CONTEMPORARY AMERICAN ZIKA VIRUS ISOLATES ELICIT DIFFERENTIAL GROWTH KINETICS AND INTERFERON RESPONSES IN HUMAN ASTROCYTOMA CELLS

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master's of Science (M.Sc.)

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November 2017

Acknowledgements

First and foremost, I would like to thank my supervisor Dr. Selena M. Sagan, for accepting me into her laboratory and supporting me throughout the duration of my studies. Her willingness to share her scientific knowledge, while patiently affording me the freedom to explore my interests was crucial to my education and development. She went well beyond her prescribed duties to provide advice and encouragement to all her students, and for this, I will forever be grateful.

I would also like thank to my colleagues Sophie Cousineau, Ashley Campbell, Nate Taylor, Julie Magnus, Annabelle Vlaun, as well as the members of the Richer Lab for providing a welcoming work environment. I would like to specifically thank my co-author and friend Trisha Barnard, who I was very fortunate to have worked with. Additionally, I would like to offer a special thank you to Jasmin Chahal and Patrick Lypaczewski for their friendship and generosity over the past few years. Importantly, I would like to thank my loving companion Annie Bernier for her patience, compassion, and support.

I am grateful to my Thesis advisory committee members, Dr. Marc Fabian and Dr. Chen Liang for their pertinent scientific guidance. I also would like to acknowledge the generous financial support provided by the Max E. Binz Fellowship and the McGill University Department of Microbiology and Immunology, which made my research possible.

Finally, although I am aware that an expression of gratitude would neither be expected nor acknowledged; I nevertheless, offer a sincere thank you to my Father and Mother. My success is a direct product of you many sacrifices and unwavering support. I dedicate this accomplishment to you.

Preface

This document is written in the traditional monograph style in accordance with the McGill University "Preparation of a Thesis" guidelines. It also complies with the thesis guidelines with regards to font size, line spacing, and margins. The thesis is presented in chapter format: Chapter 1 provides the relevant background for this project in the form of a literature review; Chapter 2 introduces the rational and outlines the specific objectives of this study; Chapter 3 describes the methods and materials used in the experimental design; Chapter 4 presents the main results of the investigations and the corresponding figures; and Chapter 5 discusses the findings in context of recent discoveries and concludes the thesis. My supervisor Dr. Selena M. Sagan, provided editorial support in the preparation of this thesis. Other specific contributions are acknowledged at the beginning of each chapter.

Table of Contents

List of Tables

Chapter 3:

List of Figures

Chapter 1:

Chapter 2:

Chapter 4:

List of Abbreviations

Abbreviation	Full Term
AF	African
BCA	bicinchoninic acid
BR	Brazilian
CDN	Canadian
cDNA	complementary Deoxyribonucleic acid
CMC	carboxymethyl cellulose
CO_2	carbon dioxide
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
CPE	cytopathic effect
EMEM	Eagle's minimum essential medium
ER	endoplasmic reticulum
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GAS6	Growth arrest-specific 6
hNPCs	human Neural Progenitor Cells
IFITM1/3	Interferon-induced transmembrane protein 1/3
IFN	Interferon
IgM	Immunoglobulin M
IRF9	Interferon regulatory Factor 9

ISG	interferon stimulated genes
ISGF3	interferon-stimulated gene factor 3
ISRE	interferon-sensitive response element
JAK	Janus kinase
MDA-5	melanoma differentiation-associated protein 5
mRNA	messenger RNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
MxA	myxovirus (Influenza Virus) Resistance 1
NS	non-structural
OAS-1	2'-5'-oligoadenylate synthetase 1
PCR	polymerase chain reaction
PFU	plaque forming units
PR	Puerto Rican
prM	pre-membrane
(p)STAT1	(phospho-) signal transducer and activator of transcription 1
pSTAT2	(phospho-) signal transducer and activator of transcription 2
PVDF	polyvinylidene difluoride membranes
RIG-I	retinoic acid-inducible gene I
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
(RT-)qPCR	(reverse transcription-) quantitative polymerase chain reaction
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM	standard error of the mean
sfRNAs	subgenomic flaviviral RNAs
Sp.	species
UTR	untranslated region
ZIKV	Zika Virus

Abstract

The recent emergence and rapid geographic expansion of Zika virus (ZIKV) poses a significant challenge for public health. Although historically causing only a mild febrile illness, recent ZIKV outbreaks have been associated with more severe neurological complications such as Guillain-Barré syndrome and fetal microcephaly. In this investigation, we demonstrate that the contemporary American ZIKV isolates replicate to higher titers at early time points post-infection and are less cytopathic than the historical African ZIKV strain in a human astrocytoma cell line. Interestingly, we found that that contemporary epidemic isolates differentially induce type I and type III IFN-mediated signaling. Specifically, the contemporary strain elicited a robust induction of several anti-viral ISGs and induced STAT1 and STAT2 protein expression and phosphorylation to a greater extent than both the historical African, and the early Asian isolates. This research suggests that ZIKV isolates may have evolved mechanisms to increase viral replication kinetics without increasing cytopathicity, while also highlighting the importance of cell-intrinsic factors in studies of viral fitness.

Résumé

L'émergence récente et l'expansion géographique rapide du virus Zika (ZIKV) posent un défi important pour la santé publique. Bien qu'historiquement causant seulement une maladie fébrile légère, les récentes épidémies de ZIKV ont été associées à des complications neurologiques plus graves telles que le syndrome de Guillain-Barré et la microcéphalie fœtale. Dans cette étude, nous démontrons que les isolats de ZIKV américains contemporains produisent des charges virales plus élevées au début de l'infection et sont moins cytopathogènes que la souche ZIKV africaine historique dans une lignée cellulaire d'astrocytome humain. De plus, nous avons constaté que les isolats épidémiques contemporains induisent différemment la signalisation médiée par l'IFN de type I et de type III. En effet, la souche contemporaine provoque une robuste induction de plusieurs ISG antiviraux et induit une expression et une phosphorylation des protéines STAT1 et STAT2 dans une plus grande mesure que les isolats africains historiques et que les premiers isolats asiatiques. Cette recherche suggère que les isolats de ZIKV peuvent avoir évolué des mécanismes augmentant la cinétique de la réplication virale sans augmenter les effets cytopathogènes, tout en soulignant l'importance des facteurs intrinsèques aux cellules lors d’études de la capacité réplicative du virus.

Chapter 1: Background

This chapter serves as a **comprehensive review of the relevant literature** regarding Zika virus (ZIKV) and the host innate immune response to ZIKV infection. Sections of this literature review, are adapted with permission from an article published by **Maran Michael Rajah**: (**Rajah MM**, Pardy RD, Condotta SA, Richer MJ, and Sagan SM "ZIKA Virus: Emergence, Phylogenetics, Challenges and Opportunities". (2016) ACS Infectious Diseases, 2(11):763-772). Copyright (2016) American Chemical Society. The segments from the published article used in this review were written by **MMR** with editorial support from Dr. Selena M. Sagan. The phylogenetic tree presented in this chapter was generated by Dr. Selena M. Sagan.

Zika Virus: A Literature Review

1.1 History and Emergence of Zika Virus

Zika Virus (ZIKV) is an emerging pathogen that has recently become a significant global health concern due to its rapid geographical expansion and pathogenesis associated with infection. The virus was initially described in 1947 in the Zika forest region of Uganda, where it was isolated from the blood of sentinel rhesus macaques (1). Serological surveys conducted in West Africa demonstrated the presence of ZIKV antibodies in human populations; however, the first human infection was not reported until 1964 (2, 3). For the latter half of the 20th century, the virus remained in relative obscurity with only isolated human cases reported until the first serious outbreak in 2007 **(Figure 1.1)** (4). During this outbreak, over 73% of the population of Yap Island, Federated States of Micronesia, became infected with ZIKV in a period of 4 months (5, 6). This was followed by a major outbreak in 2013 in French Polynesia; and together, these outbreaks represented the first significant transmission of ZIKV outside of its original endemic regions (6). Since then, ZIKV has been introduced into the Western Hemisphere causing a large epidemic in South America, with localized epidemics in Argentina, Columbia, Brazil, El Salvador, Guatemala, Paraguay, Venezuela, as well as outbreaks in the Southern United States and Singapore (**Figure 1.1)** (6-8). While ZIKV typically causes a mild, self-limiting febrile illness, its explosive spread across South America and its recent association with more severe pathogenesis make ZIKV infection a serious public health concern. The rapid emergence of the virus could be a consequence of recently acquired polymorphisms, thus the current epidemic must be contextualized through phylogenetic analysis.

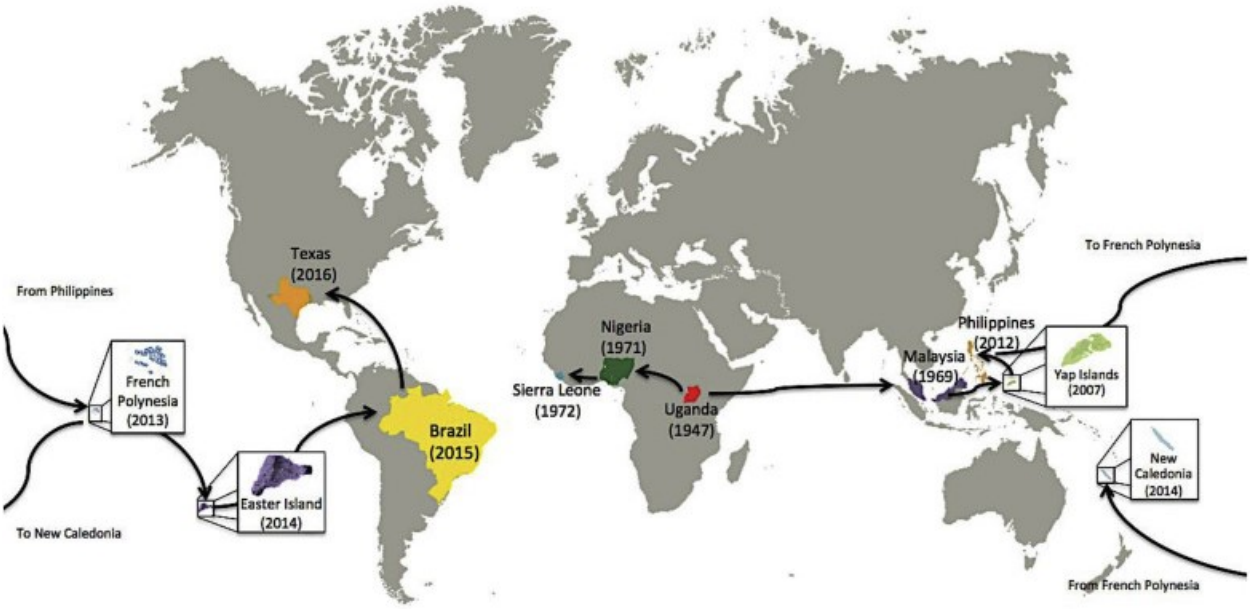

Figure 1.1 The emergence of ZIKV and potential pathways of global spread. The expansion of ZIKV from its equatorial endemic region to causing progressive epidemics in Micronesia (2007), French Polynesia (2013), and Latin America (2015-2016). Adapted from Journal of Autoimmunity, Vol 68, Chang et al., The Zika outbreak of the 21st century, Pages 1-13, Copyright (2016), with permission from Elsevier. https://doi.org/10.1016/j.jaut.2016.02.006 (6).

1.2 ZIKV Phylogenetics

ZIKV is an arbovirus that belongs to the *flavivirus* genus of the *Flaviviridae* family; along with several other clinically relevant human pathogens (e.g., Dengue, West Nile, and yellow fever viruses). Phylogenetic analysis of ZIKV demonstrates the existence of two distinct lineages: African and Asian **(Figure 1.2)**. The African lineage is the oldest of the two lineages, and several isolates have continuously circulated throughout central Africa since 1947 (1, 2). The Asian lineage was known to be endemic to a narrow Southeast Asian equatorial belt since the mid-20th century (6). Interestingly, the contemporary epidemic (2015) isolates have a greater sequence homology to the P6-740 (Malaysia, 1966) strain than any of the African strains (9), suggesting that they are evolutionary descendants of the early Asian lineage **(Figure 1.2)** (9, 10). Furthermore, the epidemic strains in the Americas are more closely related to the H/PF/2013

French Polynesian epidemic strain than the FSM/2007 Micronesia (Yap Island) epidemic strain, suggesting that these sub-lineages may have evolved independently from a common ancestor **(Figure 1.2)** (9). Sequence analyses suggests that the ZIKV outbreak in Singapore (2016), while also descended from the Asian lineage, evolved independently from a strain that was previously circulating in Southeast Asia (9). This suggests that the recent global pandemic is likely a result of several parallel evolutionary changes that occurred in historically circulating ZIKV strains (9). Understanding the phylogenetic differences between the ZIKV lineages and sub-lineages will answer important questions regarding the recent evolutionary changes may have contributed to increased viral pathogenesis and transmission seen in the contemporary outbreaks.

