by alpha/beta interferon priming of dendritic cells. Journal of virology 79, 1487-1499.
131. Ryzhikov, A.B., Ryabchikova, E.I., Sergeev, A.N., Tkacheva, N.V., 1995. Spread of Venezuelan equine encephalitis virus in mice olfactory tract. Archives of virology 140, 2243-2254.

132. Sanmartin-Barberi, C., Groot, H., Osorno-Mesa, E., 1954. Human epidemic in Colombia caused by the Venezuelan equine encephalomyelitis virus. The American journal of tropical medicine and hygiene 3, 283-293.
133. Savarino, A., Boelaert, J.R., Cassone, A., Majori, G., Cauda, R., 2003. Effects of chloroquine on viral infections: an old drug against today's diseases? The Lancet infectious diseases 3, 722-727.
134. Sawicki, D.L., Perri, S., Polo, J.M., Sawicki, S.G., 2006. Role for nsP2 proteins in the cessation of alphavirus minus-strand synthesis by host cells. Journal of virology 80, 360-371.
135. Sawicki, D.L., Perri, S., Polo, J.M., Sawicki, S.G., 2006. Role for nsP2 proteins in the cessation of alphavirus minus-strand synthesis by host cells. Journal of virology 80, 360-371.
136. Schafer, A., Brooke, C.B., Whitmore, A.C., Johnston, R.E., 2011. The role of the blood-brain barrier during Venezuelan equine encephalitis virus infection. Journal of virology 85, 10682-10690.
137. Scherr, M., Venturini, L., Battmer, K., Schaller-Schoenitz, M., Schaefer, D., Dallmann, I., Ganser, A., Eder, M., 2007. Lentivirus-mediated antagomir expression for specific inhibition of miRNA function. Nucleic acids research 35, e149.
138. Schmid, S., Fuchs, R., Kielian, M., Helenius, A., Mellman, I., 1989. Acidification of endosome subpopulations in wild-type Chinese hamster ovary cells and temperature-sensitive acidification-defective mutants. The Journal of cell biology 108, 1291-1300.
139. Schoneboom, B.A., Catlin, K.M., Marty, A.M., Grieder, F.B., 2000. Inflammation is a component of neurodegeneration in response to Venezuelan equine encephalitis virus infection in mice. Journal of neuroimmunology 109, 132-146.
140. Schoneboom, B.A., Fultz, M.J., Miller, T.H., McKinney, L.C., Grieder, F.B., 1999. Astrocytes as targets for Venezuelan equine encephalitis virus infection. Journal of neurovirology 5, 342-354.
141. Seth, P., Husain, M.M., Gupta, P., Schoneboom, A., Grieder, B.F., Mani, H., Maheshwari, R.K., 2003. Early onset of virus infection and up-regulation of cytokines in mice treated with cadmium and manganese. Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine 16, 359-368.
142. Seth, P., Mani, H., Singh, A.K., Banaudha, K.K., Madhavan, S., Sidhu, G.S., Gaddipati, J.P., Vogel, S.N., Maheshwari, R.K., 1999. Acceleration of viral replication and up-regulation of cytokine levels by antimalarials: implications in malaria-endemic areas. The American journal of tropical medicine and hygiene 61, 180-186.
143. Sharma, A., Bhattacharya, B., Puri, R.K., Maheshwari, R.K., 2008. Venezuelan equine encephalitis virus infection causes modulation of inflammatory and immune response genes in mouse brain. BMC genomics 9, 289.
144. Sharma, A., Bhomia, M., Honnold, S.P., Maheshwari, R.K., 2011. Role of adhesion molecules and inflammation in Venezuelan equine encephalitis virus infected mouse brain. Virology journal 8, 197.
145. Sharma, A., Maheshwari, R.K., 2009. Oligonucleotide array analysis of Toll-like receptors and associated signalling genes in Venezuelan equine encephalitis virus-infected mouse brain. The Journal of general virology 90, 1836-1847.

146. Sharma, A., Raviv, Y., Puri, A., Viard, M., Blumenthal, R., Maheshwari, R.K., 2007. Complete inactivation of Venezuelan equine encephalitis virus by 1,5-iodonaphthylazide. Biochemical and biophysical research communications 358, 392-398.
147. Shope, 1976. Alphaviruses, 2 edn. Raven Press: New York.
148. Shubladze, A.K., SIa, G., Gavrilov, V.I., 1959. [Virological studies on laboratory cases of Venezuelan equine encephalomyelitis]. Voprosy virusologii 4, 305-310.
149. Simmons, J.D., White, L.J., Morrison, T.E., Montgomery, S.A., Whitmore, A.C., Johnston, R.E., Heise, M.T., 2009. Venezuelan equine encephalitis virus disrupts STAT1 signaling by distinct mechanisms independent of host shutoff. Journal of virology 83, 10571-10581.
150. Singh, A.K., Sidhu, G.S., Friedman, R.M., Maheshwari, R.K., 1996. Mechanism of enhancement of the antiviral action of interferon against herpes simplex virus-1 by chloroquine. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research 16, 725-731.
151. Singh, V.K., Damewood, G.P.t., Friedman, R.M., Maheshwari, R.K., 1987. Tunicamycin enhances virus replication and inhibits antiviral activity of interferon in mice: correlation with natural killer cells. Journal of experimental pathology 3, 19-33.
152. Sperber, K., Chiang, G., Chen, H., Ross, W., Chusid, E., Gonchar, M., Chow, R., Liriano, O., 1997. Comparison of hydroxychloroquine with zidovudine in asymptomatic patients infected with human immunodeficiency virus type 1. Clinical therapeutics 19, 913-923.
153. Spertzel, R.O., Crabbs, C.L., Vaughn, R.E., 1972. Transplacental transmission of Venezuelan equine encephalomyelitis virus in mice. Infection and immunity 6, 339-343.
154. Steele, K.E., Davis, K.J., Stephan, K., Kell, W., Vogel, P., Hart, M.K., 1998. Comparative neurovirulence and tissue tropism of wild-type and attenuated strains of Venezuelan equine encephalitis virus administered by aerosol in C3H/HeN and BALB/c mice. Veterinary pathology 35, 386-397.
155. Steele, K.E., Seth, P., Catlin-Lebaron, K.M., Schoneboom, B.A., Husain, M.M., Grieder, F., Maheshwari, R.K., 2006. Tunicamycin enhances neuroinvasion and encephalitis in mice infected with Venezuelan equine encephalitis virus. Veterinary pathology 43, 904-913.
156. Strauss E.G., Strauss J.H., 1986. Structure and Replication of Alphavirus Genome. Plenum Press: New York.
157. Strauss, J.H., Strauss, E.G., 1994. The alphaviruses: gene expression, replication, and evolution. Microbiological reviews 58, 491-562.
158. Strauss, J.H., Wang, K.S., Schmaljohn, A.L., Kuhn, R.J., Strauss, E.G., 1994. Host-cell receptors for Sindbis virus. Archives of virology. Supplementum 9, 473-484.
159. Takatsuki, A., Arima, K., Tamura, G., 1971. Tunicamycin, a new antibiotic. I. Isolation and characterization of tunicamycin. The Journal of antibiotics 24, 215-223.
160. Tandon, R.N., Feuillette, A.R., Mahouy, G., Badillet, G., Friedman, R.M., Maheshwari, R.K., 1988. Interferon protects mice against an extracellular infection of Aspergillus fumigatus. Annals of the New York Academy of Sciences 544, 409-411.
161. Tatfeng, Y.M., Ihongbe, J.C., Okodua, M., Oviasogie, E., Isibor, J., Tchougang, S., Tambo, E., Otegbeye, T., 2007. CD4 count, viral load and parasite density of HIV positive individuals undergoing malaria treatment with dihydroartemisinin in Benin City, Edo state, Nigeria. Journal of vector borne diseases 44, 111-115.
162. Tricou, V., Minh, N.N., Van, T.P., Lee, S.J., Farrar, J., Wills, B., Tran, H.T., Simmons, C.P., 2010. A randomized controlled trial of chloroquine for the treatment of dengue in Vietnamese adults. PLoS neglected tropical diseases 4, e785.