1.3 Transmission of ZIKV

Transmission occurs through both sylvatic and urban cycles involving a variety of *Aedes* species mosquitos and either non-human primates or humans as the amplifying reservoir, respectively **(Figure 1.3)**. It is not yet clear whether other mammals or mosquito species can serve as an amplifying reservoir; but viral replication in cell culture has been demonstrated in a variety of animal cell lines (11). Recent reports have also described ZIKV replication in testicular tissue and excretion in semen suggesting that male-to-female sexual transmission is also a route of ZIKV infection (7, 12-14) **(Figure 1.3)**. There has also been recent case of a suspected female-to-male sexual transmission; however, further investigations are needed to better understand ZIKV infection of the human genital tract and viral shedding in vaginal fluid (15). Furthermore, there is mounting evidence that ZIKV is transmitted from mother to fetus during the course of pregnancy (16, 17) **(Figure 1.3)**. Although transmission of ZIKV through breast milk has not been documented, it has been demonstrated to contain high viral loads suggesting this may

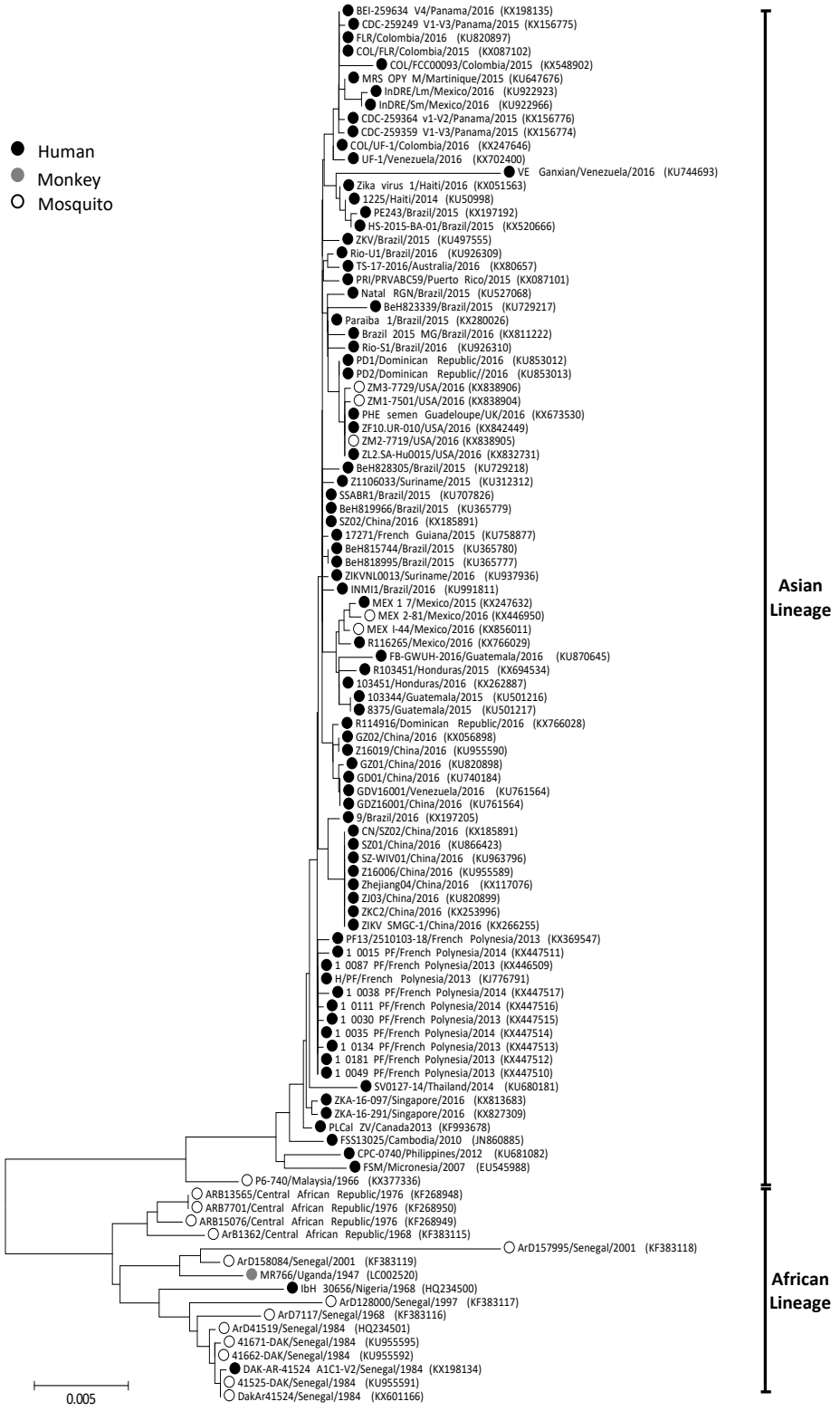

Figure 1.2 Phylogenetic Analysis of ZIKV. Phylogenetic analysis of ZIKV. Translated amino acid sequences of 94 ZIKV polyproteins were aligned using ClustalW. Trees were constructed by neighbor joining of pairwise amino acid distances with the program MEGA7 (according to

the distance scale provided). Bootstrap resampling was used to determine robustness of branches; values of ≥50% (from 1000 replicates) are shown. Human (black circles), monkey (gray circles), and mosquito (open circles) isolates are indicated. Isolate name, country of origin, and year of isolation, as well as the unique accession numbers for each sequence, are indicated. Adapted with permission from (**Rajah MM** *et al.* "ZIKA Virus: Emergence, Phylogenetics, Challenges and Opportunities". (2016) ACS Infectious Diseases, 2(11):763-772). Copyright (2016) American Chemical Society. DOI 10.1021/acsinfecdis.6b00161 (9).

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represent another potential route of transmission (18, 19). A deeper understanding of the amplifying reservoirs for ZIKV and routes of transmission will be critical to preventing further spread and containing the current (2013-2016) epidemic; which has been associated with more severe clinical symptoms.

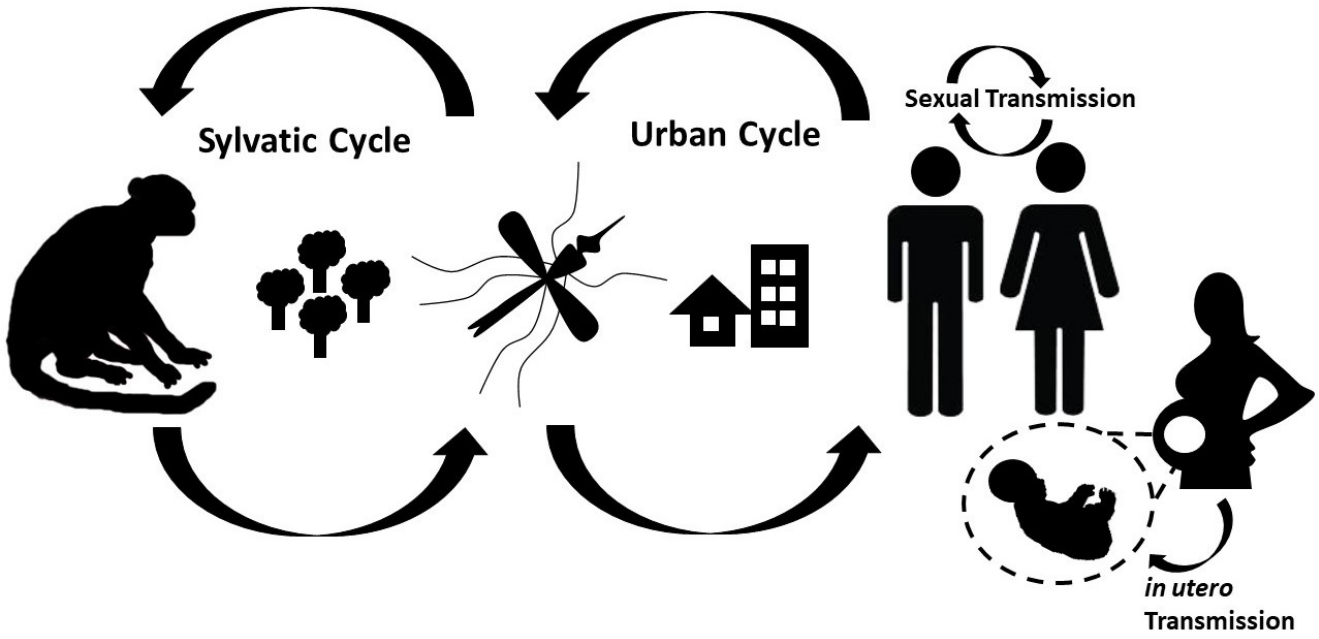

Figure 1.3 Routes of ZIKV transmission. Schematic representation of the pathways of ZIKV transmission including the sylvatic and urban cycles, sexual transmission, and vertical transmission *in utero.*

1.4 Clinical Features of ZIKV Infection

ZIKV replication is first thought to occur in human primary dermal fibroblasts, epidermal keratinocytes and immature dendritic cells at the site of inoculation (20). From the site of the mosquito bite, ZIKV spreads to the draining lymph node where it is amplified and disseminated through the bloodstream to peripheral tissues and visceral organs. ZIKV is detectable in the blood within the first 10 days of infection with viral loads peaking at the onset of symptoms, typically between 2-12 days post-infection (21-23). ZIKV infection has been reported to cause a self-limiting illness that is mostly asymptomatic, but can present with mild symptoms in up to 20% of cases (17). These symptoms typically include fever, maculopapular rash, headache, joint and muscle pain, fatigue and conjunctivitis (5, 6, 21). During the Yap Island outbreak, other symptoms including myalgia, headaches, retro-orbital pain, edema, and vomiting were also reported (5). The presentation of a mild febrile illness can be misdiagnosed as Dengue or Chikungunya virus infection due to their similar clinical presentation (24). In areas of co-circulation of these pathogens, multiple infections are likely very common (25, 26); however, it remains to be seen whether there are synergistic effects due to coinfection, vaccination, or past infections (27-30). In the recent outbreaks, beginning with the 2013 French Polynesia outbreak, ZIKV has been linked to an increase in more severe neurological complications including Guillain-Barré syndrome and fetal microcephaly. The factors contributing to this sudden rise in ZIKV-associated neurological symptoms currently remain unexplained and research efforts are directed at identifying host, pathogen, and environmental factors linked to these complications.

1.4.1 Guillain-Barré Syndrome

Guillain-Barré syndrome is a severe neurological autoimmune disease causing acute or sub-acute flaccid paralysis that is attributable to peripheral nerve damage (31, 32). During the French Polynesia outbreak, there were 48 reported cases of Guillain-Barré syndrome and 88% of the affected patients reported a symptomatic ZIKV infection prior to the onset of neurological symptoms; this was comparatively higher than the 5 cases per annum reported over the previous 4 years (32). A case-controlled study on 42 of these patients demonstrated that all of them had neutralizing antibodies directed against ZIKV (32). Although none of these patients died, 50% of them were still unable to walk without assistance 3 months after discharge. A similar pattern was observed in the 2013-2016 epidemic in Latin America and the Caribbean where the incidence of Guillain-Barré syndrome in seven different countries was reported to be 2 to 9.8-fold higher than baseline (33). Although fatalities among Guillain-Barré syndrome patients are rare, the severity and burden of the disease on the health care system as well as the affected patients and their families suggest that research is needed to better understand the underlying pathology and improve diagnosis and treatment (31, 34).

1.4.2 Fetal Microcephaly

One of the most alarming aspects of the recent outbreaks has been the association of ZIKV infection with an increase in congenital malformations, including fetal microcephaly (a neurodevelopmental disorder that results in malformation of the brain and head), which can result in severe life-long limitations for the child and family (35). Reports from the Brazilian Ministry of Health suggest that there is a 20-fold increase in incidence of microcephaly that

coincides with the current ZIKV epidemic (36). Retrospective studies also suggest that the 2013 French Polynesian outbreak correlated with an increase in the rate of congenital fetal microcephaly (37). Recent studies have strongly linked ZIKV infection during pregnancy to microcephaly, including the detection of viral RNA in amniotic fluid, ZIKV-specific IgM antibodies in the cerebrospinal fluid of microcephalic neonates (indicative of active central nervous system infection), and reports that ZIKV attenuates growth of human neural progenitor cells (hNPCs) (6, 38-40). Furthermore, contemporary ZIKV isolates have been shown to induce premature differentiation of hNPCs; the resulting progenitor depletion leads to cortical thinning which resembles ZIKV-associated microcephaly (41). Although questions still remain about the type of exposure and whether symptomatic or asymptomatic infection poses the greatest risk to the fetus, the first trimester of pregnancy has been identified as the gestational period at major risk for microcephaly (37, 42, 43). This is further supported by a recent study that demonstrated ZIKV-induced apoptosis in first trimester human trophoblasts as well as detrimental effects on trophoblast differentiation (44). In addition to microcephaly, a number of other malformations have been reported in fetuses and newborns with congenital ZIKV infection including: intrauterine growth restriction; brain atrophy; cerebral and placental calcifications; arthrogryposis; and retinal and optic nerve abnormalities (45-48). While recent research has started to decipher some of the mechanisms through which ZIKV can lead to fetal abnormalities, a concerted effort is required to further understand all the factors associated with ZIKV-induced pathogenesis. With recent investigations suggesting that a single polymorphism in the viral pre-membrane protein contributes to fetal microcephaly in mouse models (49), research into the molecular aspects of ZIKV will be necessary to fully understand viral pathogenesis.

1.5 ZIKV Genome

ZIKV is a single-stranded positive-sense RNA virus with a single open reading frame located in between two highly-structured 5' and 3' untranslated regions (UTRs). The genome encodes a single polyprotein that is processed by host and viral proteases into three structural proteins (capsid, pre-membrane (prM), and envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (50) **(Figure 1.4)**. The structural proteins are primarily responsible for forming the virion: the capsid binds to the viral RNA to form a nucleocapsid, the envelope glycoproteins facilitates receptor binding and cell entry, and the prM protein mediates virion maturation during viral assembly and egress (17). The NS1 glycoprotein has multiple oligomeric forms and is involved in viral replication and evading the host immune response. NS2A, NS2B, and NS4A are small hydrophobic proteins that are thought to mediate the assembly of the viral replication complex, with NS2B also acting as a cofactor for NS3 protease. In the related Dengue virus, both the NS1 and NS2A proteins have also been implicated in infectious particle assembly (51-53). The NS3 protein has protease, nucleotide triphosphatase, and helicase activities that mediate polyprotein processing, viral RNA capping, and genome replication and RNA synthesis (54, 55). The NS4A protein in related *flaviviruses*, has been shown to be involved in remodelling of cellular membranes to facilitate the formation of the viral replication complex (56, 57). The function of NS4B is currently unknown, but it has been implicated in alteration of the cellular membranes, viral replication, pathogenesis, and evasion of antiviral signalling in other *flaviviruses* (58). Finally, NS5 is the RNA-dependent RNA polymerase that is required for viral RNA synthesis. NS5 also possesses methyltransferase activities which are involved in viral RNA capping and 2'-O-methylation (59). The intimate

involvement of the multi-functional viral proteins in the ZIKV life cycle makes them major determinants of overall viral fitness.

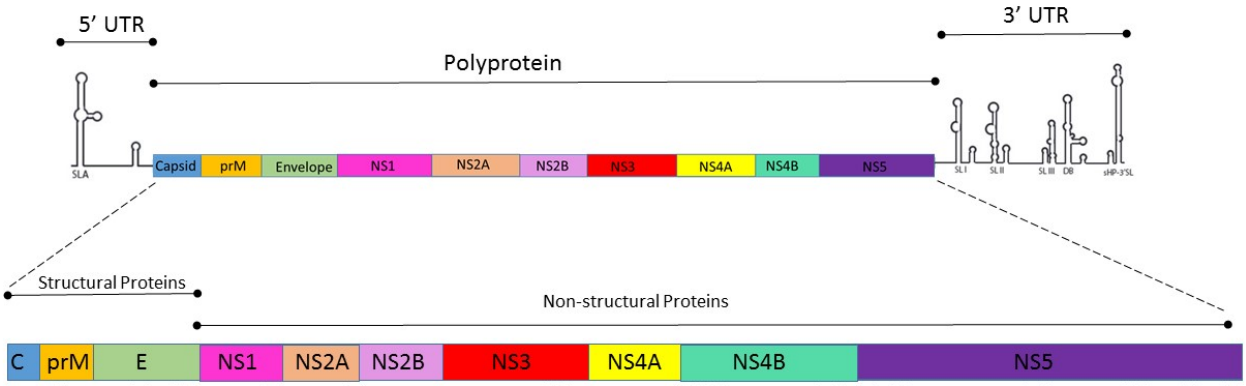

Figure 1.4 Schematic representation of the ZIKV genome. Schematic representation of the ~11 kb ZIKV genome including the 5' and 3' untranslated regions (UTRs), the polyprotein contains three structural proteins (capsid, pre-membrane, envelope), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5).

1.6 ZIKV Life Cycle

The life cycle of ZIKV resembles that of other members of the *flavivirus* genus **(Figure 1.5)**. The mature infectious ZIKV virions enter the cell through receptor-mediated endocytosis (60). Endosomal acidification results in viral fusion with the endosomal membrane and release of the viral RNA into the cytoplasm (60). The viral RNA is then translated into a single polyprotein which is co- and post-translationally processed by host and viral proteases (60, 61). Viral replication takes place on altered cellular membranes and progeny viral RNAs are packaged and bud into the endoplasmic (ER) lumen to form immature viral particles (60). Conformational changes in the immature virions, elicited by changes in pH and the host protease furin, as they

egress through the *trans*-Golgi network, completes the maturation process (60). Mature virions exit the cell through the secretory pathway (61).

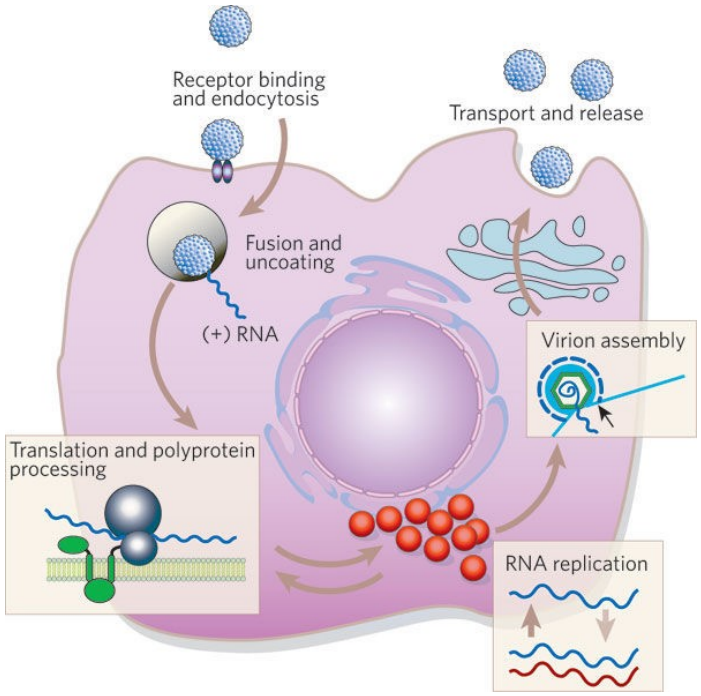

Figure 1.5 *Flavivirus* life cycle. The *flavivirus* life cycle begins with the virus entering the cell through receptor-mediated endocytosis. Acidification of the endosome results in conformational changes in the virion and the release of the viral RNA into the cytoplasm. The viral RNA is translated into a single polyprotein, which is then processed by host and viral proteases into the mature structural and non-structural proteins. The non-structural proteins form the replication complex which mediate viral RNA replication, and progeny viral RNAs are packaged into virions formed by the structural proteins. Mature virions are released through the cells secretory pathway. Reprinted with permission from Macmillan Publishers Ltd: [Nature] (Lindenbach BD, Rice CM. 2005. Unravelling hepatitis C virus replication from genome to function. Nature 436:933-8), copyright (2005). doi:10.1038/nature04077 (61).

1.7 Innate Immune Reponses to ZIKV Infection

The interferon (IFN) system is a crucial component of the innate immune response in mammalian cells that helps to restrict viral infection. Briefly, viral pathogen-associated

molecular patterns, such as viral RNA, are sensed by cellular pathogen recognition receptors, located on the cell surface, endosomal membranes, and within the cytosol. Pathogen sensing ultimately converges on transcription of type I IFNs (62). Type I IFNs are secreted and bind to their cognate receptors on the cell surface to initiate a signaling cascade that results in transcription of IFN-stimulated genes (ISGs) that limit viral replication (62). In order to establish a productive infection, ZIKV must be able to circumvent the host innate immune response. An important countermeasure employed by ZIKV is through the use of subgenomic flaviviral RNAs (sfRNAs), which accumulate during infection when RNA elements resist degradation by a host cellular exonuclease (63). The sfRNAs are able to dampen the IFN response by antagonizing retinoic acid inducible gene I (RIG-I) mediated activity, and to a lesser degree, melanoma differentiation-associated protein 5 (MDA-5) activity (64). Additionally, several ZIKV proteins have been implicated in evasion of the host cell's innate immune response, which ultimately supports ZIKV infection (65). However, limited studies have been performed to date that address the mechanisms by which ZIKV interacts with the IFN system. These studies will provide an important perspective on viral immune evasion and ZIKV pathogenesis.

1.7.1 Type I IFN response to ZIKV infection

During viral infection, viral components are recognized by host pattern recognition receptors resulting in the induction of type I IFNs (IFNα/β), which then bind to a common type I IFN receptor (65). This in turn, stimulates the Janus kinases (JAK) which phosphorylate signal transducer and activator of transcription (STAT) 1 and 2 resulting in the formation of the interferon-stimulated gene factor 3 (ISGF3) complex, composed of STAT1-STAT2 dimers and interferon regulatory factor 9 (IRF9) (65). The translocation of the ISGF3 complex to the nucleus

and subsequent binding to the IFN-stimulated response elements (ISRE) regulates the expression of over 300 ISGs (65). Several of these ISGs including interferon-induced transmembrane proteins (IFITM) 1 and 3, and Viperin have been implicated in restricting ZIKV replication and preventing virus-induced cell death (66-68).

Recent research has demonstrated that ZIKV non-structural proteins can impede the type I IFN response. The ZIKV NS1 and NS4B proteins have been demonstrated to inhibit RIG-I-like receptor signaling contributing to suppression of type I IFN production (69). Additionally, in the related dengue virus, the NS4B protein has also been implicated in inhibiting STAT1 phosphorylation (70). Furthermore, several groups have now shown that the ZIKV NS5 protein is able to attenuate antiviral signaling by targeting the STAT2 protein for proteasomal degradation (71-73). However, an independent investigation has shown that ZIKV infection of monocyte-derived dendritic cells resulted in the induction of the STAT proteins, but also the inhibition of their phosphorylation, with no STAT2 degradation, suggesting that this effect may be host-cell specific (65, 74). Other studies have implicated the NS2B and NS3 proteins in reducing the phosphorylation of STAT1 by facilitating the degradation of JAK (69). Future research will surely uncover novel mechanisms by which ZIKV proteins antagonize the host type I IFN response. Additionally, furthering our understanding of how ZIKV interacts with the type II and type III IFN signalling pathways will help elucidate the complex interactions between the innate and adaptive immune responses to ZIKV infection.

1.7.2 Type II and III IFN responses to ZIKV infection

The type II (IFNγ) and type III IFN (IFNλ) responses are also important component of the host antiviral defence system. The type III IFN signaling pathway is analogous to the type I IFN

signalling pathway and likely induces a similar set of ISGs that inhibit ZIKV replication (75). The importance of type III IFN is demonstrated by the protection it offers against ZIKV infection when it is secreted by placental trophoblasts (76). Furthermore, a study conducted on the related West Nile virus, demonstrated that type III IFN tightens the blood-brain barrier to prevent vascular leakage and viral neuroinvasion (77). These investigations suggest that the type III IFN response may be a crucial factor in limiting ZIKV related pathogenesis. As such, further information into how the virus can circumvent type III IFN signaling will complement our current understanding of how ZIKV is able to evade host innate immune responses.

The type II IFN (IFNγ) response is unique because its expression is limited to cells of the immune system where it plays a role in mediating the transition between the innate and adaptive immune responses, including macrophage activation and the recruitment of immune cells to the infection site (78). The IFNγ receptor is expressed on a wide variety of cells, and receptor binding results in the activation of JAK and the subsequent phosphorylation of STAT1 (65). Phosphorylated STAT1 homodimers are translocated to the nucleus where they induce the transcription of IFNγ-stimulated genes (65). Recent studies suggest that there is an increase in transcription of IFNγ-stimulated genes following ZIKV infection; possible due to viral proteins preferentially degrading STAT2, which consequently results in an increase in the ratio of STAT1 homodimers available to induce IFNγ-stimulated genes (65, 71). Interestingly, in contrast to the type I and type III IFNs, type II IFN signalling seems to increase ZIKV replication in placental and glioblastoma cell lines (65, 71). However, other investigations suggest that ZIKV replication is negatively affected in human lung carcinoma and foreskin fibroblast cells when treated with IFNγ (20, 73). These conflicting findings suggest that the pro-viral effect of IFNγ is

cell-type specific, and future studies will be needed to further clarify these interactions during ZIKV infection.

1.8 In Vitro Models of ZIKV Pathogenesis

In vitro and *ex vivo* models are useful for dissecting the molecular mechanisms of viral infection, fitness, replication, and host-virus interactions. To date, *in vitro* studies have been focused on determining suitable cell types for ZIKV infection and replication, as well as those involved in transplacental transmission and neural damage to investigate ZIKV pathogenesis. The link between ZIKV infection and fetal microcephaly has been demonstrated by detection of viral RNA in the placenta, amniotic fluid, and brain, as well as ZIKV IgM-specific antibodies in the cerebrospinal fluid of microcephalic neonates (6, 35, 38-40, 79). Recent studies suggest that ZIKV is able to infect and replicate in primary human placental cells (cytotrophoblasts, endothelial cells, fibroblasts and Hofbauer cells) from mid- to late gestation, as well as villus explants from first-trimester human placentas, suggesting two possible routes of ZIKV transmission to the fetus (placental and para-placental) (80, 81). Furthermore, ZIKV replication in Hofbauer cells (placental macrophages) was associated with induction of type I IFNs, pro-inflammatory cytokines, and antiviral gene expression, but minimal cytopathic effects, suggesting that these cells may allow ZIKV to gain access to the fetal compartment and could play a role in viral dissemination (81). In contrast, primary human trophoblasts (the barrier cells of the placenta) from full term placentas are refractory to ZIKV infection and produce type III IFNs that are postulated to protect trophoblast and non-trophoblast cells from infection during late stages of pregnancy (76).

Several groups have investigated the link between ZIKV infection and neural pathogenesis *in vitro* using human neural progenitor cells (hNPCs), neurons and cerebral organoids (three-dimensional, self-organized, stem-cell derived models that recapitulate the first trimester of human neurodevelopment) generated from human induced pluripotent stem cells (40, 41, 82-84). These reports indicate that ZIKV can infect hNPCs with high efficiency resulting in increased cell death, dysregulation of the cell cycle, as well as impaired growth and morphogenesis of healthy neurospheres (40). Furthermore, ZIKV-infected hNPCs release infectious viral particles, presenting a challenge for the development of therapeutics to halt or block the impact of infection (40, 82-84). Importantly, these findings suggest a potential mechanism of ZIKV-induced microcephaly as hNPCs are essential for the development of the cortex and brain. Additionally, current reports have suggested that ZIKV exhibits a unique tropism for astrocyte cells which may influence progression of neuropathogenesis (85, 86). In addition to improving our understanding of ZIKV infectivity, viral fitness and replication, cell culture models are important tools to explore the innate immune response to ZIKV infection.