163. Tsvetanova, B.C., Kiemle, D.J., Price, N.P., 2002. Biosynthesis of tunicamycin and metabolic origin of the 11-carbon dialdose sugar, tunicamine. The Journal of biological chemistry 277, 35289-35296.
164. Turell, M.J., Parker, M.D., 2008. Protection of hamsters by Venezuelan equine encephalitis virus candidate vaccine V3526 against lethal challenge by mosquito bite and intraperitoneal injection. The American journal of tropical medicine and hygiene 78, 328-332.
165. Vandenbroucke, E., Mehta, D., Minshall, R., Malik, A.B., 2008. Regulation of endothelial junctional permeability. Annals of the New York Academy of Sciences 1123, 134-145.
166. Viard, M., Ablan, S.D., Zhou, M., Veenstra, T.D., Freed, E.O., Raviv, Y., Blumenthal, R., 2008. Photoinduced reactivity of the HIV-1 envelope glycoprotein with a membrane-embedded probe reveals insertion of portions of the HIV-1 Gp41 cytoplasmic tail into the viral membrane. Biochemistry 47, 1977-1983.
167. Vigerust, D.J., McCullers, J.A., 2007. Chloroquine is effective against influenza A virus in vitro but not in vivo. Influenza and other respiratory viruses 1, 189-192.
168. Vilcarromero, S., Aguilar, P.V., Halsey, E.S., Laguna-Torres, V.A., Razuri, H., Perez, J., Valderrama, Y., Gotuzzo, E., Suarez, L., Cespedes, M., Kochel, T.J., 2010. Venezuelan equine encephalitis and 2 human deaths, Peru. Emerging infectious diseases 16, 553-556.
169. Vogel, P., Abplanalp, D., Kell, W., Ibrahim, M.S., Downs, M.B., Pratt, W.D., Davis, K.J., 1996. Venezuelan equine encephalitis in BALB/c mice: kinetic analysis of central nervous system infection following aerosol or subcutaneous inoculation. Archives of pathology & laboratory medicine 120, 164-172.
170. Vogel, P., Petterson, D.S., Berry, P.H., Frahn, J.L., Anderton, N., Cockrum, P.A., Edgar, J.A., Jago, M.V., Lanigan, G.W., Payne, A.L., Culvenor, C.C., 1981. Isolation of a group of glycolipid toxins from seedheads of annual ryegrass Lolium rigidum Gaud.) infected by Corynebacterium rathayi. The Australian journal of experimental biology and medical science 59, 455-467.
171. Waarts, B.L., Bittman, R., Wilschut, J., 2002. Sphingolipid and cholesterol dependence of alphavirus membrane fusion. Lack of correlation with lipid raft formation in target liposomes. The Journal of biological chemistry 277, 38141-38147.
172. Warfield, K.L., Swenson, D.L., Olinger, G.G., Kalina, W.V., Viard, M., Aitichou, M., Chi, X., Ibrahim, S., Blumenthal, R., Raviv, Y., Bavari, S., Aman, M.J., 2007. Ebola virus inactivation with preservation of antigenic and structural integrity by a photoinducible alkylating agent. The Journal of infectious diseases 196 Suppl 2, S276-283.
173. Watts, D.M., Callahan, J., Rossi, C., Oberste, M.S., Roehrig, J.T., Wooster, M.T., Smith, J.F., Cropp, C.B., Gentrau, E.M., Karabatsos, N., Gubler, D., Hayes, C.G., 1998. Venezuelan equine encephalitis febrile cases among humans in the Peruvian Amazon River region. The American journal of tropical medicine and hygiene 58, 35-40.
174. Watts, D.M., Lavera, V., Callahan, J., Rossi, C., Oberste, M.S., Roehrig, J.T., Cropp, C.B., Karabatsos, N., Smith, J.F., Gubler, D.J., Wooster, M.T., Nelson, W.M., Hayes, C.G., 1997. Venezuelan equine encephalitis and Oropouche virus infections among Peruvian army troops in the Amazon region of Peru. The American journal of tropical medicine and hygiene 56, 661-667.
175. Weaver, S.C., Barrett, A.D., 2004. Transmission cycles, host range, evolution and emergence of arboviral disease. Nature reviews. Microbiology 2, 789-801.
176. Weaver, S.C., Ferro, C., Barrera, R., Boshell, J., Navarro, J.C., 2004. Venezuelan equine encephalitis. Annual review of entomology 49, 141-174.