Chapter 2: Introduction

This chapter serves as an introduction for the project and outlines the **rationale** and the **specific objectives** of the investigation. The laboratory's principal investigator, Dr. Selena M. Sagan, formulated the research question and the global aims of the project. The specific aims and the experimental design of the project were planned through a collaborative effort between **MM Rajah** and Dr. Sagan.

2.1 Rationale

The recent emergence and rapid geographic expansion of the mosquito-borne Zika Virus (ZIKV) has become a significant burden on the global health infrastructure (9). ZIKV circulated throughout Africa as well as in Southeast Asia over the latter half of the twentieth century, where it caused sporadic infections resulting in mild febrile illness (4, 6). The first major transmission of ZIKV outside of its endemic zone occurred in 2007, where 73% of the population of Yap Island, Federated States of Micronesia contracted the virus within a four-month period (5, 6). However, the clinical manifestations of ZIKV infection during the Yap island epidemic were relatively similar to historical descriptions; resulting in mild, self-limiting febrile illness characterized by rash, arthralgia, conjunctivitis, and headaches (4, 5). Interestingly, the continued geographic expansion of the ZIKV epidemic coincided with reports of novel neurological pathogenesis, starting with the 2013 French Polynesian epidemic, which saw a drastic increase in reports of Guillain-Barré syndrome (32).

Following the French Polynesian outbreak, the trans-Pacific transmission of ZIKV has resulted in several epidemics throughout the Americas; the most salient being the Brazilian epidemic (2013 – 2016) which coincided with a 20-fold increase in incidence of congenital malformations including fetal microcephaly from 2014 to 2015 (36). Notably, the ZIKV outbreaks in South America were also associated with neurological symptoms, and several investigations now suggest a connection between ZIKV infection and the development of Guillain-Barré syndrome as well as congenital birth defects, including fetal microcephaly (6, 12, 35, 87-89). Several factors including the increased mobility of infected individuals, an expansion in the range of *Aedes sp.* mosquitoes, and the immunological naivety of recently afflicted human populations likely contributed to the rapid emergence of these recent ZIKV epidemics (90). **In**

addition to epidemiological factors, novel genetic polymorphisms acquired by contemporary outbreak strains may also influence ZIKV pathogenicity and dissemination. Studies conducted in human neurospheres, cerebral organoids, and primary astrocytes, as well as in murine models of infection have demonstrated differences in neurotropism, pathogenicity, and the antiviral response between Asian and African lineage isolates (84, 90, 91). Furthermore, a recent investigation uncovered that a single nucleotide substitution in the prM region of the viral polyprotein increased ZIKV infectivity in human and mouse neural progenitor cells, and led to significant fetal microcephaly in mice, resulting in greater mortality of neonatal mice (49). Another single amino acid substitution acquired by contemporary strains in the NS1 protein, has been demonstrated to enhance ZIKV infectivity during transmission from mammalian hosts to mosquito vectors; and thus may have contributed to the increased viral dissemination seen in the recent outbreaks (92). **Thus, characterizing the difference in viral fitness between the contemporary American epidemic strains to the pre-epidemic strains would provide an evolutionary context to emergence and spread of ZIKV.** Developing a more thorough understanding of ZIKV biology would also be an important step in addressing the current lack of vaccines or antiviral treatment options for ZIKV infection.

2.2 Research Objectives and Hypothesis

This investigation explores strain-specific differences in viral fitness, viral replication, and the antiviral response to ZIKV infection in an astrocytoma cell lines. We chose this cell line because astrocytes are the most abundant cell type in the central nervous system and preform a diverse array of tasks including axon guidance, synaptic support, as well as controlling the blood brain barrier and blood flow (93). Additionally, ZIKV has been shown to have a particular affinity for

astrocytes during the early stages of viral infection of the developing brain (86). We compared two contemporary American ZIKV isolates from the 2015-2016 outbreaks in Puerto Rico and Brazil ($ZIKV^{PR}$ and $ZIKV^{BR}$) to an earlier 2013 Asian lineage strain ($ZIKV^{CDN}$) and to the historical Uganda 1947 isolate ($ZIKV^{AF}$). **We hypothesized that the contemporary American ZIKV strains would display greater viral fitness than either of the historical African or early Asian isolates in human astrocytoma cells (Figure 2.1)**. The specific objectives of this investigation are:

Specific Aim 1: To compare replication and cytopathicity of the historical African ZIKV strain, the early Asian isolate, and the contemporary American epidemic isolates in U-251 MG human astrocytoma cells.

Specific Aim 2: To investigate the induction of antiviral gene expression between the historical African ZIKV strain, the early Asian isolate, and the contemporary American epidemic isolates in the U-251 MG cell line.

Specific Aim 3: To investigate differences STAT1 and STAT2 mRNA and protein expression between historical and contemporary isolates in the U-251 MG cell line.

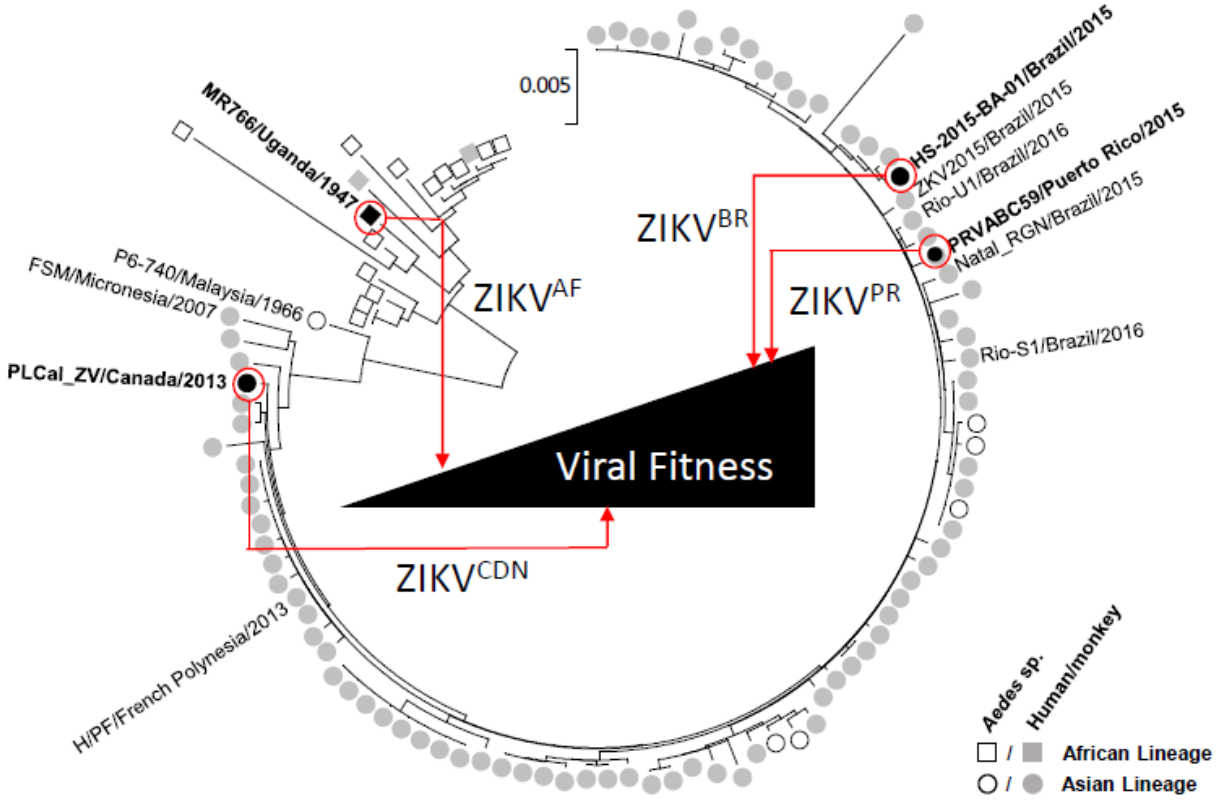

Figure 2.1 Graphical Hypothesis. The ZIKV isolates investigated in the project are indicated (black) with their corresponding predicted viral fitness. The ancestral Malaysian strain (P6-740/1966) and the epidemic strains from Micronesia (FSM/2007) and French Polynesia (H/PF/2013) are also depicted on the tree. African and Asian lineage viruses are represented with squares and circles, respectively.

Chapter 3: Materials & Methods

This chapter provides a detailed description of the **materials** and **methods** used in this study. Laboratories and researchers who provided cell lines and viruses for the investigation are acknowledged in the text.

3.1 Phylogenetic Analysis

Translated amino acid sequences of 50 ZIKV polyproteins were aligned using ClustalW. The accession number of the ZIKV strains used in this analysis are indicated in the Figure. Trees were constructed by neighbor joining of pairwise amino acid distances with the program MEGA7 (according to the distance scale provided) (73). Bootstrap resampling was used to determine robustness of branches; values of ≥50% (from 1000 replicates) were used.

3.2 Cells and Viruses

African green monkey kidney (Vero) cells, human embryonic kidney (293T) cells, and human astrocytoma (U-251 MG) cells were kindly provided by Martin J. Richer (McGill University), Connie Krawczyk (McGill University), and Anne Gatignol (Lady Davis Research Institute), respectively. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids, 1% L-glutamine, and 1% penicillin/streptomycin at 37°C/5% CO_2.

An infectious cDNA of ZIKV strain MR-766 ($ZIKV^{AF}$; Genbank accession: HQ234498.1) was kindly provided by Matthew Evans (Mount Sinai, USA) (94). $ZIKV^{AF}$ viral stocks were generated by transfection of 293T cells with the infectious cDNA using Lipofectamine 2000 (Life technologies) followed by a single passage in Vero cells. ZIKV isolate PLCal_ZV ($ZIKV^{CDN}$; Genbank accession: KF99378) was generously provided by David Safronetz (National Microbiology Laboratories, Canada) (95). Isolates PRVABC59 ($ZIKV^{PR}$; Genbank accession: KU501215) and HS-2015-BA-01 ($ZIKV^{BR}$; Genbank accession: KX520666) were provided by Tom Hobman (University of Alberta, Canada) and Mauro Teixeira

(Universidade Federal de Minas Gerais, Brazil), respectively. The passages history of all the ZIKV isolates used in this study are listed in (**Table 3.1)**.

Table 3.1 The passage history of the ZIKV isolates compared in this study

Strain Name	Lineage	Source Host	Location and Year of Isolation	Passage History
MR 766	African	Rhesus monkey	Uganda-1947	146x Suckling mice brains, 1x C6/36 cells, 1x Vero cells
PLCal_ZV	Asian	Human	Canada (Thailand Import)-2013	4x Vero cells
PRVABC59	Asian	Human	Puerto Rico-2015	3x Vero cells
HS-2015-BA-01	Asian	Human	Brazil-2015	3x C6/36 cells, 1x Vero cells

3.3 Preparation of Viral Stocks

All ZIKV stocks were prepared by passage in Vero cells, and infections were performed at MOI of 0.5 with an inoculum that contained virus diluted in unsupplemented Eagle's minimum essential medium (EMEM; Wisent Inc.). Two hours post-infection, the virus inoculum was removed and replaced with cell growth media containing 15 mM HEPES (Wisent Inc.) and 2% FBS. The supernatant was harvested at 2-3 days post-infection and clarified by centrifugation at 4^{o}C for 10 min at 3000×g. The supernatant was decanted into a fresh conical tube before being aliquoted into single use vials and stored at -80^{o}C.

Virus stocks were titrated by plaque forming unit (PFU) assay on Vero cells. Briefly, 500 uL of 10-fold serially diluted virus stocks were incubated for 2 h on Vero cell monolayers in a 12-well plate format. The virus inoculum was removed, and the cells were overlaid with EMEM,

1.2% Carboxymethyl cellulose (CMC) (Sigma-Aldrich), 2% heat-inactivated FBS, and 1% penicillin/streptomycin. At 4 days post-infection, cells were fixed with 5% formaldehyde and stained with 0.1% crystal violet (Sigma-Aldrich) to visualize plaques

3.4 ZIKV Infections

U-251 MG cells were seeded at a density of 3×10^6 cells per 10-cm^2 plate the day before infection. Cells were infected at an MOI of 0.01, 10, or 3 in 5 mL virus inoculum containing unsupplemented EMEM, for the multi-step growth curve, one-step growth curve, and innate immunity experiments, respectively. Following a 2 h absorption at 37°C/5% CO_2, the viral inoculum was removed, and 7 mL of fresh virus media was added. At each specified time point, the supernatant was collected and clarified as previously described and frozen for titration. Viral titers were determined by plaque assay.