177. Weaver, S.C., Salas, R., Rico-Hesse, R., Ludwig, G.V., Oberste, M.S., Boshell, J., Tesh, R.B., 1996. Re-emergence of epidemic Venezuelan equine encephalomyelitis in South America. VEE Study Group. Lancet 348, 436-440.

178. Weidner, J.M., Jiang, D., Pan, X.B., Chang, J., Block, T.M., Guo, J.T., 2010. Interferon-induced cell membrane proteins, IFITM3 and tetherin, inhibit vesicular stomatitis virus infection via distinct mechanisms. Journal of virology 84, 12646-12657.

179. Wengler, G., 2002. In vitro analysis of factors involved in the disassembly of Sindbis virus cores by 60S ribosomal subunits identifies a possible role of low pH. The Journal of general virology 83, 2417-2426.

180. Wengler, G., Koschinski, A., Dreyer, F., 2003. Entry of alphaviruses at the plasma membrane converts the viral surface proteins into an ion-permeable pore that can be detected by electrophysiological analyses of whole-cell membrane currents. The Journal of general virology 84, 173-181.

181. Wengler, G., Koschinski, A., Repp, H., 2004. During entry of alphaviruses, the E1 glycoprotein molecules probably form two separate populations that generate either a fusion pore or ion-permeable pores. The Journal of general virology 85, 1695-1701.

182. White D.O., Fenner F.J., 1994. Togaviridae, 4 edn. Academic Press: San Diego.

183. White, L.J., Wang, J.G., Davis, N.L., Johnston, R.E., 2001. Role of alpha/beta interferon in Venezuelan equine encephalitis virus pathogenesis: effect of an attenuating mutation in the 5' untranslated region. Journal of virology 75, 3706-3718.

184. Yang, A.X., Mejido, J., Bhattacharya, B., Petersen, D., Han, J., Kawasaki, E.S., Puri, R.K., 2006. Analysis of the quality of contact-pin fabricated oligonucleotide microarrays. Molecular biotechnology 34, 303-315.

185. Yin, J., Gardner, C.L., Burke, C.W., Ryman, K.D., Klimstra, W.B., 2009. Similarities and differences in antagonism of neuron alpha/beta interferon responses by Venezuelan equine encephalitis and Sindbis alphaviruses. Journal of virology 83, 10036-10047.

186. Yoon, J.W., Jun, H.S., 2006. Viruses cause type 1 diabetes in animals. Annals of the New York Academy of Sciences 1079, 138-146.

187. Young, N.A., Johnson, K.M., 1969. Antigenic variants of Venezuelan equine encephalitis virus: their geographic distribution and epidemiologic significance. American journal of epidemiology 89, 286-307.

188. Yu, D., Marchiando, A.M., Weber, C.R., Raleigh, D.R., Wang, Y., Shen, L., Turner, J.R., 2010. MLCK-dependent exchange and actin binding region-dependent anchoring of ZO-1 regulate tight junction barrier function. Proceedings of the National Academy of Sciences of the United States of America 107, 8237-8241.

189. Zehmer, R.B., Dean, P.B., Sudia, W.D., Calisher, C.H., Sather, G.E., Parker, R.L., 1974. Venezuelan equine encephalitis epidemic in Texas, 1971. Health services reports 89, 278-282.

190. Zhang, K., Kaufman, R.J., 2008. Identification and characterization of endoplasmic reticulum stress-induced apoptosis in vivo. Methods in enzymology 442, 395-419.

191. Zhang, Y., Burke, C.W., Ryman, K.D., Klimstra, W.B., 2007. Identification and characterization of interferon-induced proteins that inhibit alphavirus replication. Journal of virology 81, 11246-11255.

192. Zoll, J., Erkens Hulshof, S., Lanke, K., Verduyn Lunel, F., Melchers, W.J., Schoondermark-van de Ven, E., Roivainen, M., Galama, J.M., van Kuppeveld, F.J., 2009.

Saffold virus, a human Theiler's-like cardiovirus, is ubiquitous and causes infection early in life. PLoS pathogens 5, e1000416.

List of Publications

List of manuscripts published:

1. Sharma A, **Gupta P**, Maheshwari RK. Inactivation of Chikungunya virus by 1,5 iodonapthyl azide. Virol J. 2012 Dec 4;9:301.
2. Gaidamakova EK, Myles IA, McDaniel DP, Fowler CJ, Valdez PA, Gayen M, **Gupta P,** Sharma A, Glass PJ, Maheshwari RK, Datta SK and Daly MJ. Genome destruction without loss of immunogenicity in irradiated vaccine preparations of viruses or bacteria. Cell Host Microbe. July 2012.
3. Sharma A, **Gupta P**, Glass PJ, Parker MD, Maheshwari RK. Safety and protective efficacy of INA-inactivated Venezuelan equine encephalitis virus: implication in vaccine development. Vaccine. 2011 Jan 29;29(5):953-9. Epub 2010 Nov 27.
4. Bhomia M, Balakathiresan N, Sharma A, **Gupta P**, Biswas R, Maheshwari R. Analysis of miRNAs induced by Venezuelan equine encephalitis virus infection in mouse brain. Biochem Biophys Res Commun. 2010 Apr 23;395(1):11-6. Epub 2010 Mar 19.

List of manuscripts communicated:

1. **Paridhi Gupta,** Anuj Sharma, Manish Bhomia, Jing Han, Amy Yang, Raj K Puri and Radha K Maheshwari. Identification of novel host derived factors associated with neuroinvasion of Venezuelan equine encephalitis virus in mice. (Journal of General Virology)

2. **Paridhi Gupta,** Anuj Sharma, Yossef Raviv, Mathias Viard, Robert M Blumenthal and Radha K Maheshwari. 1,5 iodonapthyl azide inactivates non-enveloped virus but does not protect mice from challenge. (Journal of virology)
3. Veena Menon[§], **Paridhi Gupta**[§], Nagaraja Balakathiresan, Anuj Sharma, Manish Bhomia, Jing Han, Raj K Puri and Radha K Maheshwari. Molecular mechanism(s) of enhanced pathogenesis of viruses by chemotherapeutic agents: public health implications (§ equal amount of work). (American Journal of Tropical Medicine and Hygeine)

List of manuscripts in preparation:

1. **Paridhi Gupta,** Anuj Sharma, Jing Han, Raj K Puri and Radha K Maheshwari. Comparative host responses associated with completely and partially neurovirulent Venezuelan equine encephalitis virus infection in mice.

List of Oral Abstracts Presented

1. Development of Second Generation Inactivated Alphavirus Vaccine. **Paridhi Gupta**, Anuj Sharma, Shelley P Honnold, Manoshi Gayen, Elena K Gaidamakova, Yossef Raviv, Mathias Viard, Kevin B. Spurgers, Robert Blumethal, Michael Parker, Pamela J Glass, Michael J Daly and Radha K Maheshwari. American Society of Microbiology Biodefense and Emerging Infectious Diseases, Feburary 2013.

2. Novel Approaches for the Inactivation of Viruses for the Development of Second Generation Vaccine. **Paridhi Gupta**, Anuj Sharma, Shelley P Honnold, Manoshi Gayen, Elena K Gaidamakova, Yossef Raviv, Mathias Viard, Kevin B. Spurgers, Robert Blumethal, Michael Parker, Pamela J Glass, Michael J Daly and Radha K Maheshwari. 2nd World Congress on Virology, August 2012.

3. Identification of potential host derived factors associated with neuroinvasion and neurovirulence by Venezuelan equine encephalitis virus, **Paridhi Gupta**, Anuj Sharma, Manish Bhomia, Jing Han, Amy Yang, Raj K. Puri and Radha K. Maheshwari, 10th Annual meeting of American Society of Microbiology Biodefense and Emerging Infectious Diseases, February 2012.

4. Comparative host gene responses contributing towards neuroinvasion and neurovirulence by Venezuelan equine encephalitis virus, **Paridhi Gupta**, Anuj Sharma, Veena Menon, Manish Bhomia, Jing Han, Raj K. Puri and Radha K. Maheshwari 2011 Chemical and Biological Defense Science and Technology Conference, November 2011

5. Gene expression microarrays identified host derived factors associated with neuroinvasion of Venezuelan equine encephalitis virus in mice, **Paridhi Gupta**, Veena Menon, Anuj Sharma, Manish Bhomia, Jing Han, Raj K. Puri and Radha K. Maheshwari. The American Society for Virology 30th Annual Meeting, July 2011.