3.5 Cell Viability

Cell viability was monitored using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay similar to what has been previously described (96). Briefly, cells were plated at density of 5000 cells per well in a flat-bottom 96-well plate and allowed to adhere overnight. Each virus strain was diluted in virus storage media, and 10 wells per strain were infected with 100 µL of the virus dilution at an MOI of 1. At each of the specified time points, 10 µL of a 0.005 g/mL MTT salt (Sigma-Aldrich) in EMEM solution was added to each well and incubated for 4 h at 37°C. After 4 h, 100 µL of solubilization solution (10% SDS in 0.01 M HCl) was added to each well and the plates were left overnight at 37°C. The plates were then read at 550 nm with a reference wavelength of 650 nm on a PowerWave X340 (Bio-

Tech Instruments, INC.) plate reader. The Optical density was defined as: **Optical Density = ((Uninfected Absorbance – Infected Absorbance)/ (Uninfected Absorbance)) * 100%.** The average absorbance of 10 wells was used in all cases and viability experiments were carried out in triplicate. The data was expressed in % Cytopathicity, which was defined as: **% Cytopathicity = 100% – % Optical Density.**

3.6 Quantitative PCR Analysis

Total RNA from mock and ZIKV-infected cells were harvested by Trizol extraction according to manufacturer's instructions and RNA concentration was determined using a NanoDrop 2000 spectrophotometer. RNA was reverse transcribed using the High Capacity cDNA Reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. qRT-PCR was performed in duplicate in 96-well PCR plates (Bio-Rad) using SensiFAST SYBR Lo-ROX mix (Bioline) in a Bio-Rad CFX96 Touch Real-Time System under standard cycling conditions. Relative mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method using GAPDH as an internal control, and plotted as fold change by normalizing to mock-infected samples (97). All primer sequences used in this study are listed in (**Table 3.2)**.

3.7 Antibodies and Immunoblotting

Total cell lysates from U-251 MG were collected and washed twice with PBS following a 24 h infection with an MOI of 3. The samples were lysed on ice in radioimmunoprecipitation assay (RIPA) buffer (150 mM sodium, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 50 mM Tris; pH 8) supplemented with complete protease inhibitors (Roche) for 20 min and then quantified by bicinchoninic acid (BCA) protein assay. Twelve

Table 3.2 List of Primers used in this study.

Gene	Primers	Sequence (5' -3')
GAPDH	GAPDH-FOR	GAAGGTGAAGGTCGGAGTC
	GAPDH-REV	GAAGATGGTGATGGGATTTC
ISG56	ISG56-FOR	GAAGCAGGCAATCACAGAAA
	ISG56-REV	TGAAACCGACCATAGTGGAA
IFN-β	IFNβ1-FOR	TCAGTGTCAGAAGCTCCTGTGG
	IFNβ1-REV	CTGCTGCAGCTGCTTAATCTCC
IFN-λ1	IFNL1-FOR	GCAGGTTCAAATCTCTGTCACC
	IFNL1-REV	AAGACAGGAGAGCTGCAACTC
MxA	hMxA-FOR	GTGGCTGAGAACAACCTGTG
	hMxA-REV	GGCATCTGGTCACGATCCC
OAS-1	hOAS-1-FOR	GATCTCAGAAATACCCCAGCCA
	hOAS-1-REV	AGCTACCTCGGAAGCACCTT
ISG15	hISG15-FOR	TCCTGGTGAGGAATAACAAGGG
	hISG15-REV	GTCAGCCAGAACAGGTCGTC
ISG54	hISG54-FOR	ATGTGCAACCTACTGGCCTAT
	hISG54-REV	TGAGAGTCGGCCCATGTGATA
STAT2	hSTAT2-FOR	GACGCTGTAGCAACTCTGTGA
	hSTAT2-REV	GAAGGAGCTGAAGGGACTGA
STAT1	hSTAT1-FOR	ACAGCAGAGCGCCTGTATTG
	hSTAT1-REV	CAGCTGATCCAAGCAAGCAT
Viperin	Viperin-FOR	CGTGGAAGAGGACATGACGGAAC
	Viperin-REV	CCGCTCTACCAATCCAGCTTC
IFITM1	IFITM1-FOR	CCCCCAGCACCATCCTTC
	IFITM1-REV	ACCCCGTTTTTCCTGTATTATCTGT
IFITM3	IFITM3-FOR	TGTCCAAACCTTCTTCTCTCC
	IFITM3-REV	CGTCGCCAACCATCTTCC

micrograms of each sample was incubated at 95°C for 5 min in 1X SDS Blue Loading Buffer (New England BioLabs) under reducing conditions. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (PVDF) for immunoblotting. The following antibodies were used in this study: rabbit anti-pSTAT1 (Tyr701) (Cell Signaling; 9167S), rabbit anti-STAT1 (Cell Signaling; 9172S), rabbit anti-pSTAT2 (Tyr689) (Millipore; 07-224MI), rabbit anti-STAT2 (Cell Signaling,

72604), and mouse anti-GAPDH (6C5) (Fisher; MAB374MI). For the MG132 experiments, cells were infected with ZIKV (MOI = 3) and at 12 h post-infection, were treated with 20 μM MG132 (Sigma) or dimethyl sulfoxide (DMSO) for 12 h followed by lysis and immunoblotting as described above.

3.8 Statistical Analysis

All statistical analyses were performed using Prism 6 software (Graphpad). Viral titers from growth curves were normalized by taking the $\log_{10}$ of each measurement, and comparisons were made using a repeated-measure two-way analysis of variance (ANOVA) with Tukey's multiple-comparison test. For the qPCR data, ΔΔCT values were compared using a one-way ANOVA followed by Tukey's multiple comparison test.

Chapter 4: Results

This chapter presents the **results** of the investigation and corresponding **figures**. The phylogenetic analysis that forms the research question, was conducted by the laboratory's principal investigator, Dr. Selena. M Sagan. The results and figures featured in this chapter are derived from a manuscript in preparation co-authored by **MM Rajah**: (**Maaran Michael Rajah***, Trisha R. Barnard*, and Selena M. Sagan, "Contemporary American Zika virus isolates elicit differential growth kinetics and IFN responses in human astrocytomas and lung epithelial cells". (2017). *Manuscript in Preparation).* The majority of the experiments **(>90%)** presented in this chapter were designed and performed by **MM Rajah**. Trisha R. Barnard provided support on the MG132 experiments and western blot quantifications.

4.1 Phylogenetic and amino acid variance across ZIKV isolates selected for comparative analyses.

Our phylogenetic analysis suggested that the strains identified in the 2015-2016 epidemic are more closely related to the H/PF/2013 French Polynesia strains than the FSM/2007 Micronesia (Yap Island) strain, suggesting that these sub-lineages may have evolved independently from a common ancestor **(Figure 4.1)**. Notably, the ZIKV outbreaks in French Polynesia and South America were the first to be associated with neurological symptoms, including Guillain-Barré syndrome and fetal microcephaly (6, 17, 87). As such, we sought to perform a comparative analysis of historical and contemporary ZIKV isolates to study the impact of genetic polymorphisms on pathogenesis and the induction of the innate immune response to infection in relevant cell culture models. To do so, we chose a panel of ZIKV isolates including: Uganda 1947 (MR766, $ZIKV^{AF}$); an early Asian lineage strain, isolated from a Canadian traveller who returned from Thailand viremic in 2013 (PLCal_ZV, $ZIKV^{CDN}$); and two isolates from the 2015-2016 outbreak in Puerto Rico 2015 (PRVABC59, $ZIKV^{PR}$) and Brazil 2015 (HS-2015-BA-01, $ZIKV^{BR}$). These isolates represent strains isolated from 1947 to 2015 from different geographic locations, and our phylogenetic analysis reveals their positions within the African or Asian lineages **(Figure 4.1)**.

4.2. Contemporary American ZIKV isolates have a unique plaque morphology and rapid growth kinetics in U-251 MG cells.

While preparing viral stocks we noticed differences in the plaque morphology on Vero cells between the four ZIKV isolates **(Figure 4.2A**). The historical $ZIKV^{AF}$ and early Asian $ZIKV^{CDN}$ strains produced plaques with indefinite borders, whereas the plaques from the contemporary

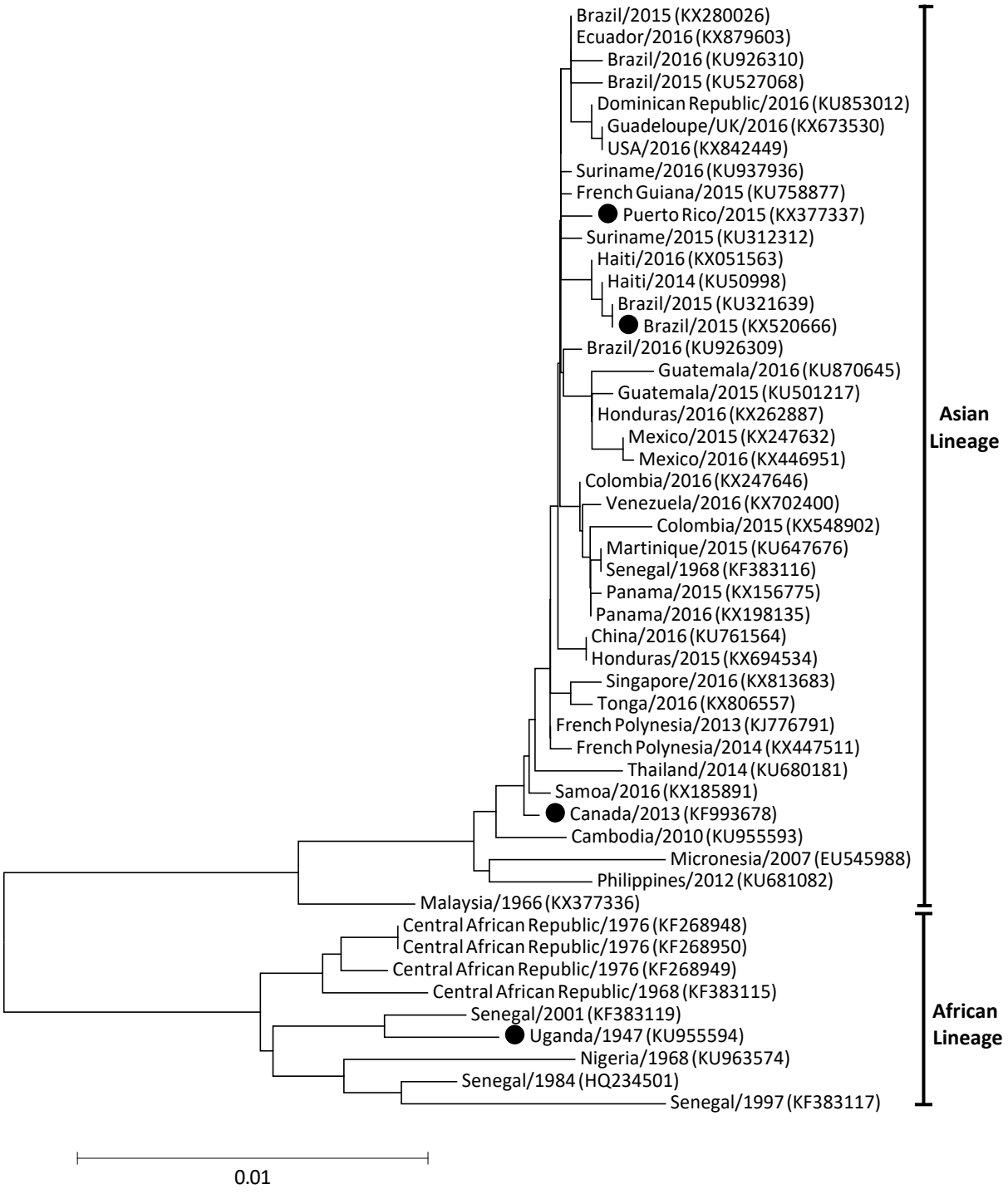

Figure 4.1 Phylogenetic analysis of selected ZIKV strains. Neighbor joining phylogenetic tree showing the position of the viral isolates used in this study (indicated by black circles) in the global ZIKV diversity. African and Asian lineages are indicated as well as country of origin, year of isolation, and GenBank accession number.

American ($ZIKV^{PR}$ and $ZIKV^{BR}$) isolates were very clearly defined **(Figure 4.2A)**. We speculated that the differences in plaque morphology could be a result of strain-specific differences in growth kinetics, cytopathicity, or differential induction of innate immune responses. Thus, we proceeded to investigate potential strain-specific differences in the U-251 MG astrocytoma cell line because of the biological relevance of astrocytes to ZIKV-induced neuropathology (85, 86).

We first sought to determine the viral growth kinetics for each of the four ZIKV isolates via plaque assay. Viral replication was assessed using a multi-step (**Figure 4.2B)** and one-step growth curve (**Figure 4.2C)** at an MOI of 0.01 and 10, respectively. In U-251 MG cells, at an MOI = 0.01, both the $ZIKV^{PR}$ and $ZIKV^{BR}$ contemporary American isolates grew to high titers very quickly, reaching peak titers (between 10^7 and 10^8 PFU/mL) at 48 h post-infection, with no significant differences in viral titer between the two isolates (**Figure 4.2B**). In contrast, the historical $ZIKV^{AF}$ and the early Asian $ZIKV^{CDN}$ isolates produced significantly lower titers early (approximately 10^6 PFU/mL at 48 h post-infection), and did not reach peak titers until 96-120 h post-infection **(Figure 4.2B)**.