6. Identification of host derived factors involved in neuroinvasion by Venezuelan equine encephalitis virus, **Paridhi Gupta**, Anuj Sharma, Veena Menon, Manish Bhomia, Jing Han, Raj K. Puri and Radha K. Maheshwari. Chemical and Biological Defense Science and Technology Conference, November 2010.

List of Poster Abstracts Presented

1. Identification of potential host derived factors associated with neuroinvasion and neurovirulence by Venezuelan equine encephalitis virus, **Paridhi Gupta**, Anuj Sharma, Manish Bhomia, Jing Han, Amy Yang, Raj K. Puri and Radha K. Maheshwari, 10th Annual meeting of American society of Microbiology Biodefense and emerging infectious diseases, February 2012.
2. Second-Generation Inactivated Vaccines Protect Mice from Lethal Venezuelan Equine Encephalitis Virus Infection, Kevin B. Spurgers, Shelley P. Honnold, Russell R. Bakken, Jeffrey W. Cohen, Lori T. Rowan, Cathleen M Lind, **Paridhi Gupta**, Anuj Sharma, Radha Maheshwari and Pamela J. Glass, 10th Annual meeting of American society of Microbiology Biodefense and emerging infectious diseases, February 2012.
3. Radioprotective Mn-DP-Pi Complexes Preserve Immunogenicity of Inactivated VEEV during Gamma Irradiation: A New Approach for Vaccine Development. Manoshi Gayen, Elena K Gaidamakova, **Paridhi Gupta**, Anuj Sharma, Pamela J Glass, Michael J Daly and Radha K Maheshwari1. 10th Annual meeting of American society of Microbiology Biodefense and emerging infectious diseases, February 2012.
4. Novel targeted approaches for virus inactivation: Towards better vaccine development. Anuj Sharma, **Paridhi Gupta**, Shelley P Honnold, Elena K Gaidamakova, Manoshi Gayen , Yosef Raviv, Mathias Viard, Robert Blumethal, Michael Parker, Pamela J Glass Michael J Daly and Radha K Maheshwari 2011 Chemical and Biological Defense Science and Technology Conference, November 2011.

5. Comparative host gene responses contributing towards neuroinvasion and neurovirulence by Venezuelan equine encephalitis virus, **Paridhi Gupta**, Anuj Sharma, Veena Menon, Manish Bhomia, Jing Han, Raj K. Puri and Radha K. Maheshwari 2011 Chemical and Biological Defense Science and Technology Conference, November 2011
6. Gene expression microarrays identified host derived factors associated with neuroinvasion of Venezuelan equine encephalitis virus in mice, **Paridhi Gupta**, Veena Menon, Anuj Sharma, Manish Bhomia, Jing Han, Raj K. Puri and Radha K. Maheshwari, The American Society for Virology 30th Annual Meeting, July 2011.
7. Identification of potential host derived factors associated with neuroinvasion of Venezuelan equine encephalitis virus in mice, **Paridhi Gupta**, Veena Menon, Anuj Sharma, Manish Bhomia, Jing Han, Raj K. Puri and Radha K. Maheshwari, USU Research Week, May 2011.
8. Identification of host derived factors involved in neuroinvasion by Venezuelan equine encephalitis virus, **Paridhi Gupta**, Anuj Sharma, Veena Menon, Manish Bhomia, Jing Han, Raj K. Puri and Radha K. Maheshwari 2010 Chemical and Biological Defense Science and Technology Conference, November 2010.
9. A novel strategy for Inactivation of Encephalomyocarditis Virus: Implications for Development of Vaccines against Infections of Livestock. **Paridhi Gupta**, Anuj Sharma, Viard Mathias, Yossef Raviv, Robert Blumenthal and Radha K. Maheshwari, USUHS Research Week, 2010.
10. Complete inactivation of Venezuelan Equine Encephalitis Virus by 1, 5 iodonaphthyl-azide: A novel approach for vaccine development **Paridhi Gupta**, Anuj Sharma, Pamela J. Glass,

Michael D. Parker and Radha K. Maheshwari, American Society of Microbiology Biodefense and emerging infectious diseases, 2010.