To further investigate the differences in viral replication kinetics between the isolates, we produced one-step growth curves using an MOI of 10 **(Figure 4.2C)**. The contemporary American isolates rapidly generated high viral titers in the U-251 MG cells, peaking between 12 h to 24 h post-infection **(Figure 4.2C)**. In contrast, the historical $ZIKV^{AF}$ and early Asian $ZIKV^{CDN}$ strains did not reach peak viral titers until 48-72 h post-infection **(Figure 4.2C)**. This further demonstrates that historical (pre-epidemic) ZIKV isolates ($ZIKV^{AF}$ and $ZIKV^{CDN}$) have delayed growth kinetics when compared with the contemporary American isolates ($ZIKV^{PR}$ and $ZIKV^{BR}$). Interestingly, the $ZIKV^{AF}$ isolate grew to higher titers than the $ZIKV^{CDN}$ isolate at

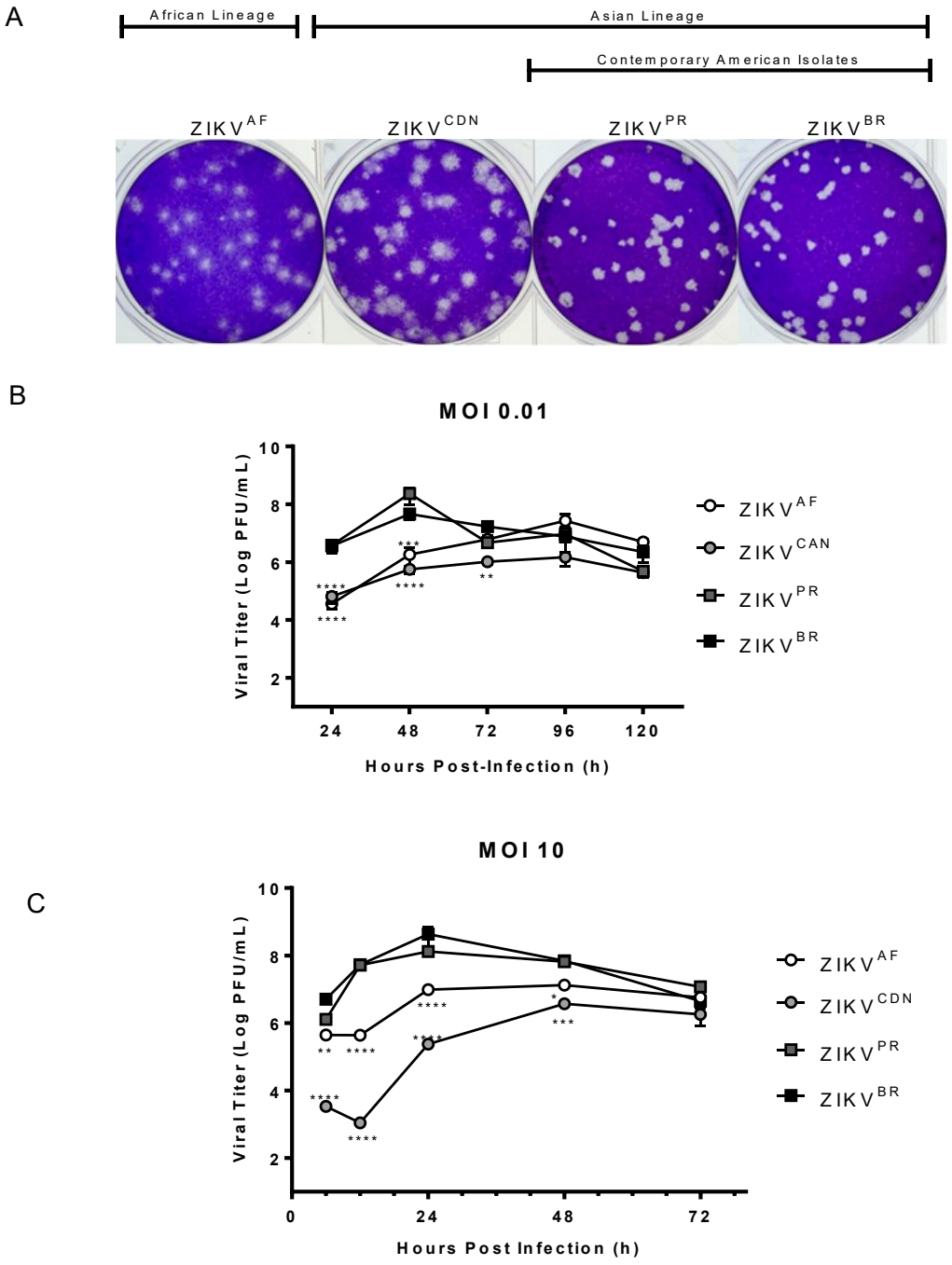

Figure 4.2 Contemporary American ZIKV isolates demonstrate unique plaque morphology and more rapid growth kinetics in U-251 MG cells. (A) Representative images of Vero cell plaque assays of the indicated ZIKV isolate. Cell culture supernatants were collected at the indicated time points and viral titer was determined by plaque assay. U-251 MG cells were infected with ZIKV at (B) MOI = 0.01 or at (C) MOI = 10. Values represent mean ± SEM of at least three independent experiments. Astericks indicate significant differences in viral titer relative to $ZIKV^{BR}$: *p < 0.05, ** p < 0.01, *** p < 0.001, **** p<0.0001.

early time points, suggesting that there is also a difference in replication kinetics between the two examined pre-epidemic strains **(Figure 4.2C)**.

4.3. Asian lineage isolates elicit lower cytopathic effects than the historical African isolate in U-251 MG cells.

Due to the more rapid growth kinetics of the contemporary American isolates ($ZIKV^{PR}$ and $ZIKV^{BR}$) vs. the pre-epidemic isolates ($ZIKV^{AF}$ and $ZIKV^{CDN}$) in cell culture, we sought to investigate if there was a correlation between the viral growth kinetics and their ability to induce cytopathic effects (CPE). Thus, we investigated whether there were any strain-specific differences in cytopathicity in the U-251 MG cells using the MTT assay **(Figure 4.3)**. Following an infection with an MOI = 1, we found that the CPE elicited by all four strains plateaued at approximately 72 h post-infection **(Figure 4.3)**. Infection with the $ZIKV^{AF}$ strain elicited the highest CPE in the human astrocytomas, with approximately 80% cytopathicity at 72 h post-infection **(Figure 4.3)**. In comparison to $ZIKV^{AF}$ strain, both $ZIKV^{PR}$ and $ZIKV^{BR}$ contemporary American isolates elicited lower CPE (approximately 65%) **(Figure 4.3)**. Interestingly, The $ZIKV^{CDN}$ isolate elicited a similar CPE to the contemporary American isolates **(Figure 4.3)**. This suggests that the main difference in terms of CPE elicited by the isolates, is between the African and Asian lineages, and not between the pre-epidemic isolates and contemporary American isolates. Overall, there appears to be an inverse correlation between viral titers and CPE; where the contemporary American isolates, which elicit reduced CPE, generate higher viral titers at early time points, while the historical $ZIKV^{AF}$ isolate had greater CPE and lower viral titers at early time points.

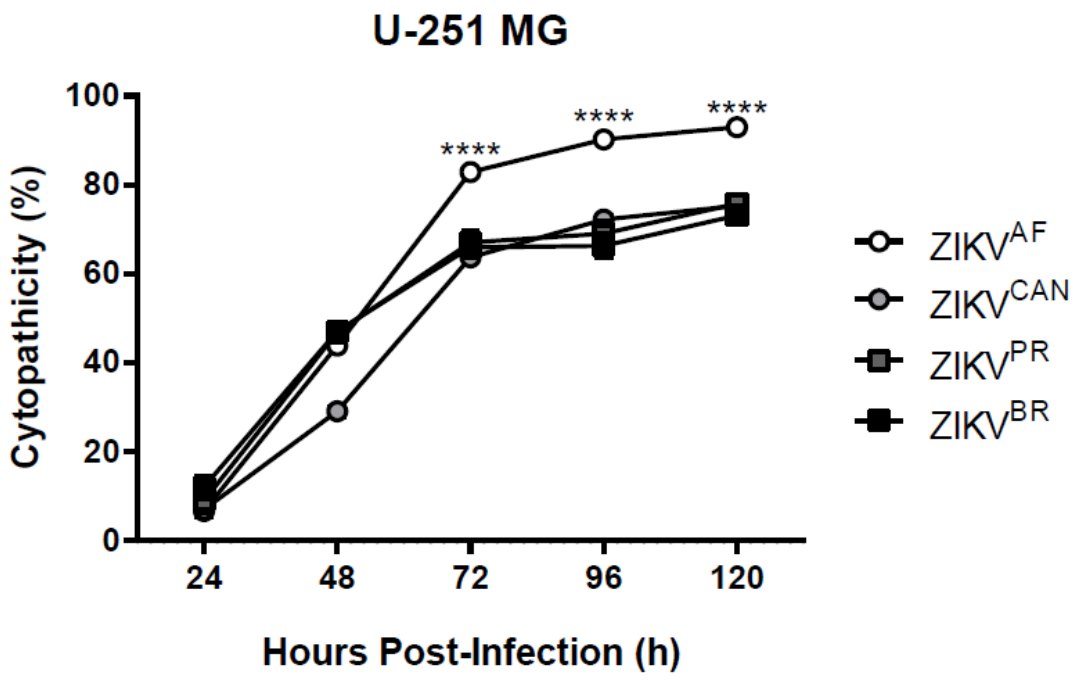

Figure 4.3 Asian lineage ZIKV isolates elicit comparatively reduced cytopathic effects when compared with the African lineage isolate in U-251 MG cells. U-251 MG cells were infected with ZIKV at MOI = 1 and cell viability was determined by MTT assay at the indicated time points. Values represent mean ± SEM of at least three independent experiments. Asterisks indicate significant differences in cytopathicity relative to $ZIKV^{BR}$, **** $p<0.0001$.

4.4. ZIKV isolates elicit similar induction of type I and III IFNs in U-251 MG cells.

Our observation that the contemporary American isolates had distinct plaque phenotypes, more rapid growth kinetics, and reduced CPEs when compared with the historical ZIKV isolates, prompted us to test whether this may be due to differences in their induction of antiviral responses in cell culture. Since type I and III IFNs have been reported to limit ZIKV replication (74), we set out to characterize differences in IFN induction across ZIKV isolates using RT-qPCR analysis. We conducted these studies at an MOI of 3 as previously reported for similar studies of IFN and antiviral signaling pathway in ZIKV infection (73). We observed substantial induction of type I (IFNβ) and type III (IFNλ1) IFN gene expression by all four ZIKV isolates at 24 h and 48 h post-infection **(Figure 4.4A and B)**. However, we did not observe significant IFN

induction before these time points (data not shown), consistent with previous reports that *flaviviruses* are late inducers of IFN (98). We noticed that in U-251 MG cells, all ZIKV isolates induced similar levels of type I IFN at 24 h post-infection **(Figure 4.4A)**. At 48 h, the pre-epidemic strains seem to induce a higher level of type I IFN, but this was not statistically significant **(Figure 4.4A)**. When we examined type III IFN induction we found that the contemporary American strains induced significantly higher levels of mRNA than the early Asian strain at 24 h post-infection; however, there was no statistical difference when the contemporary strains were compared to the historical African strain (**Figure 4.4B**). The robust III IFN induction elicited by the contemporary strains at 24 h post-infection, is in concordance with their generation of significantly higher viral titers **(Figure 4.4C)**. There was no significant difference in type III IFN expression between any of the strains at 48 h post-infection, although the pre-epidemic strains seem to have higher overall induction **(Figure 4.4B)**. Due to the strong CPE observed at the 48 h time point post-infection, we chose to limit further analysis to the 24 h time point post-infection.

4.5 Contemporary American isolates induce more robust ISG expression in U-251 MG cells.

We next investigated whether the differences observed in IFN induction resulted in differential expression of important antiviral ISGs that have been implicated in sensing or responding to *flavivirus* infections, including ISG54, ISG56, ISG15, MxA, OAS-1, Viperin, IFITM1 and IFITM3 by RT-qPCR analyses **(Figure 4.5A-H)** (75). ISG induction by ZIKV isolates did not necessarily coincide with type I or III IFN induction. Interestingly, the contemporary American