11. Analysis of Gene Expression Profile in Tunicamycin treated Mice Infected with Venezuelan Equine Encephalitis virus: Correlation with Increased Pathogenesis. Veena Menon, **Paridhi Gupta,** Anuj Sharma, Manish Bhomia and Radha K. Maheshwari. USUHS Research Week, 2010.

12. Analysis of MicroRNAs induced by Venezuelan Equine Encephalitis virus infection in mouse brain. Bhomia M, Balakathiresan N, Sharma A, **Gupta P**, Biswas R, Maheshwari R. Presented at annual meeting of microRNA in Human Disease and Development, 2010.

13. Expression profile of microRNAs in mouse brain upon VEEV infection. Manish Bhomia, Nagaraja Balakathiresan, Anuj Sharma, **Paridhi Gupta**, Roopa Biswas and Radha K Maheshwari. Presented at annual meeting of American Society of Microbiology Biodefense and emerging infectious diseases, 2010.

14. Inhibition of Venezuelan equine encephalitis virus by artificial microRNA targeting virus polymerase. Manish Bhomia, Anuj Sharma, **Paridhi Gupta** and Radha K Maheshwari. Presented at Annual Meeting for American Society of Virology, 2009.

15. Inhibition of Venezuelan Equine Encephalitis Virus Replication by Short Interfering RNA Directed Against the Viral Polymerase Manish Bhomia, Anuj Sharma, **Paridhi Gupta** and Radha Maheshwari. Presented at annual meeting of American Society of Microbiology Biodefense and emerging infectious diseases, 2008.

List of Scholarships Received

1. Selected for oral abstract presentation and received student travel award during Chemical and Biological Defense Science and Technology Conference, November 2011.

2. Selected for oral abstract presentation and received student travel award during The American Society for Virology 30th Annual Meeting, July 2011.

Brief Biography of the Candidate

Paridhi Gupta completed her Bachelor of Technology in Biotechnology from Uttar Pradesh Technical University, Lucknow in 2005. She has been a merit scholarship recipient during her bachelors degree for several years. After that, she pursued Master of Engineering in Biological Sciences from Birla Institute of Technology and Science, Pilani (Pilani campus). As part of her dissertation during her masters degree, she was involved with studies in the field of structural biology at Central Drug Research Institute, Lucknow for a period of 6 months. Subsequently, she was selected for a vertical transfer to PhD program at Birla Institute of Technology and Science, Pilani under a collaborative program at Uniformed Services University of the Health Sciences, USA. Since 2007, she has been involved with studying the various host responses triggered upon Venezuelan Equine Encephalitis Virus for identifying therapeutic drug development targets and evaluation of vaccine development strategies. She has also won several scholarships and travel awards for her research work over the years.

Brief Biography of the Supervisor

Prof. Radha K Maheshwari, received his Bachelor in Science degree in Biological Sciences from Lucknow University, India followed by a Masters of Science degree in Biological Sciences from Birla Institute of Technology and Sciences, Pilani in 1970. Thereafter, he pursued his PhD from Central Drug Reseach Institute, Lucknow, India in the field of Virology. He moved to United States of America to under post- doctoral fellowship at National Institutes of Health in 1977. Soon he joined Uniformed Services University of the Health Sciences and has been working there as a Professor of Pathology. He has been appointed as an adjunct BITS faculty member to coordinate and grade the research activity of these students with the preceptor USUHS faculty. Over the past years, he has established highly successful and productive international collaborations with India for the promotion of science as well as institutional capacity building. He has instituted useful, innovative, and comprehensive teaching and research programs with several Government as well as private institutions in India. He has published more than 125 scientific papers in refereed journals with international recognition, under the auspices of this program. These have included, but are not limited to research on: malaria, radiation effects, wound healing, uncontrolled hemorrhage, organisms that may be involved in bioterrorism, cancer, and environmental pollutants. He has mentored several PhD as well as masters students in his laboratory over the years.