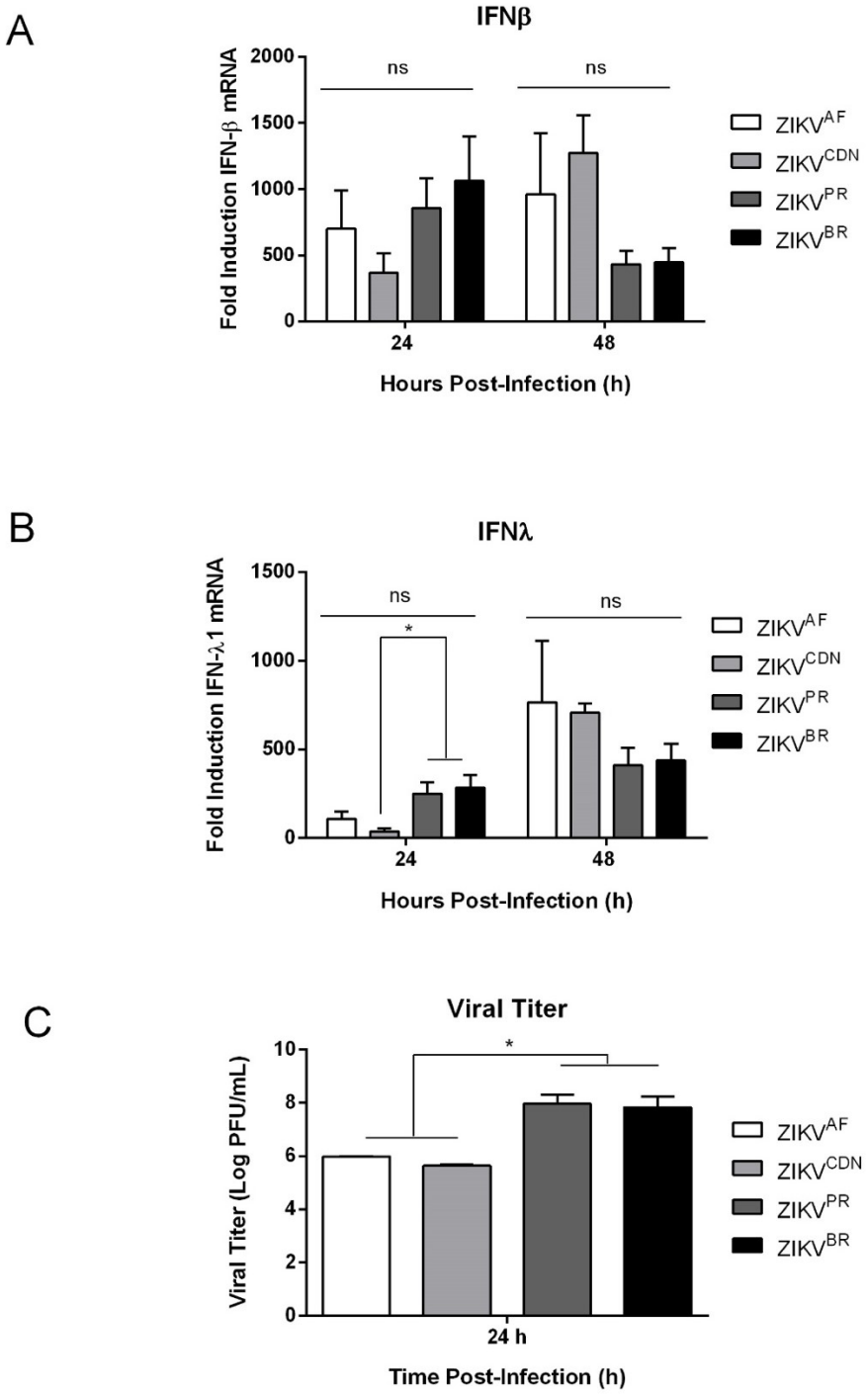

Figure 4.4 ZIKV isolates elicit similar induction of type I and III IFNs in U-251 MG cells. U-251 MG cells were infected with ZIKV at MOI = 3 for 24 h or 48 h and (A) IFNβ and (B) IFNλ1 gene expression was quantified by RT-qPCR. (C) Cell culture supernatants were collected at 24 h to determine viral titers after infection with ZIKV isolates at MOI = 3 by plaque assay. Values represent mean ± SEM of at least three independent experiments. *$p < 0.05$.

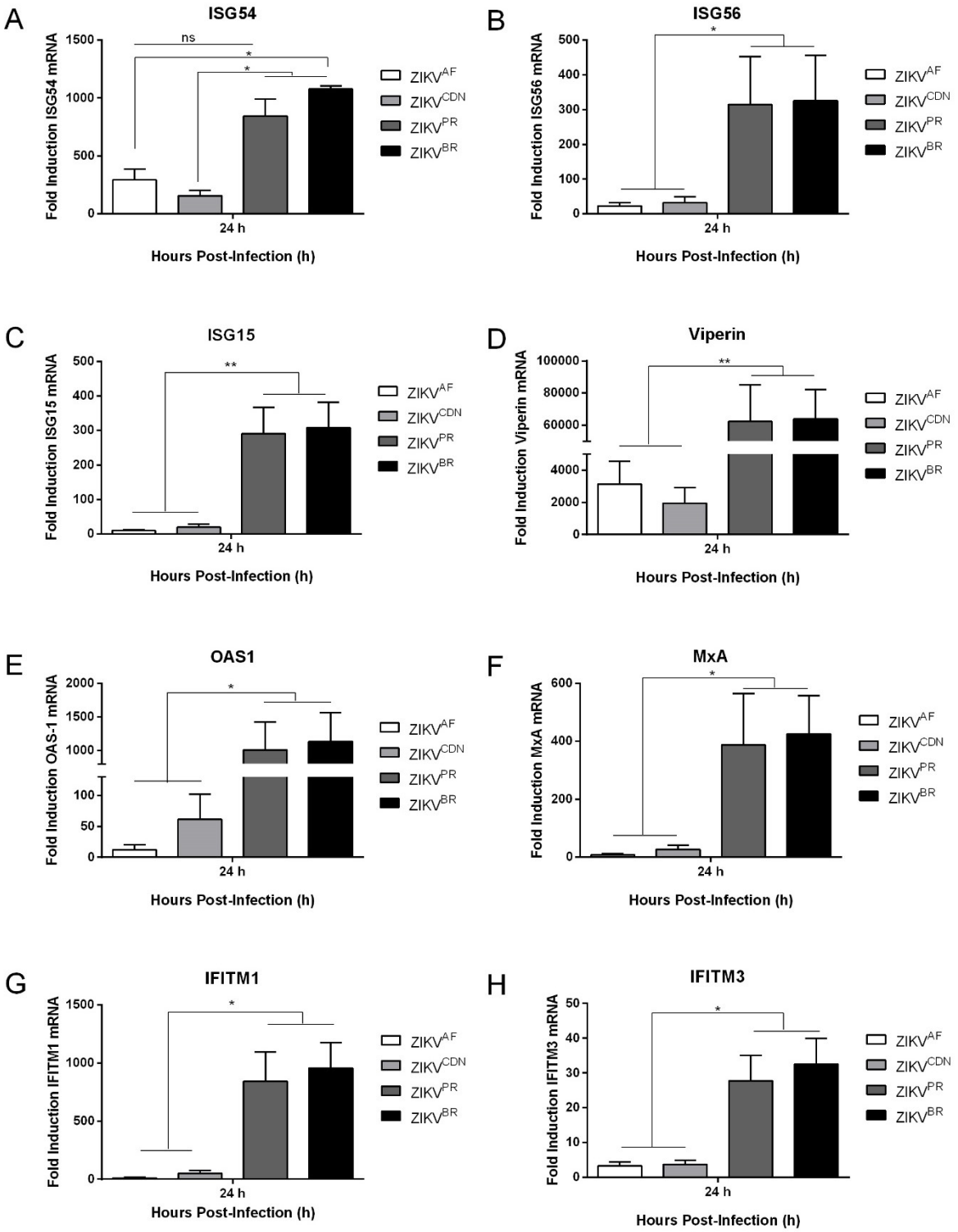

Figure 4.5 ZIKV isolates differentially induce the expression of several ISGs in U-251 MG cells. U-251 MG cells were infected with ZIKV at MOI = 3 for 24 h and induction of (A) ISG54, (B) ISG56, (C) ISG15, (D) Viperin, (E) OAS-1, (F) MxA, (G) IFITM1, and (H) IFITM3 gene expression was quantified by RT-qPCR. Values represent mean ± SEM of at least three independent experiments. *$p < 0.05$, ** $p < 0.01$.

ZIKV isolates induced a significantly higher expression of almost all of the selected ISGs than either of the pre-epidemic ($ZIKV^{AF}$ and $ZIKV^{CDN}$) isolates **(Figure 4.5)**. The only exception was for ISG54 mRNA induction, where there was no significant difference between $ZIKV^{AF}$ and $ZIKV^{PR}$ **(Figure 4.5A)**.

4.6 Differential regulation of STAT protein expression and phosphorylation by the contemporary American isolates.

Finally, we wanted to investigate whether the contemporary ZIKV isolates differentially regulate STAT1 and STAT2 mRNA and protein levels. First, we investigated the induction of STAT1 and STAT2 mRNA expression by RT-qPCR analyses **(Figure 4.6A)**. At the mRNA level, infection with all four ZIKV isolates induced both STAT1 and STAT2 gene expression in the U-251 MG cells **(Figure 4.6A and B)**; however, the contemporary American ZIKV isolates induced significantly higher STAT1 and STAT2 gene expression than either the historical African or early Asian lineage isolates **(Figure 4.6A and B)**. This trend was similar to what was observed in the induction of ISGs **(Figure 4.5)**.

Given previous reports that the ZIKV NS5 protein can induce the degradation of the STAT2 protein without affecting STAT1 protein levels (72, 73), we compared the STAT1 and STAT2 protein levels by western blot in the U-251 MG cells following infection (MOI = 3) with each of the four ZIKV isolates **(Figure 4.6C and D)**. While cells infected with the historical African and early Asian isolates had similar total STAT1 protein levels as the mock-infected cells, and we observed greater induction of STAT1 protein expression by the contemporary American isolates **(Figure 4.6C)**, which correlates with the STAT1 mRNA induction **(Figure 4.6A)**. Additionally, in contrast to previous reports (72, 73), we observed an induction, rather

than

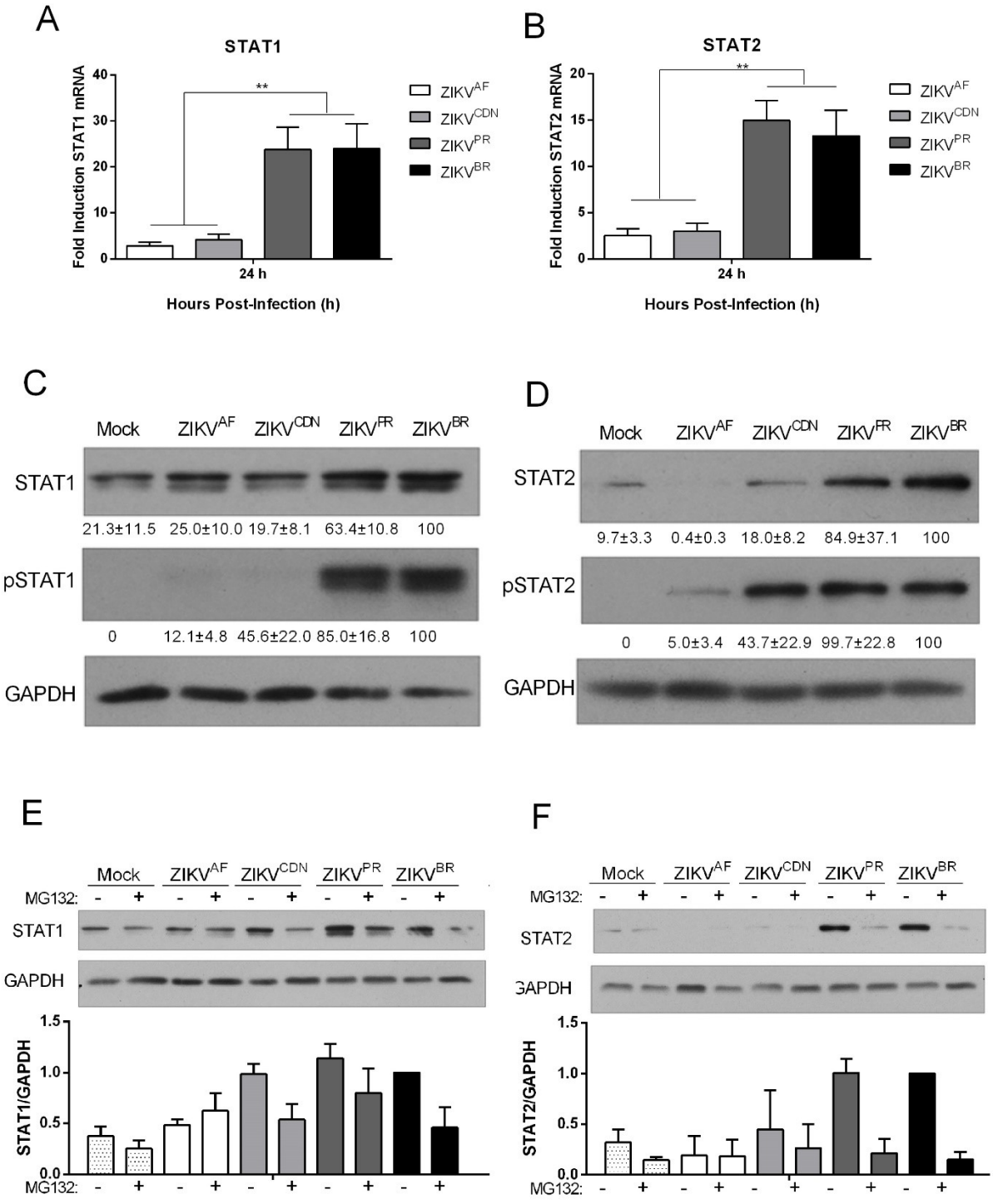

Figure 4.6 ZIKV isolates differentially regulate STAT1 and STAT2 responses in U-251 MG cells. U-251 MG cells were infected with ZIKV at MOI = 3 for 24 h and induction of (A) STAT1 and (B) STAT2 mRNA was analyzed by RT-qPCR analysis. Values represent mean ± SEM of at least three independent experiments. ** $p < 0.01$. (C) STAT1 and (D) STAT2 protein expression

was analyzed in mock and ZIKV-infected (MOI = 3) U-251 MG cells at 24 h post-infection, where protein abundance is represented as a ratio of STAT/GAPDH or phosphoSTAT (pSTAT)/GAPDH. STAT and pSTAT protein levels were quantified with respect to $ZIKV^{BR}$ STAT protein levels. MG132 experiments were conducted in U-251 MG cells infected with ZIKV (MOI = 3) for 12 h followed by treatment with 20 μM MG132 or DMSO for 12 h after which (E) STAT1 and (F) STAT2 protein expression was analyzed by western blot. Protein abundance is represented as a ratio of STAT/GAPDH normalized relative to the STAT/GAPDH ratio in the $ZIKV^{BR}$ control (DMSO) treated condition. Representative blots are shown from one of three independent experiments and quantification of at least three independent experiments is indicated below each of the representative blots.

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than a reduction in the STAT2 protein levels during infection with the contemporary American ZIKV isolates, when compared to the pre-epidemic ZIKV isolates ($ZIKV^{AF}$ and $ZIKV^{CDN}$) and mock-infected cells **(Figure 4.6D)**. Both $ZIKV^{PR}$ and $ZIKV^{BR}$ robustly induced STAT2 protein expression in the human astrocytomas **(Figure 4.6D)**, which again correlates with the STAT2 mRNA induction by these contemporary isolates **(Figure 4.6B)**. However, infection with $ZIKV^{CDN}$ only resulted in a slightly lower level of STAT2 protein expression than the contemporary American strains **(Figure 4.6D)**, despite a significantly lower mRNA induction **(Figure 4.6B)**. Interestingly, we observed a reduction in STAT2 protein expression in cells infected with $ZIKV^{AF}$ when compared with mock-infected cells, which is in agreement with previous reports suggesting that ZIKV induces STAT2 degradation **(Figure 4.6D)**(72, 73). However, the differential STAT1 and STAT2 protein expression observed in the contemporary American isolates does not appear to be due to increased STAT1 or STAT2 protein turnover by $ZIKV^{AF}$ or $ZIKV^{CDN}$ when compared with $ZIKV^{PR}$ and $ZIKV^{BR}$, as proteasome inhibition did not result in significant stabilization of STAT1 or STAT2 **(Figure 4.6E-F)**. Interestingly, proteasome inhibition using MG132 actually resulted in a decrease in both STAT proteins in the cells infected with the $ZIKV^{PR}$ and $ZIKV^{BR}$ isolates **(Figure 4.6E-F)**.

Finally, since IFN signaling occurs via phosphorylation of STAT proteins, we investigated whether the ZIKV isolates differentially regulate STAT1 and STAT2 phosphorylation **(Figure 4.6C-D)**. Despite some variability across experiments, all four ZIKV isolates induced phosphorylation of both STAT1 and STAT2 in the U-251 MG cells; however, the contemporary isolates induced phosphorylation of both STAT1 and STAT2 to a greater extent. Overall, infection with the contemporary American ZIKV isolates resulted in increased STAT1 and STAT2 protein expression and phosphorylation, when compared with the historical ZIKV isolates in human astrocytoma cells.

Chapter 5: Discussion and Conclusion

The first part of this chapter **discusse**s the results in the context of the current understanding of ZIKV pathogenesis in astrocytes. The reader is encouraged to refer to the literature review in Chapter 1, for more information on the *in vitro* models of ZIKV infection and pathogenesis. The discussion is followed by the **overall conclusion** of the thesis, where the main finding are summarized and potential avenues for future research are explored. Editorial assistance for this chapter was provided by Dr. Selena M. Sagan and Trisha R. Barnard.

Discussion

The recent expansion of the previously obscure ZIKV, beyond its historical endemic range in equatorial Africa and Southeast Asia has piqued scientific and public health interest in emerging viral diseases. Phylogenetic analyses and investigations into recently acquired genetic polymorphisms suggest that recent evolutionary changes could have contributed to the rapid emergence and novel neurological pathogenesis observed in the contemporary ZIKV outbreak in the Americas (9, 49). Furthermore, there is accumulating evidence that there may be strain-specific as well as host cell-specific differences in the innate immune response to viral infection (65). In this study, we selected four isolates from different points in ZIKV's phylogenetic history **(Figure 4.1)** to investigate differences in viral fitness and the innate immune response to infection in the human astrocytoma (U-251 MG) cell line. We compared two contemporary American ZIKV isolates from the 2015-2016 outbreaks in Puerto Rico and Brazil ($ZIKV^{PR}$ and $ZIKV^{BR}$), an earlier Asian lineage isolate ($ZIKV^{CDN}$), and the historical Uganda 1947 isolate ($ZIKV^{AF}$).

Interestingly, we observed that the contemporary American ZIKV isolates replicate much more rapidly and to higher titers when compared to the early Asian and the historical $ZIKV^{AF}$ isolate in the human astrocytoma cells. A current perspective on the neurotropic potential of ZIKV suggests that infected placental macrophages could potentially carry the virus into the developing brain through a "Trojan-horse" mechanism and then spread the virus (81). Once the virus is in the brain, other investigations conducted in mice and primary human tissue samples suggest that ZIKV preferentially infects astrocytes (85, 86). In concordance with these studies, all of the ZIKV strains examined in this investigation were able to infect and replicate in human astrocytoma cells. This affinity for astrocytes is likely due to their high expression of AXL, a

putative receptor for ZIKV infection, which interacts with Gas6 to promote ZIKV infection (99, 100). An independent study that examined strain-specific differences in the infection of primary astrocytes reported that an African lineage ZIKV strain (Central African Republic, 1989) infected more cells and produced higher viral titers than an early Asian strain (French Polynesia, 2013) (101). This phylogenetic trend in infectivity mirrors our results herein, where the $ZIKV^{AF}$ strain produced higher viral titers than the early Asian ($ZIKV^{CDN}$) isolate in the astrocytoma cell culture model. However, we found that the most notable difference was not between African and Asian lineages, but between the contemporary American strains and the pre-epidemic (2015) strains **(Figure 4.2B-C)**. Examining the differential affinity displayed by the different strains in binding to viral entry receptor(s) early in the infectious cycle, may help elucidate the mechanism by which the contemporary strains elicit increased infection kinetics. The AXL/GAS6 viral entry receptor is one such potential target for further investigation.

We also noticed that all three Asian lineage isolates were less cytopathic than $ZIKV^{AF}$ in U-251 MG cells **(Figure 4.3)**. The reduced cytopathicity displayed by the Asian lineage strains could potentially serve to increase the duration of infectious particle production, effectively turning the astrocytes into sites of increased viral replication which disseminate virus to other cell types in the brain; ultimately resulting in increased neurological pathogenesis. This difference in virulence and pathogenesis between African and Asian lineage isolates has also been observed in *in vivo* models where mice infected with African lineage isolates presented with severe short episodes of neural malfunction and a higher mortality rate (90). In contrast, mice infected with Asian lineage isolates had lower mortality rates, but exhibited a wider range of neurological complications for a longer duration (90). Another independent study also found that the historical African strain caused more brain damage and higher postnatal lethality than a

contemporary American isolate (Mexico, 2016) (102). However, it should be noted that some of these findings are contradicted by an investigation which suggests that a contemporary Brazilian isolate elicited a stronger deleterious effect than an African isolate in human brain organoids (84). Nevertheless, the current understanding of strain-specific differences in ZIKV pathogenesis leans towards the African strains being more virulent than their Asian counterparts (90, 102). Although we found that the contemporary American isolates were just as cytopathic as their early Asian lineage counterparts, their increased replication potential may translate into more severe neurological pathogenesis over the course of an infection. The unique virulence of the contemporary epidemic strains has also been demonstrated in neonatal mice where a contemporary American isolate (Venezuela, 2016) was shown to have increased replication as well as more severe neurological complications than an earlier Asian lineage isolate (Cambodia, 2010) (49). It is tempting to speculate that the reduced cytopathicity displayed by the contemporary Asian strains may allow them to induce pathogenesis while maintaining cell viability, whereas the highly virulent African strains are more prone to induce massive CPE that could result in miscarriage. However, it must be noted that the disease phenotype of the African lineage strains in humans has not been thoroughly investigated to date (90).

To further understand the difference in viral growth kinetics and cytopathicity between the contemporary and historical strains, we examined the induction of type I and III IFN as well as several important antiviral ISGs **(Figures 4.4 and 4.5)**. The contemporary American isolates did not produce significantly more IFNβ or IFNλ1 mRNA in the astrocytoma cells; however, they elicited a more pronounced ISG response. Notably, viperin, an ISG which has been shown to restrict ZIKV infection, was induced much more robustly by the contemporary American isolates (67). Although the type I IFN response has been reported to be important for protection

against *flaviviruses* in astrocytes (103), the robust IFN response elicited by the contemporary isolates in U-251 MG cells was unable to limit viral replication, since the contemporary isolates replicated to comparatively higher titers than the pre-epidemic (pre-2015) strains. This suggests that there are both strain-specific and cell-intrinsic factors that influence IFN and ISG induction, and subsequently, viral replication. In particular, the fact that the contemporary American isolates are able to replicate to higher titers early in the course of infection, despite the induction of a robust ISG response, suggests that these isolates may have a unique affinity for this cell type. Additionally, it is likely that the ISG response is triggered either by alternative pathways or different type I/III IFN subtypes, since the pattern of ISG induction does not mirror the pattern of IFNβ or IFNλ1 induction (75). However, there may still be an efficient interference mechanism employed by the contemporary strains as the IFN mRNA fold induction is quite low when considering the approximate 100-fold difference in infectious titers between contemporary and pre-epidemic strains **(Figure 4.4)**. Future studies that examine strain-specific differences in amount of ZIKV RNA in infected cells would provide valuable information on the relationship between the innate immune response and ZIKV RNA replication.

Interestingly, the contemporary American ZIKV isolates elicited a greater induction of IFITM1 and IFMIT3 gene expression. This is of particular interest because it has been previously reported that IFITM3 can prevent ZIKV-induced cell death by restricting viral access and takeover of the endoplasmic reticulum (66, 68). As such, the greater induction of IFITM3 by the contemporary American isolates in the astrocytomas could potentially explain how they are able to elicit reduced CPE when compared to the $ZIKV^{AF}$ isolate. However, it should be noted that the $ZIKV^{CDN}$ isolate induced IFITM3 to a similar extent to the highly cytopathic $ZIKV^{AF}$, but had similar cytopathicity to the contemporary American isolates.

Furthermore, IFITM1 and IFITM3 are important antiviral factors that have been shown to restrict early replication of ZIKV (66). However, herein we find that the contemporary American isolates replicate efficiently at early time points post-infection, despite strong induction of IFITM1 and IFITM3 gene expression. Since the localization of IFITM3 has been shown to be important for its antiviral function (66), it is possible that there are isolate-specific differences in IFITM3 localization in ZIKV-infected cells that should be further explored in future studies.

Several groups have now demonstrated that the ZIKV NS5 protein is able to bind to and induce degradation of STAT2 to inhibit type I IFN signaling in multiple cell types (71-73). Consistent with these reports, we observed reduced STAT2 protein expression in U-251 MG cells infected with $ZIKV^{AF}$ when compared with mock-infected cells (**Figure 4.6**). However, since inhibition of the proteasome did not result in significant stabilization of STAT2 (**Figure 4.6F**), ZIKV NS5 interactions with STAT2 in U-251 MG cells may be different than previously reported in other cell types (73). There are likely cell-type specific differences in how ZIKV affects STAT2, and reports carried out in Vero cells, which are deficient in type I IFN signaling, are likely not physiologically relevant when investigating the STAT response to infection (72). In addition to the potential cell intrinsic differences in STAT1 and STAT2 expression observed herein, there are likely strain-specific differences in how ZIKV affects STAT2, since we observed that infection with the contemporary American ZIKV isolates induced robust expression of the STAT2 protein in the U-251 MG cells. Additionally, the increased phosphorylation of STAT1 and STAT2 by the contemporary isolates may explain why there is more robust induction of ISG expression by the contemporary American isolates. In addition to influencing STAT1 and STAT2 phosphorylation, it is possible that the contemporary American isolates may differentially affect STAT1/STAT2 dimerization and/or nuclear translocation which

could also affect IFN signaling and downstream ISG expression. Future investigations should be focused on examining differences in viral fitness and innate immune response between contemporary and pre-epidemic strains in IFN-stimulated cells, which will help further elucidate the relationship between the strains and the JAK/STAT pathway. Additionally, comparing the infectious titers and viral replication of the ZIKV stains in immunodeficient cell lines could also clarify if the differences in replication between the contemporary and pre-epidemic isolates at early time points is directly influenced the innate immune response.

Overall Conclusion

The rapid geographic spread of ZIKV and the novel neurological pathogenesis associated with pandemic strains, have made ZIKV an urgent global public health concern. The current lack of antiviral therapeutics and vaccination strategies makes research into the biology of the virus a priority. This investigation is centered on understanding the link between recent ZIKV evolution and the development of novel pathogenesis. The physiologically relevant human astrocytoma cell culture model used herein provides important information on ZIKV infectivity, fitness, and the antiviral immune response. Our data suggests that the contemporary American ZIKV isolates may have evolved mechanisms to increase viral replication kinetics without increasing cytopathicity in the U-251 MG cells. Further research is needed to identify the viral proteins and the specific amino acid polymorphisms that are responsible for these phenotypes. Reverse genetic approaches will be useful tools for uncovering the specific amino acid polymorphisms acquired by the contemporary epidemic strains that contributed to their increase in viral fitness. *In vitro* models of infection also provide information on the host-cell specific responses to infection and may ultimately help explain the placental and neurotropism of ZIKV. Future

studies should thus consider the relationship between cell-intrinsic and strain-specific factors when examining ZIKV-host interactions. Furthermore, this work can be translated to examine strain-specific differences in pathogenicity in small animal models, which may be a more medically relevant context. Thus, this work advances our knowledge of the ZIKV biology and pathogenesis and may provide a foundation for understanding the evolutionary changes that may have contributed to the emergence of ZIKV as a significant global pathogen in the past decade.

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